Title

Mission N-Possible: Influence of Inorganic Nitrogen Forms on Small Grain Crop Carbon Assimilation

Author

Kasemsap, Pornpipat

Publication Date

2024

Mission N-Possible: Influence of Inorganic Nitrogen Forms on Small Grain Crop Carbon Assimilation

By

PORNPIPAT KASEMSAP
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Horticulture and Agronomy

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

Arnold J. Bloom, Chair

Jorge Dubcovsky

Daniel J. Kliebenstein

Committee in Charge

2024

Table of Contents

This dissertation is dedicated to farmers and scientists,

whose sweat, tear, and countless hours in the sun

help advance our understanding of the natural world

and nourish the inhabitants of this hungry planet where I call home

“The essence of intercultural education is the acquisition of empathy-the ability to see the world as others see it, and to allow for the possibility that others may see something we have failed to see, or may see it more accurately. The simple purpose of the exchange program...is to erode the culturally rooted mistrust that sets nations against one another.
The exchange program is not a panacea but an avenue of hope.”
J. William Fulbright

This journey was only possible because of
Thailand – United States Educational Foundation (Fulbright Thailand)

Land Acknowledgement Statement

We should take a moment to acknowledge the land on which we are gathered.
For thousands of years, this land has been the home of Patwin people.
Today, there are three federally recognized Patwin tribes: Cachil DeHe Band of Wintun Indians of the Colusa Indian Community, Kletsel Dehe Wintun Nation, and Yocha Dehe Wintun Nation.

The Patwin people have remained committed to the stewardship of this land over many centuries.
It has been cherished and protected, as elders have instructed the young through generations.
We are honored and grateful to be here today on their traditional lands.

Acknowledgement

These first few pages that precede the hardcore academic dissertation content are what I love reading the most. I started writing this section first and finished it last right before I wrapped up the dissertation. Please pardon my English writing—grammar and styles, although it has fortunately already improved significantly over the years in the US. As opposed to the rest of this dissertation, I wrote this section primarily with my heart, rather than my head. It also has not been polished by any large language model or AI (including "Arnold Intelligence")! These pages, where we recall sweet moments in school and express gratitude to those who has supported us, in my opinion, "humanize" the work we do as a researcher.

"*People here want to help you succeed*"— Arnold told me casually on the first day that we ever met in person in Davis. I came to UC Davis to primarily learn about how to do "sciences", but I ended up learning so much about "people" who are the true forces behind our scientific advances. This section reminds me that besides academic research outputs, the student itself is the main product of the whole process, which couldn't have been possible without an army of supporters. Please let me acknowledge and share with you, the people who have helped me grow to become a person, whom I am, whom I love, and whom I am proud of today. They are my personal "Avengers" (since we are right where Marvels was born).

Arnold Jeffrey Bloom—"*You do sciences, and I'll find funding to support you*", Arnold told me whenever I asked him if I should apply for more funding, and he has kept his word. Arnold is never slow to reply to emails or calls. Even during the time when he was on sabbatical in Munich for 9 months in the first year I got to Davis, I have never felt that I lacked any support I needed. Thank you for encouraging me to seek help, for letting me take so many Spanish classes as I please, for supporting me to join and initiate countless professional development activities, and for just regularly checking in on me as simple as "*how are you?*". Arnold makes me feel valued, respected, and appreciated as a person. Every time I feel like I don't want to do sciences any longer, Arnold inspires me with examples how fun and impactful doing sciences can be. I wish to grow up into my 80s still having fun doing whatever I do in the future as much as Arnold has with sciences. "*If it's not fun, why would you do it?*" he said. I could perhaps write a few full articles on how working with Arnold is like. But the conclusion will always remain the same: I wish everyone had a mentor as supportive as Arnold in their life. Despite not (always) agreeing with me, Arnold would always provide constructive feedback and support me to grow, personally and professionally. Thank you, Arnold, for being part of my life.

Jorge Dubcovsky—I am grateful and excited to have had Jorge on my guidance as well as my dissertation committee. I have been trying very hard to be as responsive and reliable on emails as Jorge. Still trying. I remember clearly a time when Jorge slowly explained to me how "HIF" works using a pair of chopsticks to represent a chromosome and another time when he saved me from tough questions about wheat breeding when we presented talks in Norwich. Jorge's passion, energy, and enthusiasm that radiate in every direction whenever he talks about sciences are eternally contagious. Thank you, Jorge.

Dan Kliebenstein— A few Spring quarters ago, Plant Biology Graduate Group hosted a seminar on surviving graduate school led by a graduating doctoral student, Jenna, and several professors, including Dan. I still remember vividly the two take-home wisdoms: be comfortable with failure and curate a social circle of supporters. I tried my best to follow his advice. A lot of time I've learned about Dan's scientific approaches through students and scientists working in his lab. I thank Dan for serving on my dissertation committee, for his fresh perspectives to look at complex data, and for his "spicy" and scientifically inspiring tweets that I've enjoyed immensely throughout the years. Thank you, Dan.

Dr. Z—I sent Maciej an email before I came to UC Davis to which he replied 6 years later. But, I was proactive and already recruited him to join my Qualifying Exam committee. I have learned so much from Maciej's kindness and wisdom, both on sciences and on life. Thank you! (and in advance for more questions that I have yet to ask)!

Matthew Gilbert—Matthew served as my academic advisor. We met at least once a year, but in reality, we crossed path a bit more frequently because I just had so many questions for him. Matthew always finds creative and practical ways to help me look for answers. Thank you for guiding me through this journey, Matthew.

Patrick H. Brown—Patrick shared valuable advice for my research plan when he served on my Qualifying Exam committee and during his Plant Nutrition course. I thank him for encouraging me to go to the Plant Nutrition conference, for biking with me to see the local farmers, and for connecting me the scientific community of Plant Nutritionists.

Donald Strong—I had a pleasure to take Prof. Strong’s class on Global Change Ecology, which was one of the most inspiring classes I’ve ever taken. I thank him for challenging my intellectual vitality and for serving on my Qualifying Exam committee.

Mark Lundy—I thank Mark for all the data-driven tools and approaches he and his team have incorporated into the Small Grains program at UC Davis. I learned so much from our conversations during the time I developed my proposal and prepared for my Qualifying Exam.

Diane Beckles—I thank Diane Beckles for her kindness and practical advice she has always shared with me since before I joined UC Davis. Thank you, Dr. Beckles!

Asmundsonians—There was a time when the fire alarm went off and we all left the building to gather on a lawn nearby. That was when I realized how many people work in Asmundson Hall. From feeling a bit scared of how old the building is and how leaky it gets when the summer heat overwhelms the cooling system, I fell in love with this workplace and its people. I thank my fellow building mates for joining my social initiatives, for enriching my day-to-day work with joyful conversations in the corridors, and for sharing highs and lows of our life as a scientist. I am glad the listserv we started is still being used and I hope it continues to help connect people together. Special shoutouts in no specific order to Ella, Shu, Cody, Karin, Emma, Bixuan, Shaoyun, Baohua, Matt, Céline, Amanda, Rosalie, Jackie, Yaxin, Dan S., Margaret and the IT support team. Particularly, I also would like to thank Bruce who has always been making sure that everything is tidy. Thank you for sharing refreshing conversations whenever I stayed late to work.

Wheat experts of Robbins—I’ve only eaten wheat, but not grown any until I started this project. Yet, I was in good hands of the amazing team of wheat geneticists and breeders from Dubcovsky lab. In particular, I would like to thank: Junli who never (hopefully) gets tired of answering my questions on genetic analyses, Nestor who helped me get familiar with wheat during my first preliminary experiment that eventually got us a big grant!, Youngjun and Tyson for sharing all the tips on how I could grow wheat successfully, Josh who helped me understand all the seed quality matrices and got me in touch with the lovely team at the California Wheat Commission (Particularly, Teng for his help with seed protein measurements!), Alejandra and Gilad who shared their years of sciences with me during the time we went to ASTA conference in San Diego together, Priscilla who has provided great suggestions during our wheat writing group which was a great success because everyone has graduated!, Saarah, whom I had a pleasure to work with on so many other projects and I will always be inspired by her genuine care for other people, and her standard of excellence in sciences, Xiaoqin for all the empowering conversations we've had in the Robbins hallway and in the ENH greenhouses. I also thank my writing group affiliates from TCAP, Ella, Brittney, and Mikayla who have shared tons of helpful comments that improved my writing significantly.

Graduate Group team—The magnificent Lisa, Kelly, and Jeremiah. I wish we all had someone who listen to us as attentive and caring as my grad group coordinators. Thank you for always being on top of everything and for your continued support. Just hearing Lisa's voice already calms me down whenever I feel anxious about this complex world. I also thank Astrid for her leadership, especially during the COVID-19 pandemic. I am glad we started and continue to hold the townhall meetings with the graduate group members. Her efforts to foster connections within the grad group are much appreciated.

The protectors of the Plants—My whole experiments would have not been possible and successful without great support from the wonderful staff members of Environmental Horticulture and Controlled Environment Facilities. Particularly, I thank Jianguo, Ron, Bill, Davith, and all the student assistants who have helped me taking care of my wheat in the greenhouses. Thank you for sharing all your plant care magics with me! I thank Kevin, Fred, Andre, Joel and all the past staff members of CEF who have troubleshooted innumerable technical challenges and kept my plants alive and well under elevated CO_2 for years. I genuinely appreciated your continued and steadfast support.

The staff members of Plant Sciences—I have crossed path and experienced kindness from so many staff members of Plant Sciences over the years. Particularly, on purchasing, lab safety, hiring, and IT. Specifically, I would like to express my sincere thanks to Karen, the late Dena, Raj, Tom, and

Janet who have helped me significantly with all the questions and support in any matter I have had to deal with. Big thanks also go to the IT team of 150 Asmundson for solving all my challenges online.

The lab safety team of Plant Sciences—During my time at Davis, I served as a lab safety coordinator for almost the entire time. Without the tremendous help from Karin, Tony, and Kristy, I would not have known what to do with all the chemicals, safety protocols, and compliance. Particularly, I thank Karin for her support especially during the unsettled times of COVID-19. Her calm manner and wealth of knowledge have been incredibly comforting and much appreciated.

SAS25 teaching team—Teaching Science & Society 25 "*Global Climate Change*" with you all was the highlight of my time at Davis!: Kamyar, Anna, Sarah, Nina, Alan, Natalia, Taylor, Kahui, Pallawi, Margaret. Thank you for sharing your teaching tips, lesson plans, grading advice, and for your patience with how long I usually take to grade papers. Thanks, Braintrust!

Outdoor Adventure—I joined Outdoor Adventure because I was hoping to organize a trip for my grad group, which did not happen because of COVID-19. But, in the process, I got to do a whole week of swift water rescue, CPR, wilderness first-aid and whitewater rafting guide training, and later regularly volunteer with OA. Thank you for welcoming me into the community and into the wild with your enthusiasm and passion for nature. Special thanks go to Beck, Lizzy, Matt, Zach N., and all my BGT folks! Thanks, Zach Dashner, for introducing me to this wonderful community where I feel at home. To more rocks that I'll hit on the river. Go OA!

SISS student advisors—As an international student, one of my sources of uncertainties is immigration. Without expertise and kind help from the staff members of SISS, I would have had way more sleepless nights. I am grateful for the continued support from the SISS advising team, especially Lorena, Meredith, Robert, Andrew, Matthew, Tammy, Stephanie, and Wesley, who have all helped me with SO MUCH of my paperwork throughout the years.

Fulbright Thailand and Institute of International Education—Before the SISS era, I was fortunate to have been advised by the wonderful team of advisors at IIE. I would like to thank Tom, Laurie, Miranda, Jemina, and Liza who have always made me feel well-taken care of throughout my time under their care. I still remember clearly the advice Tom gave me (as my first ever advisor).

Thanks, Tom! I thank Katie and the staff members of TUSEF as well as my fellow grantees who have supported me throughout this whole journey, especially Jump and Win! Go California Fulbrighters!

Innovation Institute for Food and Health—My colleague Katie said that this was one of the best programs she's joined at UC Davis. And she was right. I almost did not accept the fellowship because that was when the COVID-19 lockdown started. If I did not, I would have missed one of the most transformative opportunities of my life. BIG Thanks Justin Siegel for your leadership and vision for this organization, thanks Kyeema, Sarah, Dana, Marissa, and especially Veronica, for all their excellent professional development support. I am still very sad I did not get to go to New York because of the pandemic, but that did not change how wonderful this whole experience was.

Food Shot Global—One whole year with the team at Food Shot Global has changed how I see this world. I greatly appreciate the mentorship and support from Sara Eckhouse, An Wang, Will Schmitt, Rajiv Singh, Victor Friedberg. I thank all the start-ups and investment partners I have had a chance to learn from during our calls and due diligence process. For a better food system!

Davis Alt. Protein Project—I first saw a presentation by Ted at the GSA meeting and immediately knew I must be a part of this team. I am glad that we successfully developed the first ever curriculum for alternative protein for our undergraduates. I thank my colleagues for always inspiring me with their cutting-edge research and leadership, especially, Ted, Nick J., Minami, Jules, and Sophie.

Sustainable Growth Yolo and the Join Housing Taskforce team—I thank Don Gibson and all the inspiring advocates I've learned from in the past years. Thanks for your leadership!

Team Davis—I found Team Davis by chance and I'm so happy to have been inspired by our volunteers and athletes who never ever give up! I thank Jules, Debbie, Eric, Julia, and all our volunteers and parents who have supported the operations of this non-profit over the years. Running with Team Davis always makes my Sunday a very fun day!

Global Education for All—One of the highlights of my time at Davis was being part of the Steering Committee and the inaugural Student Fellows in this "Big Idea" Initiative. Thank you for trusting me to lead the student team and to represent our student body. I sincerely appreciate the unwavering support from Nancy, Janice, Robb, and all my fellow student leaders, especially Esther and Marwa.

Bob—On a fine Sunday afternoon in October, I stopped by an International Fair, and I met Bob. Bob, a retired old gentleman and a stranger, introduced me to the iHouse where he served on the board, and the community this place has helped create. I thank the iHouse and Bob for his kindness and support that help make Davis feels like home so fast.

Seed Central— François Korn has put so much effort and energy into this awesome platform that connects professionals in the seed industry with our university students and professors. I thank him and all the inspiring Ag professionals I have had a pleasure to meet through this monthly event. Free food and drinks. Free talk. Free networking. What would be a better way to spend an afternoon? I thank Monica Ivey Smith for serving as my industry mentor through the Seed Central-led mentorship program. Thank you, François, for your continued support to help early career researchers navigate the job market.

Leaders for the Future cohort 2—It feels like a century ago, but I still remember all the key lessons and business training I learned from my colleagues and mentors during this fellowship. Thank you all for sharing and supporting each other throughout our professional development journey. This is a starting point that has helped me find my way through the job market in later years.

Grad Slam—I would like to thank the team at Grad Studies for supporting me to share my work through Grad Slam! One of the most fun times! Thanks, David, particularly, for suggesting the use of this famous quote from the movie "*Your mission, should you choose to accept it…*" to spice up my talk!

Horticulture and Agronomy Student Association—I am proud of what we have accomplished together for our diverse community of 100+ plant enthusiasts. Well-done team! I want to specifically thank Jen for initiating the Peer Mentor program; Mitch for leading the journal club; Amanjot and Steven for their help as a treasurer; the late Cristobal, Zach, Taylor, Miguel, Emily, Telha, Aaron, Evie, Allison, Claire, Ally, Anca, and Rana for spearheading and supporting the social activities; Sire, Ellie, and Evie for leading the DEI efforts, Liberty and Krista for leading the efforts to improve QE process; Lila for her awesome writing skills and help with the Seeds of Inspiration series; Umair and Moo for their website management, Dante for leading the Find A Talk project; Kimberly, Amy, Saarah and the SCOPE team who always supply tasty produce for our gatherings; Mengyuan and Randi J. for their leadership that helped build a strong foundation for the club; To all my GSA reps, thanks for stepping up to represent your community and sit through 3+ hr of meetings every month! Thanks to Youngjun and Tamara for being my peer mentor! Lastly, I thank the team at Sakata for their generous donation of vegetables and fruits for our annual BBQ; as well as UC Davis Plant Breeding Center, the campus book project, Yolo Food Bank and Davis Joint Unified School District Schools, and local farmers and seed companies for their partnership with us over the years.

"*Davis is not a place, it's a people*" —modified by me from a quote first said by Odin in Thor: Raknarok (2017). I thank my (Thai) community in Davis who has shared with me countless hours of laughs and yummy dishes. I cherish the beautiful memories we have made together. I particularly want to thank my housemates and neighbors at Solano Park and Sunset residence "Baan Phra Arthit". Korn, Jareerat, Nok, Best, Khine, May, and Ten—thank you for being there when I need support.

Davis Plant Scientists—I thank all my friends and colleagues who have consistently inspired me with their love for plants and their passion for improving our agriculture. Particularly, thanks for your support, advice, and random conversations that you may not know they were so important to me: Jessica, Ed, Yufei, my weekly Burrito squad (Clemens, Colleen, Rana), Alejandra, Best, Chenjiao (thanks for adopting my chicken babies!!!), Sam and Polulu, Laila, Juliya, Stefan, Oon, Marie, Matt D., Houston, Chad, Gitanshu, Leah, Heather, Gina, Juliana, Xinyu, Hana, Paul, Stephanie, Jake, Francisco, Kyle, Livingstone, Jorge B., Matthew E., Marcos, Thort, Karla, Lindsay, Nico, Nick R..

Wageningen Horticulturists—Going to a grad school in the Netherlands was not the first plan I had in mind back then, but it ended up being the best years of my life where I have met the sweetest people with whom I have explored the world of plants. I'm glad technology has kept us connected until these days. I thank my Horticulturist squad for their words of encouragements, waves of Happy birthdays, cute pictures of their pets and human babies (and sometimes plants), and just for being there reminding me of beautiful memories we have made together. Melpo, Yutaka and Daisuke, Lucas M., Chiara, Filippos, Ying, Yi, love you all!

A big shout out to my friends who have either stopped by to visit me in California, or have listened to my story about Davis for million times: Peson, Toon, Bighong, Jidapa, Lasse, Minh, Axel, Isma, Lucia, Ellen, Pai, Benz, Noo, Meng, Kaw, Whay, Lucas H., Jack, Marcelo, Fred N., Max, the Ehrmans. Thanks for being there for me when I need you.

Bloom lab members and affiliates—I have had so much fun being part of the Bloom lab. And it would have not been possible without the Bloomers: Anna and Max, Xiaoxiao, Nate, Itay, Fred F., and our regular guests, Bill and Tom. The work you are about to enjoy is a product of so many discussions in our lab meetings with these wonderful people.

Here is the core team that has made the magics happen. My grand project is only possible because of this wonderful team of undergraduate research assistants: Zennin G. Casl, Timothy Congleton, Zion Congrave-Wilson, Joshua Claxton, Samuel Doolittle, Anna Gomes, Ariel Herrera, Sydney Koelbel, Steven A. Kunze, Dana Lawrence, Khine Lin, Carly Miranda, Steven Moreno, Jenna O'Kelley, Elias Potashov, Francesco G. Riga, Temiloluwa Salako, Abenezer Shankute, Guilherme M. Silva, James K. Schmidt, Jordan Stefani, Lisa Tam, Kungyao Wang— You all taught me so much about team management and professional development. I am so proud of what we have accomplished, and I am grateful to grow personally, professionally, and botanically with you all in this journey!

The Strongest Ladies of Redding—All the weights and calories I have gained during my study derived from your yummy food. Thank you for always being there listening to me, showering me with love and comforts, and for driving all the way to be with me when I needed you.

Dearest Sompian Kasemsap, the first farmer I've ever known, Grandparents, Uncle Maek, Aunt Qing, Mom, Dad, and my dearest brother: I love you and miss you every day, every hour, every minute, and every second. Thank you for loving me, even when my hair was longer than what makes you comfortable (I did donate it for charity all three times, though, and I saved a lot of money!). I love you.

To all my past mentors and teachers, thanks for your patience. Arnold was right about people! Sciences and scientists cannot exist, without our community and our network of support. I thank YOU for reading our work and for supporting sciences, and especially the people who do sciences.

This work was supported in part by USDA-IWYP-16-06702, USDA-NIFA 2022-68013-36439 (WheatCAP), NSF IOS 13-58675, NSF IOS 16-55810, the John B. Orr Endowment, Thailand – United States Educational Foundation (TUSEF/Fulbright Thailand), UC Davis Department of Plant Sciences Graduate Student Researcher fellowship, Henry A. Jastro research award, Grad Innovator Fellowship from UC Davis Innovation Institute for Food and Health, and the United States-Israel Binational Agricultural Research and Development Fund.

I am eternally grateful :)

Abstract

Nitrogen fertilization of agricultural fields sustains global food security, but less than half of the fertilizer applied reaches the table. Under elevated CO_2 levels anticipated in the near future, food production suffers from crop protein decline and declining or stagnant yields, threatening human nutrition worldwide. The natural variation in the ability of crops to assimilate different soil inorganic nitrogen forms, nitrate (NO_3^-) and ammonium (NH_4^+), into protein remains unexplored and may provide solutions to this grand challenge.

This doctoral dissertation explores 3 major questions pertaining to wheat and rice, staple food crops that provide 32% of the protein in the human diet: (i) how have past genetic modifications and breeding improved grain protein concentration and yield? (Chapter 1), (ii) what is the extent of natural variation in the ability to utilize different nitrogen forms for growth and development? (Chapter 2 and 3), (iii) how does adaptation to specific nitrogen sources influence crop responses to changing climates? (Chapter 3 and 4).

Chapter 1 summarized successful breeding attempts to modify nitrogen metabolism through genes that coordinate nitrogen and carbon metabolism. Gaps in our current understanding include (1) species differences among candidate genes in nitrogen metabolism pathways, (2) the extent to which relative abundance of these nitrogen forms across natural soil environments shape crop responses, and (3) natural variation in and genetic architecture of nitrogen-mediated yield improvement. The following chapters addressed these knowledge gaps.

Chapter 2 and Chapter 3 examined the growth responses to different nitrogen forms of rice and wheat populations that represent the genetic diversity within the global germplasm. Generally, most of the populations effectively use either ammonium or nitrate at moderate levels to support vegetative growth. Such plasticity may allow plants to be more resilient to fluctuations in soil nitrogen. Genome-wide analyses identified genetic markers associated with growth under different nitrogen sources that may be employed in future breeding programs.

Chapter 3 and Chapter 4 further evaluated the extent to which soil nitrogen sources alter wheat responses to atmospheric CO_2 fluctuations using genotypes that demonstrated a preference for ammonium or nitrate, and contrasting degrees of ammonium tolerance. Nitrogen-form preference, but not ammonium tolerance, correlated with CO_2 responses. Notably, ammonium-preferring genotypes maintained higher biomass and sustained grain nitrogen concentrations, thus avoiding CO_2 acclimation, the decline in biomass stimulation after prolonged exposure to CO_2 enrichment.

Overall, this dissertation provided strategies to guide matching crop genetic adaptations with fertilizer management to improve nitrogen-use efficiency and to sustain food security under the atmospheric conditions anticipated in the future. To continue improving grain yield and quality under changing climates, breeding strategies need to focus on both carbon and nitrogen assimilation. We recommend breeding for ammonium-adapted genotypes, which may not only improve climate resilience, but also potentially accelerate development and increase yield without any penalty on grain quality.

General Introduction

"…millions of people have died in armed conflicts over the past 100 years, but, at the same time, billions of people have been fed." by the discovery of the Haber-Bosch process (Erisman et al., 2008).

Food crop production depends heavily on nitrogen fertilization of agricultural fields to archieve high yield and nutritional quality. The patent "Synthesis of Ammonia from its Elements" filed by Fritz Haber in 1908 led to the Nobel prizes in Chemistry being awarded to Haber in 1918 and to Carl Bosch and Friedrich Bergius in 1931 for development of the chemical methods necessary for scaling up this world-changing process that sustains the global food production (Erisman et al., 2008). Dinitrogen gas, ~78% of the atmosphere, is comprised of two nitrogen atoms bonded with a powerful triple covalent bond. The Haber-Bosch process uses high pressures combined with extreme heat and a catalyst to convert dinitrogen gas into ammonia, a more reactive form of nitrogen that is starting compound for both nitrogen fertilizers and most conventional explosives (Erisman et al., 2008).

Agriculture already accounts for 20% of global greenhouse gas emissions, one-quarter of which derives solely from production and management of nitrogen fertilizer (Gao & Cabrera Serrenho, 2023). Less than 50% of the fertilizer makes it into the crop plants to which it is applied (Zhang et al., 2021). During the green revolution, the development of crops with small statue allows higher proportion of biomass partitioning to the grains, known as higher harvest index. As an unintentional consequence, however, such genetic variations seem to have rendered modern crop varieties relatively insensitive to nitrogen (Li et al., 2018). Consequently, farmers require large amount of nitrogen fertilizers to sustain crop productivity, resulting in massive losses of nitrogen and a potential environmental hazard (Erisman, 2021; Galloway et al., 2021; Matassa et al., 2023). To meet the increasing food demands from rising human population growth, we need to devise better approaches to manage nitrogen.

Plants have evolved to deal with soil nutrient heterogeneity across natural ecosystems. Yet, we still have little understanding of how soil variations shape plant nitrogen responses because relatively few studies have examined the genetic basis of nutrient local adaptation (Kasemsap & Bloom, 2022). Exploitation of global crop genetic diversity in adaptations to variations in nitrogen pertaining to different soil conditions in natural habitats around the world, therefore, may have the potential to advance crop breeding for improved nitrogen management.

This doctoral dissertation explores the genetic diversity in nitrogen responses within the global germplasms of two major food crops, wheat and rice, that are critical sources of protein and calories in human diets. The present study characterized the genetic and physiological basis of nitrogen and carbon assimilation by answering 3 major questions: (i) how have past breeding and genetic modifications for nitrogen metabolism improved grain protein concentration and yield? (Chapter 1), (ii) what is the natural variation in the ability to utilize different nitrogen forms for growth and development in the two species? (Chapter 2 and 3), (iii) how does adaptation to specific nitrogen sources influence responses to changing climates? (Chapter 3 and 4). This dissertation addresses the overarching hypothesis: *genetic adaptations to specific forms of nitrogen allow plants to alter their responses to fluctuations in atmospheric climate conditions and may be beneficial to breeding for improved nutrition and climate resilience.*

Chapter 1 (Published in *Plants*) (Kasemsap & Bloom, 2022) reviewed the existing body of knowledge in regards to nitrogen metabolism and its applications in plant breeding. I highlighted key areas of research that warrant substantially more investigation: comparative studies of candidate genes between species, the influence of different inorganic nitrogen forms, and the local adaptation of crops to variations in soil nitrogen. The remaining 3 chapters attempt to fill these gaps by assessing the natural variation of biomass production under different forms of nitrogen in two major cereal crops.

Chapter 2 (Submitted for publication and deposited on *biorxiv*) (Kasemsap et al., 2024) evaluated the global rice germplasm and demonstrated that rice, often assumed to be adapted to using ammnonium (NH_4^+) as its primary nitrogen source, may actually be equally adapted to using nitrate. Genome-wide analyses identified distinct and shared sets of loci underlying responses to ammonium and nitrate forms as well as candidate genes related to such responses across diverse rice populations

Chapter 3 (Ready to be submitted for publication) characterized wheat genetic architecture of nitrogen responses in the global germplasm and bi-parental nested association mapping populations under both ambient and elevated CO_2 atmospheres. Genome-Wide Association and linkage mapping identified loci consistently associated with biomass accumulation under the different nitrogen forms and CO_2 levels.

Chapter 4 (Submitted for publication and deposited on *biorxiv*) (Kasemsap & Bloom, 2023) provided as a proof of concept of how genetic adaptations to a specific nitrogen source alter crop responses to changing climates. By accounting for genetic diversity and potential adaptation to nitrogen forms, we concluded that breeding for better utilization of NH_4^+ nutrition may sustain grain quality and prevent declines in biomass under elevated CO_2 levels, a typical acclimation response observed across multiple plant species.

References

Erisman, J. W. (2021). How ammonia feeds and pollutes the world. *Science*, *374*(6568), 685–686. https://doi.org/10.1126/science.abm3492

Erisman, J. W., Sutton, M. A., Galloway, J., Klimont, Z., & Winiwarter, W. (2008). How a century of ammonia synthesis changed the world. *Nature Geoscience*, *1*(10), 636–639. https://doi.org/10.1038/ngeo325

Galloway, J. N., Bleeker, A., & Erisman, J. W. (2021). The Human Creation and Use of Reactive Nitrogen: A Global and Regional Perspective. *Annual Review of Environment and Resources*, *46*(1), 255–288. https://doi.org/10.1146/annurev-environ-012420-045120

Gao, Y., & Cabrera Serrenho, A. (2023). Greenhouse gas emissions from nitrogen fertilizers could be reduced by up to one-fifth of current levels by 2050 with combined interventions. *Nature Food*, *4*(2), 170–178. https://doi.org/10.1038/s43016-023-00698-w

Kasemsap, P., & Bloom, A. J. (2022). Breeding for Higher Yields of Wheat and Rice through Modifying Nitrogen Metabolism. *Plants*, *12*(1), 85. https://doi.org/10.3390/plants12010085

Kasemsap, P., & Bloom, A. J. (2023). *Genetic adaptation to ammonium sustains wheat grain quality and alleviates acclimation to CO_2 enrichment* [Preprint]. Plant Biology. https://doi.org/10.1101/2023.11.21.568194

Kasemsap, P., Cohen, I., & Bloom, A. J. (2024). *Genome-wide Association Study of Rice Vegetative Biomass under Different Inorganic Nitrogen Forms—Ammonium or Nitrate*. https://doi.org/10.1101/2024.08.12.607622

Li, S., Tian, Y., Wu, K., Ye, Y., Yu, J., Zhang, J., Liu, Q., Hu, M., Li, H., Tong, Y., Harberd, N. P., & Fu, X. (2018). Modulating plant growth–metabolism coordination for sustainable agriculture. *Nature*, *560*(7720), 595–600. https://doi.org/10.1038/s41586-018-0415-5

Matassa, S., Boeckx, P., Boere, J., Erisman, J. W., Guo, M., Manzo, R., Meerburg, F., Papirio, S., Pikaar, I., Rabaey, K., Rousseau, D., Schnoor, J., Smith, P., Smolders, E., Wuertz, S., & Verstraete, W. (2023). How can we possibly resolve the planet's nitrogen dilemma? *Microbial Biotechnology*, *16*(1), 15–27. https://doi.org/10.1111/1751-7915.14159

Zhang, X., Zou, T., Lassaletta, L., Mueller, N. D., Tubiello, F. N., Lisk, M. D., Lu, C., Conant, R. T., Dorich, C. D., Gerber, J., Tian, H., Bruulsema, T., Maaz, T. M., Nishina, K., Bodirsky, B. L., Popp, A., Bouwman, L., Beusen, A., Chang, J., … Davidson, E. A. (2021). Quantification of global and national nitrogen budgets for crop production. *Nature Food*, *2*(7), 529–540. https://doi.org/10.1038/s43016-021-00318-5

Chapter 1

Breeding for higher yields of wheat and rice through modifying nitrogen metabolism

Published in *Plants* (https://doi.org/10.3390/plants12010085)

plants
MDPI

Review

Breeding for Higher Yields of Wheat and Rice through Modifying Nitrogen Metabolism

Pornpipat Kasemsap * and Arnold J. Bloom

Department of Plant Sciences, University of California at Davis, Mailstop 3, Davis, CA 95616, USA
* Correspondence: pkasemsap@ucdavis.edu

Abstract: Wheat and rice produce nutritious grains that provide 32% of the protein in the human diet globally. Here, we examine how genetic modifications to improve assimilation of the inorganic nitrogen forms ammonium and nitrate into protein influence grain yield of these crops. Successful breeding for modified nitrogen metabolism has focused on genes that coordinate nitrogen and carbon metabolism, including those that regulate tillering, heading date, and ammonium assimilation. Gaps in our current understanding include (1) species differences among candidate genes in nitrogen metabolism pathways, (2) the extent to which relative abundance of these nitrogen forms across natural soil environments shape crop responses, and (3) natural variation and genetic architecture of nitrogen-mediated yield improvement. Despite extensive research on the genetics of nitrogen metabolism since the rise of synthetic fertilizers, only a few projects targeting nitrogen pathways have resulted in development of cultivars with higher yields. To continue improving grain yield and quality, breeding strategies need to focus concurrently on both carbon and nitrogen assimilation and consider manipulating genes with smaller effects or that underlie regulatory networks as well as genes directly associated with nitrogen metabolism.

Keywords: cereal; biomass; NUE; yield component; nitrate; ammonium; adaptation

check for updates

Citation: Kasemsap, P.; Bloom, A.J. Breeding for Higher Yields of Wheat and Rice through Modifying Nitrogen Metabolism. *Plants* **2023**, *12*, 85. https://doi.org/10.3390/plants12010085

Academic Editors: John T. Hancock and Mikihisa Umehara

Received: 23 November 2022
Revised: 21 December 2022
Accepted: 21 December 2022
Published: 23 December 2022

1. Introduction

Balancing crop nitrogen and carbon status under changing environmental conditions is essential for sustaining agricultural productivity and food security. Nitrogen constitutes 1 to 2% of plant dry biomass, yet plants allocate a disproportionate amount of their energy to convert inorganic nitrogen forms, especially nitrate (NO_3^-) and ammonium (NH_4^+), into organic compounds [1]. As much as 50% of total plant protein may be ribulose-l,5-bisphosphate carboxylase/oxygenase (rubisco), the enzyme that initiates plant assimilation of CO_2 into organic carbon through C_3 carbon fixation [2]. Consequently, plant organic nitrogen and organic carbon are inextricably linked [3]. Rice (*Oryza sativa* L.) breeding has succeeded in increasing both grain yield and grain protein concentration in recent decades [4]. In contrast, long-term wheat (*Triticum aestivum* L.) breeding has achieved incremental biomass yield gain, but at the loss of grain protein content over time [5]. Plant breeders therefore actively seek to achieve two sometimes opposing goals, maximization both food productivity and quality.

Articles about breeding strategies to improve yield often discuss crop ideotype, outlining and dissecting desirable traits with the potential to achieve the highest theoretical yield or most rapid progress in genetic gains (for example, [6–11]). This review offers commentary presented in four sections: Process, Progress, Prospects and Puzzles. First, we briefly discuss crop inorganic nitrogen uptake, assimilation, and mobilization, topics for which a plethora of reviews already exist (for example, [12,13]). Second, we evaluate recent successful breeding endeavors involving genes within the nitrogen pathways that improve yield, using the framework of yield component analyses. Third, we present key trends among 40 validated genes that enhance crop yield. Highlighted are genes that influence tiller number, flowering time, and NH_4^+ assimilation. Lastly, we conclude by identifying areas for further research such as homologs across species, responses to different inorganic nitrogen forms, and complexities of natural variation and epistasis.

2. Process—Reaping What We Sow: How Soil Nitrogen Makes Its Way onto Our Plates

Plants acquire most of their nitrogen, both organic and inorganic forms, from soil, but reliance on each form varies greatly over time, with location, and under different environmental conditions [14]. Soil microorganisms mineralize organic nitrogen into NH_4^+, which subsequently becomes oxidized into NO_3^- through nitrification [15]. Plants compete with soil microbes for NH_4^+, a form which also serves as a crucial microbial energy source [16]. In temperate aerobic agricultural soils, microbial activities rapidly convert most soil nitrogen into NO_3^-, and so NO_3^- remains the dominant soil inorganic nitrogen compound available to crops [16,17].

Plant nitrogen acquisition relies on a well-coordinated network of transporters [13]. Nitrate transporters are among the most extensively studied groups of proteins and include low and high affinity systems that cover a large range of concentrations in soil; they also have additional functions beyond NO_3^- transport [4]. Ammonium transporters are considered high affinity systems because they operate under low NH_4^+ concentrations [4]. The primary inorganic nitrogen assimilation pathway involves several reactions: nitrate reductase (NR) catalyzes NO_3^- reduction into nitrite (NO_2^-), nitrite reductase (NiR) catalyzes nitrite (NO_2^-) reduction into ammonium (NH_4^+), and the concurrent actions of glutamine synthetase/glutamate synthase (GS/GOGAT) catalyze the incorporation of NH_4^+ into amino acids [18]. The resulting organic nitrogen compounds are transported, remobilized, and re-assimilated in different organs according to sink demand as a plant develops [4]. As plants mature and reach a reproductive stage, nitrogen compounds that have accumulated throughout vegetative stages are directed toward seeds, the organs vital to species survival and the harvestable part for most crops [4].

Our major focus here is wheat and rice for multiple reasons. First, these crops are the two top sources of plant protein that we consume daily according to the Food and Agriculture Organization of the United Nations (FAO) [19] (Table 1). Relative reliance on these two crops as a major protein source varies across geographical regions. Wheat contribution to human protein intake is dominant in Northern Africa (38%), Central Asia (38%), Southern Asia (26%), Western Asia (39%), and Europe (22–29%). Rice prevails in South-eastern Asia (34%), Southern Asia (21%), and Micronesia (18%). Second, wheat and rice are both C_3 plants belonging to the Poaceae family. Such relatedness may facilitate the transfer of knowledge between these two closely related species, although the genome of hexaploid wheat is 40 times larger than that of rice [20]. Third, as model species and major food crops, they both have been the subject of extensive research extending over a broad range of production areas across diverse environmental conditions worldwide [21]. Lastly, under current cultivation practices, wheat and rice may have adapted to different habitats [22], especially to distinct forms of inorganic nitrogen. Wheat is grown in aerobic soils, dominated by NO_3^-, whereas rice is grown usually under hypoxic conditions with a relatively high NH_4^+ presence in the root zone. Understanding how major food crops adapt to different forms of nitrogen should highlight nutrient management strategies to improve grain yield and quality. Three major components contribute to yield of small grain crops: number of tillers, number of grains per tiller (or grains per spike), and grain weight [23]. Number of grains per tiller may be further divided into number of panicles (or spikelets) and number of grains per panicle (or spikelet). Whereas tiller development can be influenced significantly by changes in the environment, grain characteristics are highly heritable [23,24]. Grain number and grain yield are positively correlated with crop nitrogen content [21]. Crops absorption of NO_3^- and NH_4^+ from soils and assimilation into organic forms reaches a peak at anthesis [25]. During grain production, plants remobilize stored organic nitrogen compounds and translocate them to seeds [26]. Nitrogen supply from before planting until anthesis is more strongly related to vegetative growth and yield potential, while nitrogen application post-anthesis is more strongly related to improved protein content and grain quality [25,27]. Photosynthesis, a process in which nitrogen-rich compounds play a major role, contributes biomass to fill in grain weight [21]. In other words, nitrogen is fundamental to all processes that determine final grain yield [11]. Therefore, optimizing nitrogen acquisition throughout crop development is crucial for attaining maximum yield potential.

Table 1. Average contribution of wheat and rice to daily protein intake between 2010 and 2019 [19].

Region		**Protein Supply in FAO's Food Balance Sheet (g per Capita per Day)**					
		Total Intake	**From Wheat**	**%**	**From Rice**	**%**	**% Wheat and Rice**
World		81.39	16.28	20.00	10.08	12.38	32.38
Africa	Eastern Africa	60.06	6.31	10.50	3.35	5.58	16.09
	Middle Africa	45.17	3.50	7.75	2.39	5.29	13.04
	Northern Africa	93.05	35.33	37.97	3.45	3.71	41.68
	Southern Africa	78.91	13.76	17.44	2.77	3.51	20.95
	Western Africa	63.46	5.02	7.92	7.87	12.40	20.32
America	Caribbean	67.31	9.40	13.97	9.37	13.93	27.89
	Central America	83.73	6.86	8.19	2.05	2.45	10.64
	Northern America	110.57	19.31	17.46	1.44	1.30	18.76
	South America	86.53	11.89	13.74	5.53	6.40	20.13
Asia	Central Asia	91.29	35.14	38.49	1.26	1.38	39.87
	Eastern Asia	98.35	17.29	17.58	14.89	15.14	32.72
	South-eastern Asia	69.05	4.82	6.99	23.52	34.07	41.05
	Southern Asia	62.71	16.48	26.29	13.09	20.88	47.17
	Western Asia	87.67	34.33	39.16	3.72	4.24	43.40
Europe	Eastern Europe	97.68	28.77	29.45	0.69	0.70	30.15
	Northern Europe	106.60	24.27	22.76	1.13	1.06	23.83
	Southern Europe	104.59	26.91	25.73	1.25	1.19	26.92
	Western Europe	105.43	23.54	22.33	0.83	0.79	23.12
Oceania	Australia/New Zealand	106.23	18.93	17.82	1.73	1.63	19.45
	Melanesia	65.03	8.40	12.92	3.74	5.75	18.67
	Micronesia	71.28	10.86	15.23	12.60	17.68	32.91
	Polynesia	92.76	14.25	15.36	4.17	4.50	19.86

3. Progress—Common Breeding Strategies Are Limited to Regulating Expression of Few Genes

Plant breeders achieve genetic gain in breeding populations over generations by selecting and retaining genetic materials with targeted characteristics and superior performance. A more thorough understanding of the molecular biology and genetic basis of specific traits facilitates the rapid development of more desirable genotypes, especially for traits that are controlled by a single or few loci with large effects. Yet, improving complex traits like yield and nitrogen responses remains challenging.

Will breeding for improved nitrogen uptake and assimilation also increase yield? While yield improvement can arise from factors affecting yield components besides increased nitrogen use efficiency (NUE), breeding for this trait should lead to increased biomass production and grain yield [28,29]. Nonetheless, breeding programs for yield seldom monitor nitrogen responses [30–33], and modern cultivars with higher yield demonstrate little improvement in NUE [34].

The genetic basis underlying desirable phenotypes for grain yield and quality often remain obscure, despite genetic gains through selection. For example, in Green Revolution varieties, the genetic variants and mechanisms responsible for the short stature and increased harvest index that underpin the yield boost were identified only several decades after the release of improved cultivars [35]. In rice, the recessive loss-of-function mutation of *Semi-Dwarf 1* (*SD1)* impairs an oxidase enzyme in the synthesis pathway of gibberellin, a key hormone promoting height, whereas in Green Revolution-derived varieties of wheat, mutations of *Reduced Height 1 (RHT-1)* encode modified proteins that also diminish height, but are insensitive to gibberellin-induced degradation [36–39]. Dwarfing genes improve yield through several mechanisms that act in concert to both diminish height and significantly increase biomass partitioning to the grain [36]. High harvest index, the fraction of biomass allocated to harvestable organs, is known to be strongly associated with high crop nitrogen status [40]. Unfortunately, many Green Revolution phenotypes, regardless of the mechanisms responsible for decreased gibberellin responses, also limit crop responses to nitrogen [41,42]. Plants with a dwarfing gene often have slower nitrogen uptake [42] and nitrogen accumulation relative to dry matter accumulation after anthesis, thereby decreasing NUE on a grain biomass basis in the field [41]. Insensitivity to increased nitrogen supply may be beneficial because the absence of nitrogen-promoted stem elongation makes plants more resistant to lodging [42], although at the high cost of requiring additional nitrogen fertilizer to maintain adequate yield. This case study from Green Revolution varieties underscores the challenge of improving yield through modifying nitrogen metabolism.

Generally, attempts to improve yield also alter rates or paths of metabolite production [43]. In particular, the enhanced harvest index of widely grown Green Revolution varieties diverts more biomass into harvestable grains. Nonetheless, assuming we have not reached the limits of biomass production, we may coordinate source vs. sink balance and continue to allocate additional crop assimilation of carbon and other nutrients toward yield [44]. Although efforts to increase crop source strength in terms of light capturing efficiency have been long underway [45], this goal seems elusive unless we address water and nutrient limitations [46–48]. Greater emphasis on enhancing nitrogen accumulation upon which biomass production depends may prove more effective in increasing yield in the near future [46,48]. With more extensive knowledge about the genetics of the underlying traits and advanced breeding tools, we could perhaps make even faster progress if we target both enhanced carbon and nitrogen assimilation concurrently.

An extensive body of literature is now available about the major transporters and enzymes associated with nitrogen assimilation and remobilization throughout crop growth cycles [4,13,30,49]. Characterized and cloned are key genes that govern metabolic pathways, but successful breeding applications for yield improvement that involve these genes are few, especially those that have reached the stage of commercial field trials [12].

Here, we have tabulated 40 genes that influence nitrogen metabolic pathways and improve grain yield (Table 2). Among these, regulation of gene expression seems to be the most successful approach for translating improved nitrogen metabolism into higher yields. Overexpression of genes [50] is the most common approach. Less common is knocking out [51] or silencing the genes of interest with small interfering RNA (RNAi) [52] or Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) [53] that precisely targets specific genomic regions. Relatively few studies have employed conventional breeding methods and, thus, have avoided transgenic means for introgression or incorporation of a functional allele into a breeding population. Progress in rice overall has been more rapid than in wheat [54]. Below are four different categories of genes involved in nitrogen metabolism that recent breeding efforts have manipulated to improve yield.

Table 2. Breeding applications of nitrogen metabolism genes in rice and wheat that were proven successful in improving yield.

						Yield Component Improvement					
#	Gene		Ref	Species	Breeding Application	Yield	Tillering	Grain weight	Grain number	Biomass NUE	Other/Note
1	*Nitrate transporter 1/Peptide transporter Family 6.1*	*NPF6.1*	[55]	Rice	Overexpression	✓	✓ *				* Effective panicle number. No data on grain number and weight.
2	*Nitrate transporter 1/Peptide transporter Family 6.3*	*NPF6.3 (NRT1.1A)*	[56]	Rice	Overexpression	✓				✓	Shortened maturation time
3	*Nitrate transporter 1/Peptide transporter Family 6.5*	*NPF6.5 (NRT1.1B)*	[57]	Rice	Near-isogenic line, Transgenic japonica with indica variant	✓	✓			✓	
4	*Nitrate transporter 1/Peptide transporter Family 7.1*	*NPF7.1*	[58]	Rice	Overexpression	✓	✓		✓	✓	
5	*Nitrate transporter 1/Peptide transporter Family 7.1*	*NPF7.2*	[59]	Rice	Overexpression	✓	✓				Increased root length, root number, root biomass
6	*Nitrate transporter 1/Peptide transporter Family 7.4*	*NPF7.4*	[58]	Rice	CRISPR/Cas9 mutant	✓	✓		✓	✓	
7	*Nitrate transporter 1/Peptide transporter Family 7.7*	*NPF7.7*	[60]	Rice	Overexpression	✓ *	✓		✓	✓	* Yield presented as g grain/g N. Larger panicle, Higher N content, but not amino acid suggests N accumulation
8	*Nitrate transporter 1/Peptide transporter Family 8.20*	*NPF8.20 (PTR9)*	[61]	Rice	Overexpression	✓	✓	✓	✓	✓	Highest improvement at low N
9	*High-affinity nitrate transporter 2.1*	*NRT2.1*	[62,63]	Rice	Overexpression	✓					Increased Mn accumulation
10	*High-affinity nitrate transporter 2.3b*	*NRT2.3b*	[64]	Rice	Overexpression	✓				✓	
11	*High-affinity nitrate transporter-activating protein 2.1*	*NAR2.1*	[63,65–67]	Rice	Overexpression, Transgenic with native promoter	✓				✓	
12	*Ammonium transporter 1;1*	*AMT1;1*	[68]	Rice	Overexpression	✓					
13	*Ammonium transporter 1;2*	*AMT1;2*	[69]	Rice	Double activation mutants with *GOGAT1*	✓				✓	
14	*Glutamate synthase 1*	*GOGAT1*	[69]	Rice	Double activation mutants with *AMT1;2*	✓				✓	
15	*Glutamine synthetase 1*	*GS1*	[70]	Rice	Overexpression	✓	✓		✓		
16	*Glutamine synthetase 2*	*GS2*	[70,71]	Wheat, Rice	Overexpression	✓		✓	✓		
17	*Nitrate reductase 2*	*NR2*	[72]	Rice	Transgenic japonica with indica variant	✓	✓			✓	
18	*Asparagine synthetase 1*	*ASN1*	[73]	Rice	Overexpression	✓ *				✓	* Yield increases only at low N
19	*Amino acid permease 1*	*AAP1*	[74]	Rice	Overexpression	✓	✓		✓		
20	*Amino acid permease 3*	*AAP3*	[75]	Rice	RNAi	✓	✓				
21	*Amino acid permease 4*	*AAP4*	[76]	Rice	Overexpression	✓	✓				
22	*Amino acid permease 5*	*AAP5*	[77]	Rice	RNAi	✓	✓				
23	*Nodule Inception-Like protein 1*	*NLP1*	[78]	Rice	Overexpression	✓				✓	
24	*Nodule Inception-Like protein 3*	*NLP3*	[79]	Rice	Overexpression	✓				✓	
25	*Nodule Inception-Like protein 4*	*NLP4*	[80,81]	Rice	Overexpression, Quadrupling the promoter of *NiR*	✓	✓				

Table 2. *Cont.*

#	Gene		Ref	Species	Breeding Application	Yield Component Improvement					Other/Note
						Yield	Tillering	Grain weight	Grain number	Biomass NUE	
26	*Growth-Regulating Factor 4*	*GRF4*	[42]	Rice, Wheat	Overexpression	✓	✓ *	✓	✓ *	✓	* Depending on genetic background; Tiller number not changed in *dep1*-driven dwarfism and in *NJ6-sd1*. Grain number not changed in *NJ6-sd1*
27	*Nitrogen-mediated tiller Growth Response 5*	*NGR5*	[82]	Rice	Overexpression	✓	✓	?	?	?	Did not show changes in yield components apart from tiller number for *NGR5* transgenic plants.
28	*Teosinte branched1, Cycloidea, Proliferating cell factor 19*	*TCP19*	[83]	Rice	Introgression	✓	✓		✓		
29	*NAM/ATAF1/2/CUC2 42*	*NAC42*	[55]	Rice	See *NPF6.1*						
30	*NAM/ATAF1/2/CUC2 2-5A*	*NAC2-5A*	[84]	Wheat	Overexpression	✓				✓	Increased root growth
31	*Basic Leucine Zipper 60*	*bZIP60*	[85]	Wheat	RNAi	✓			✓		Increased N uptake and NADH-GOGAT
32	*Grain Number, Plant Height, and Heading Date 7*	*Ghd7*	[86]	Rice	Overexpression	✓				✓	
33	Abnormal cytokinin response1 REpressor 1	ARE1	[87,88]	Rice, Wheat	Loss-of-function, CRISPR/Cas9 mutant	✓					Delayed senescence
34	*N-mediated heading date 1*	*Nhd1*	[89,90]	Rice	Knockout mutant	✓	✓			✓	Increased N uptake
35	*Dense and Erect Panicle 1*	*DEP1*	[91–93]	Rice	Loss-of-function, gain of function mutant	✓			✓		
36	*Dull Nitrogen Response 1*	*DNR1*	[94]	Rice	Loss-of-function mutant	✓			✓		Lower tiller number. Total biomass/grain weight not reported.
37	*Dehydration-Responsive Element-Binding Protein 1C*	*DREB1C*	[95]	Rice, Wheat	Overexpression	✓		✓	✓	✓	Early flowering, Higher photosynthesis, Higher N uptake, Higher harvest index
38	*Nuclear Factor Y A-B1*	*NFYA-B1*	[96]	Wheat	Overexpression	✓	✓				Higher N uptake, Higher grain N content, More lateral root growth
39	*Rice Dof Daily fluctuations 1*	*RDD1*	[97]	Rice	Overexpression	✓		✓	✓		Higher harvest index, Altered uptake of multiple nutrients, Early flowering
40	MicroRNA 396	MIR396	[98]	Rice	Knock-out mutant	✓		✓	✓	✓	15% yield increases under low N, Larger panicle

3.1. Nitrogen Transporters (17 Genes)

Modern phylogenetic studies classify major families of nitrogen transporters in land plants based on their substrate: nitrate, ammonium, or peptides [99]. Most characterizations of transporters are in *Arabidopsis* and rice [100]; relatively limited information is available for transporters in wheat [30,101,102]. Nitrate transporters (11 genes) have received more attention and their potential for yield improvement have been evaluated more thoroughly than transporters of ammonium and organic nitrogen [13,33]. Perhaps this derives from NH_4^+ being a nitrogen source that only dominates in a few agricultural production systems and from the ability of its counterpart NH3 to move freely through membranes following electrochemical gradients [1]. Ammonium transporters (2 genes) may also prove to be more elusive as a target for yield improvement because of the potential for toxicity from excessive accumulation of free NH_4^+ in tissues as discussed below [33,103]. Nonetheless, coupling NH_4^+ uptake with assimilation by concurrent modification of *AMT1;2* and *GOGAT* can drive yield improvement [69].

Modification of amino acid transporters (*AAP*, 4 genes), despite receiving less attention than that of NO_3^- transporters [12], is another effective strategy for increasing grain yield. These transporters, in contrast to those that transport NO_3^- or NH_4^+, are key players in remobilizing assimilated organic nitrogen compounds, although their exact functions remain largely unknown in cereals [104]. Organic nitrogen transport within plants directly relates to grain nutritional quality at maturity [26]. Of particular interest is that variation in the promoter regions across germplasm suggest tight expression regulation and local adaptation that may help plants cope with fluctuations in soil nitrogen gradients [75–77].

Overall, while we have some understanding of how transporters contribute to uptake and transport of each nitrogen form across membranes and might improve plant nitrogen acquisition, modification of these transporters has had limited success in crop yield enhancement [33].

3.2. Nitrogen Assimilatory Enzymes (5 Genes)

Assimilation of inorganic into organic nitrogen in plants is well-regulated at transcriptional, translational, and post-translational levels [105,106]. The enzymes GS and GOGAT are central to nitrogen metabolism, but attempts to alter yield by modifying genes coding for these enzymes have achieved only little success. Previous modification to *GS1* increased nitrogen partitioning to grain and nitrogen harvest index, but not vegetative yield nor overall shoot nitrogen accumulation [107]. Failure to successfully modify *GS1* on its own may derive from the critical functions for which this gene is responsible [108]. By contrast, modifying *GS2* can lead to wheat yield improvement in stressful environments [71]. Alteration to *GOGAT* expression to boost yield was only achieved through changing expression levels of transcription factors upstream of the enzyme (see discussion below). Thus, successful breeding applications coupled *GOGAT* with changes to ammonium transporter AMT1;2 [69] or simultaneously modulated *GS1* and *GS2* [70]. Because *GS1* and *GS2* are involved in crop growth at different developmental stages [109,110], selecting the appropriate developmental time to express each of these enzymes was critical for a positive result [70].

3.3. Nodule INception-like Proteins That Sense NO_3^- and Regulate Downstream Genes (3 Genes)

Legumes when associated with certain bacteria can generate organic nitrogen from dinitrogen gas N_2 in air, a process named symbiotic nitrogen fixation [111]. *Nodule INception (NIN)* genes govern legume root nodule initiation and symbiotic nitrogen fixation [112]. *NIN-like proteins (NLPs)* that are homologs to *NINs* found in non-leguminous crops have critical roles in regulating nitrogen signaling and downstream genes within nitrogen metabolism [113].

Multiple highly conserved *NLPs* are found in *Arabidopsis* [112], wheat [114], and rice [112]. *Arabidopsis NLPs* function as transcription factors, and *NLP7* also serves as a biosensor responsive to intracellular NO_3^- supply [115]. Rice *NLPs* generally serve as activators that control expression of nitrogen responsive genes. For example, *NLP4* regulates expression of genes underlying key enzymes in nitrogen assimilation pathways [80], thereby affecting activities of *NiR* [81] and *NR* [116]. Some *NLPs* also shows nitrogen form-specific responses with NO_3^- being the major form to which rice *NLPs* are most responsive. While either NO_3^- or NH_4^+ can trigger expression of *NLP3*, only NO_3^- induces its nuclear retention [79]. Overexpression of these *NLPs* in rice stimulate yield, whereas reduced expression of *NLPs* inhibits growth. To date, yield improvements of wheat from modifications of *NLPs* are lacking, although nitrogen starvation upregulates *NLP7* [114] and *NLP4* [117].

3.4. Transcriptional Factors and microRNA That Regulate Other Genes (15 Genes)

Transcriptional factors bind to the promoter of target genes to regulate downstream gene functions [118]. System biology is steadily clarifying how a large network of transcription factors regulate nitrogen metabolism and how key transcription factors control expression of multiple proteins in the pathways simultaneously [119]. Whereas modifying expression of individual transporters and enzymes has had only modest success in improving crop performance, altering transcription factors that orchestrate simultaneously systematic changes in multiple nitrogen-related genes may have profound effects on biomass accumulation and grain quality. For example, overexpressing rice *DREB1C*, which regulates nitrogen assimilation genes, increased nitrogen assimilation and photosynthesis significantly, resulting in increased grain number, grain weight, and harvest index [95].

Together these changes resulted in 68.3% higher yield than wildtypes and in a 13 to 19 days earlier flowering time [95]. Light- and nitrogen-mediated *OsDREB1C* controlled over 9000 genome-wide putative binding sites, including five gene targets in the carbon and nitrogen metabolism pathways: *rubisco small subunit 3 (OsRBCS3)*, *OsNR2*, *nitrate transporter 2.4 (OsNRT2.4)*, *OsNRT1.1B*, and *flowering locus T-like 1 (OsFTL1).* Previous attempts to engineer several individual genes from this list never reached as high a yield gain as manipulating the transcriptional factor gene *OsDREB1C* alone. For instance, overexpression of transporters led to higher accumulation and efflux of excessive nitrogen because plants were not able to assimilate more nitrogen into protein [120].

Manually coordinating individual genes underlying nitrogen sources and sinks to complete a whole pathway therefore remains a challenge. Although we still have limited understanding about the regulation and function of transcription factors, modifying a single transcription factor appears more effective than manipulating individual genes and proteins in a pathway [121]. This highlights the complexity and tight regulation of nitrogen metabolism. As more genotypic and phenotypic data become available across diverse plant species, the roles of transcriptional factors in nitrogen metabolism should become clearer. Editing genetic networks, rather than individual candidate genes that regulate the balance between carbon and nitrogen metabolism may prove to be a more promising approach for increasing yield.

4. Prospects—Fine-Tuning Yield Component Responses to Transient Nitrogen Supply Can Maximize Yield

Successful manipulation of genes regulating nitrogen metabolism (Table 2) is contingent upon more advanced understanding of how nitrogen acquisition influences growth and vice versa. We now have a better understanding on how nitrogen, especially NO_3^-, drives hormonal and physiological changes underlying canopy architecture and development [122]. However, the influence nitrogen has on yield components and the tradeoffs among subcomponents are still uncertain. In this next section, we summarize recent findings, focusing on tiller number, flowering time, and NH_4^+ assimilation as key links between carbon and nitrogen metabolism and, therefore, highly relevant to nitrogen-driven yield improvement.

4.1. Tiller Production Contributes to Higher Yields through Multiple Nitrogen-Mediated Signaling Pathways

Tiller number, a key determent of effective number of panicles that contribute to grain filling and grain yield, is the most responsive of all yield components to nitrogen [122]. Tiller number is a routinely measured yield component because its assessment is straightforward. Out of the 40 genes reported to improve yield in this review (Table 2), 19 genes are associated with higher tiller number.

Increasing nitrogen supply generally increases tiller production [82] whereas limiting supply decreases tiller production [122]. Soil NH_4^+ concentration correlates linearly with tiller number in rice [123] and nitrogen fertilization levels explain 66% to 96% of the variation in tillering rate, which is significantly correlated with the final grain yield [23]. Similarly, increased nitrogen levels also boost tiller production in wheat [124]. Changing canopy architecture by optimizing nitrogen inputs and increasing tiller number per unit area thus enhances biomass source strength and grain yield in rice [125], and both yield and grain protein content in wheat [126].

Changes in tillering number derive from the interplay between multiple opposing nitrogen-mediated hormonal shifts [122]. High nitrogen availability induces cytokinins to increase tillering, but also induces auxins and strigolactones to inhibit tillering [127]. In rice, multiple amino acid transporters balance the opposite actions of auxins and cytokinins: *OsAAP1* and *OsAAP4* regulate auxin and cytokinin signaling [74,76], whereas *OsAAP5* only influences cellular cytokinin levels [77]. *microRNA393* (*OsmiR393*), in turn, lowers sensitivity to auxin signaling and increases tillering [128].

Feedback mechanisms between hormones and nitrogen ensure optimized developmental responses to fluctuating external nitrogen pools. As nitrogen supply increased, a negative feedback mechanism driven by *DNR1* reduced auxin functions to upregulate genes for tiller production and nitrogen metabolism, thereby repressing nitrogen uptake and assimilation as well as tiller production [94]. Conversely, a nitrogen shortage downregulated *DNR1*, promoting nitrogen acquisition and tiller development [94].

The complex balancing acts of gibberellin, which explain why Green Revolution plant varieties maintain lower height and high yield, but require high nitrogen fertilizer inputs, have been reviewed in great detail elsewhere [106]. In brief, gibberellin and its counterparts DELLA proteins, which are named after their conserved chain of amino acids D-E-L-L-A, have two fates. Under high nitrogen availability, gibberellin can either inhibit tiller development by degrading gibberellin's downstream transcriptional factor *NGR5* protein or promote tiller production via a positive feedback mechanism driven by nitrogen itself to increase nitrogen assimilation and upregulate *NGR5,* which represses tiller inhibitory genes. Likewise, DELLAs may sustain tiller promotion by interfering with gibberellin-driven NGR5 destruction [82] or decrease nitrogen accumulation by downregulating nitrogen assimilation genes [42], thereby indirectly limiting nitrogen-driven tiller development. Because most Green Revolution-derived high-yielding cultivars already contain dwarfing genes conferring high DELLA abundance, breeders can further increase tiller production and yield even at low nitrogen levels by increasing NGR5 abundance directly, suggesting a potential decoupling of tillering from nitrogen supply [82].

Modification of transcriptional factors further enhances yield by tipping the balance of proteins and promoting nitrogen-driven tiller production. The coordination for carbon and nitrogen is systematically regulated by the transcription factor *GRF4* [82] and its upstream repressor MIR396 [94], both of which modulate nitrogen acquisition and growth via *DNR1* [94] and modulate nitrogen assimilation genes to counterbalance the inhibitory effects of DELLA [42]. Therefore, increased *GRF4* expression alters the balance of GRF4-DELLA, thus enhancing nitrogen assimilation, tiller development, and grain yield [42].

Nitrogen influence on tiller development via the brassinosteroid signaling pathway also remains an active area of research. High NO_3^- levels decrease rice expression of *TCP19*, which represses *Dwarf and Low-Tillering (DLT)*, a gene involved in brassinosteroid signaling and tillering promotion, thereby inhibiting tiller bud outgrowth [83]. *OsTCP19* overexpression lines exhibit brassinosteroid-deficient phenotypes similar to *dlt* mutants [83]. In wheat, overexpression of *Dwarf4 (DWF4)*, which encodes a key enzyme in brassinosteroid synthesis, also increases both nitrogen assimilation and tiller number [129]. Furthermore, the proteins of rice *DLT* and *MONOCULM1 (MOC1),* which regulate tiller production, are both under control of *NGR5* [82].

Interestingly, there are tradeoffs among yield subcomponents. For example, not all yield improvement is associated with increased reproductive tiller number. In fact, fewer tillers is a key characteristic proposed as an ideal canopy architecture for high yields [130]. Mutants with loss-of-function *dnr1* or reduced DNR1 abundance develop fewer tillers, but increase auxin, accelerate nitrogen uptake, and exhibit higher yields [94]. In the case of *DREB1C* overexpression, transgenic rice plants with higher yields have fewer panicle numbers, but instead produce elongated panicles with increased grain weight and number of grains within each panicle [95]. Such coordination between source and sink components appear to shift if carbohydrate supplies increase because these transgenic plants also have higher photosynthetic rates and accumulate more biomass at heading stage. Additionally, reduced branching may also result from a shortened development period to be discussed in the next section. Altogether, regulations of nitrogen-mediated tiller development highlight the importance of evaluating all yield components that contribute to actual yield changes.

4.2. *Optimized Flowering Time Maximizes Nitrogen and Carbon Assimilation in Agricultural Settings*

Adjustments of flowering time or heading date is an evolutionary adaptation that maximizes seed yield and survivability over generations [131]. Flowering time optimized for each environment can enhance grain yield in staple food crops [132]. The transition from vegetative to reproductive developmental stages determines total nitrogen accumulation over the vegetative growth period [25,32] and shifts the emphasis of nitrogen metabolism to remobilization and reassimilation in maturing grains [26]. While photosynthesis per unit leaf area may remain unchanged, cumulative increases in leaf area, light interception, overall growth period, and vegetative biomass accumulation—all responsive to nitrogen inputs—may together increase yields [133]. Suboptimal or excess nitrogen supply often, respectively, accelerate or slow the transition to reproductive phase [134]. The precise extent to which nitrogen supply influences cereal flowering time, however, is uncertain [135].

The genetics underlying vernalization and photoperiod pathways in cereals are well-characterized [136], but their interactions with nitrogen remain an open question. Multiple genes regulate flowering time and its influence on grain yield [132]. Indeed, genes underlying developmental timing like *Photoperiod* (*Ppd*) and *Vernalization* (*Vrn*) appear to co-locate with Quantitative Trait Loci (QTL) associated with NUE [137], suggesting a potential connection with nitrogen metabolism. Several recent studies have identified genes with pleiotropic effects that change both nitrogen responsiveness and crop developmental timing via senescence and flowering time. These include *NPF6.3*, *GS2*, *Nhd1*, *Ghd7*, *ARE1*, *miR396* (Table 2). Specifically, transcription factors *Nhd1*, *Ghd7* and *DREB1C* have direct control on genes involved in determining heading date [86,90,95]. Connections of other candidate genes with developmental timing require further validation.

An appropriate flower timing is essential for avoiding stressful conditions and maximizing favorable conditions for seed production [131]. Most genes in Table 2 promote a longer growing season. Prolonged vegetative growth generally allows crops to accumulate and assimilate more nitrogen before a crop reaches maturity and senescence, potentially resulting in increased NUE and biomass accumulation. However, a longer growth season may also increase the chance of experiencing abiotic and biotic stresses [131]. Only *NPF6.3*, *DREB1C*, and *RDD1* accelerate a transition to the reproductive stage and still show a yield improvement [56,95,97]. For example, *OsDREB1C* significantly enhanced yield, despite a 2–3 weeks shorter growth period [95]. The ability to accumulate higher biomass under a shorter timeframe indicates a higher capacity for carbon and nitrogen assimilation. Nevertheless, early flowering time in rice with photo-insensitive alleles was previously shown to be associated with reduced grain filling, fewer panicles, and subsequently lower yield [138].

Developmental changes driven by variations in the growth environment determine the extent to which yields can be improved. Varying outcomes from different modifications may derive from the environmental interactions underlying nitrogen influence on growth and development. Late season tiller production may not produce a fertile fluorescence and thus contributes only to vegetative biomass production [35]. Tillers initiated early in the season also tend to have higher yields than late tillers [139]. High tiller production combined with longer maturation time generally contributes to higher rice yield [140]. For *OsDREB1C* modifications, overexpressing plants grown under long days and temperate climates flower about 50 to 70 days later and have higher yield improvement rates than plants under other experimental conditions [95]. Photoperiod pathways seem to be likely candidates that connect nitrogen responsiveness with flowering time, although no known mechanisms have been confirmed to date [134]. Understanding how carbon and nitrogen assimilation intersect and their environmental interaction in the context of crop developmental timing will be crucial in matching crop demands with resource supplies.

4.3. GOGAT as an Indirect Target—A Case Study from Editing Transcriptional Factors ARE1, Nhd1, and bZIP60

GOGAT, when coupled with GS, catalyze the assimilation of NH_4^+ into glutamate, an amino acid central to nitrogen and carbon metabolism [141]. Based on genetic map synteny, a meta-analysis of cereal QTL studies on NUE identified *GOGAT* as a candidate gene that is conserved among major food grain crops (rice, wheat, sorghum, maize) [137]. Although editing *GOGAT* directly has little influence on yield, coupling *GOGAT* with *AMT1;2* proved effective in enhancing yield [69]. Modifying transcription factors upstream of *GOGAT* also has been successful (Table 2): *ARE1* and *Nhd1* both suppress *Fd-GOGAT* [87,89,90], while *bZIP60* suppresses *NADH-GOGAT* [85].

Eliminating suppression of *GOGAT* via these transcription factors, improves yields significantly. Enhancement of *GOGAT* function seems to be a plausible approach for raising yield because glutamate links carbon and nitrogen metabolism [141]. The role of transcriptional factors suggests that we have yet to characterize additional players involved in the assimilation of NH_4^+ into organic nitrogen. Identification and modification of other pathways similar to *GS/GOGAT* -driven NH_4^+ assimilation, in that they influence both nitrogen acquisition and remobilization, may prove most effective for improving yields.

5. Puzzles—Knowledge Gaps about Modifying Nitrogen Metabolism for Yield Improvement

The current body of literature proffers open questions that require further investigation. In particular, studies that compare homeologs across species, crop responses to different inorganic nitrogen forms, and quantitative genetics underlying crop adaptation to natural soil nitrogen gradients should accelerate yield improvement through modified nitrogen metabolism.

5.1. Differences among Homologs across Species Remain Elusive

Comparative studies among species offer unique insights into finding related genes underlying desirable traits [142] such as for genes involved in C_4 carbon fixation [95]. Nevertheless, transfer of successful breeding strategies across species remains challenging, even decades after fully characterizing most elements in nitrogen metabolism pathways [143].

To date, studies have identified more candidate genes and generated more breeding applications related to NUE in rice than in wheat [22,54] (Table 2). Translating insights from rice to wheat require herculean efforts, largely because of differences in genomic size and structure [22,144,145]. New mutant resources [146] and transgenic tools [147], however, increase the feasibility of characterizing candidate genes across a polyploid genome. Novel approaches like CRISPR-Cas9 system also further allows more precise editing of targeted loci of interest [148]. Even cross-species gene modifications such as transforming rice with wheat *TaGS1* have proved successful in enhancing rice yield [149].

Multiple yield-determining genes are shared among rice, wheat, maize, and barley [22,150]. Identification of orthologous genes offers an alternative to introducing foreign genetic materials. Here, we discuss three examples: *GOGAT*, *DREB1C* and *Ghd1*. First, *GOGAT* is well-conserved in rice, wheat, sorghum and maize [137]. Editing transcription factors regulating *GOGAT*, however, seems more effective than modifying individual genes on their own (see discussion above). Second, rice *OsDREB1C*, whose overexpression increased yields up to 68.3%, has an ortholog in wheat *TaDREB1C*, whose overexpression results in 22.6% more grain yield than wildtypes [95]; the reason for the large differences in yield enhancement among species is not yet understood. Third, *Ghd7* in rice and its ortholog *VRN2* in wheat [86] have a high potential to improve agricultural performance. Both genes are well-studied and control flowering time in their respective species [151–153]. To date, however, there is no clear evidence on how *VRN2* integrates signaling from nitrogen into regulation of flowering time in wheat. Given the promising yield enhancements attained with *Ghd7* in rice, *VRN2* might also provide major increases in wheat yields, but this is still unknown.

5.2. Insights on How Inorganic Nitrogen Forms Affect Crop Responses Are Lacking

Each form of inorganic nitrogen, NH_4^+ or NO_3^-, triggers specific crop responses [154]. In particular, an exposure to high concentration of soil NH_4^+ is generally toxic to most plants because root absorption of NH_4^+ may exceed the capacity of the plants to sequester the NH_4^+ in vacuoles or assimilate it into organic forms [103]. As free NH_4^+ accumulates within plant tissues, it can dissipate pH gradients through which mitochondrial and chloroplastic electron transport generate ATP [1]. To avoid such ill effects, plants generally assimilate NH_4^+ in roots and transport organic nitrogen compounds to other organs [103]. Optimizing root NH_4^+ accumulation and assimilation can enhance plant NH_4^+ tolerance and overall nitrogen acquisition [155]. By contrast, plants can store relatively large amounts of free NO_3^- without ill effect [1], and it serves as major signaling molecules for a number of metabolic pathways [156].

Although the importance of each inorganic form as a nitrogen source in crop production is well established [157], information is still meager on how each form induces or suppresses expression of nitrogen responsive genes or how changes in these genes in turn affect uptake and assimilation of each form. For example, NO_3^- transporter genes have a strong influence on NH_4^+ metabolism, and vice versa [158]. A more comprehensive understanding of these interactions would be crucial to designing and implementing more effective nitrogen fertilizer management strategies.

Relatively few studies compare responses to both form of inorganic nitrogen side by side, let alone evaluate the responses to a range of concentrations in diverse genetic materials. Although the model species *Arabidopsis* usually exhibited higher biomass and root production under NO_3^- nutrition, this species showed a wide range of distinct phenotypic responses and gene expression pattern when receiving NO_3^- or NH_4^+ as a sole nitrogen source [159]. Wheat growth under either form also demonstrated distinct accumulation and distribution patterns of other essential nutrients [160]. Nonetheless, we do not have sufficient information about the extent to which editing major genes in the nitrogen metabolism pathways changes responses to each inorganic form, and whether responses in wheat and rice are like those observed in *Arabidopsis*.

Most experiments to characterize genes reported in this review have only focused on a single nitrogen form or fail to designate the nitrogen form at all (Table 3). Detailed characterizations of individual nitrogen transporters may show that, not only are they responsible for uptake of both NO_3^- and NH_4^+ (for example, *NRT 2.3b* [64]), but also their functions have expanded and co-evolved to interact with other biotic and abiotic factors [161,162]. For example, *NPF6.5* not only regulates NO_3^- uptake, but is also associated with recruitment of root microbes involved in the synthesis of NH_4^+ [158]. Understanding balance in crop utilization of both inorganic nitrogen forms will help us improve our crop and fertilization management in response to changing environments [163].

Table 3. Functions, selection, and effects on nitrogen acquisition of nitrogen metabolism genes in rice and wheat that were proven successful in improving yield. Empty cells indicate the lack of information.

#	Gene	Ref	Function	Natural Variation and Selection	Effects on NH_4^+	Effects on NO_3^-
1	*NPF6.1*	[55]	NO_3^- uptake; Must be activated by *NAC42* transcriptional factor	Rare allele absent in 90.3% of rice varieties		Increased uptake/concentration
2	*NPF6.3 (NRT1.1A)*	[56]	Upregulate N utilization and flowering genes			
3	*NPF6.5 (NRT1.1B)*	[57,158]	NO_3^- uptake, transporter; Upregulate NO_3^- responsive genes	Directional positive selection. Indica has a functional variant.		
4	*NPF7.1*	[58]	Determine axillary bud outgrowth; NO_3^- uptake			Increased influx/concentration
5	*NPF7.2*	[59]	Upregulate genes in cytokinin pathway, thereby increasing cytokinin concentration; Downregulate genes in strigolactone biosynthesis, perception and signaling pathway, thereby reducing suppression on tillering.			Increased influx/concentration
6	*NPF7.4*	[58]	Determine axillary bud outgrowth, NO_3^- uptake			Increased influx/concentration
7	*NPF7.7*	[60]	Two splicing variants transport distinct N forms; Upregulate *GS1.2*, *NPF6.5*; Downregulate *Fine Culm1 (FC1)*, *Dwarf3 (D3)* to regulate tillering.		Increased influx/concentration for both variant, Higher for variant 2	Increased influx/concentration for variant 1 only
8	*NPF8.20 (PTR9)*	[61,164]	Upregulate *GS*, *AMT1;2*; Increase lateral root density		Increased uptake	
9	*NRT2.1*	[62,63]	High affinity NO_3^- transporter; Responsive only to NO_3^-; Interact with *NAR2.1*.			
10	*NRT2.3b*	[64,165]	Buffering pH; NO_3^- uptake; Increase NH_4^+ uptake even though it does not transport NH_4^+	Under selection. Expression ratio of two variants correlated with vegetative N content.		Increased uptake
11	*NAR2.1*	[63,65–67,165,166]	NO_3^- uptake, interacting with *NRT2.1, NRT2.2, NRT2.3a*			Increased uptake
12	*AMT1;1*	[68,167,168]	NH_4^+ uptake under low and high NH_4^+ conditions; N/K homeostasis		Increased uptake	
13	*AMT1;2*	[69,167]	NH_4^+ uptake and remobilization			
14	*GOGAT1*	[69]	NH_4^+ uptake and remobilization			
15	*GS1*	[70,169]	Coordinate N metabolic balance and remobilization; Confer tolerance to abiotic stresses; Must be expressed concurrently with *GS2*.			
16	*GS2*	[70,71]	Increase root N uptake before and after flowering, N mobilization and N harvest index; Prolong leaf photosynthesis post-anthesis; Increase expression of *NRT2.1* and *NPF 6.3*; In rice, must be expressed concurrently with GS1.	2 haplotypes in A genome		
17	*NR2*	[72]	Encode *NADH/NADPH-dependent NO_3^- reductase*; Interact with *NPF6.5* to control NO_3^- uptake	Diverged between indica and japonica.		Increased uptake
18	*ASN1*	[73]	Upregulate *AMT1;1, AMT1;2, AMT1;3, GS1;1, NADH-GOGAT1*		Increased uptake	
19	*AAP1*	[74,170]	Facilitate amino acid transportation to reproductive organs			
20	*AAP3*	[75]	Reduced expression promotes tiller bud elongation, relatively more than formation, via balancing basic and neutral amino acid to maintain higher cytokinin	25 haplotypes. Promoter sequence differs between indica and japonica		
21	*AAP4*	[76]	Higher expression in indica produce more tiller and grain yield	5 haplotypes. Promoter sequence differs between indica and japonica		
22	*AAP5*	[77]	Reduced expression regulate tiller bud via balancing basic and neutral amino acid to maintain higher cytokinin	11 promoter variants. Sequence differs between indica and japonica		

Table 3. *Cont.*

#	Gene	Ref	Function	Natural Variation and Selection	Effects on NH_4^+	Effects on NO_3^-
23	*NLP1*	[78]	Regulate transcription of N related genes and transcriptional factors (both NO_3^- and NH_4^+)			
24	*NLP3*	[79]	Bind to NO_3^--responsive cis-elements in promoters of N uptake and assimilation genes; Overlaps with *NLP1* and *NLP4*			
25	*NLP4*	[80,81]	Regulate expression of known N genes by binding to NO_3^-- responsive cis-element in promoter, Activate *NiR*	2 haplotypes		
26	*GRF4*	[42]	Counteracts DELLA to promote N assimilation both NO_3^- and NH_4^+; Upregulate expression of *AMT1.1, GS1.2, GS2, NADH-GOGAT2, NRT1.1B, NRT2.3a, NPF2.4, NIA1, NIA3, NiR1* and genes related to photosynthesis, C metabolism and cell division to maintain stable C:N ratio; Highest expression at low N;	3 haplotypes. Haplotype B has highest yield. Absent from elite varieties.	Increased uptake	Increased uptake
27	*NGR5*	[82]	Recruit PRC2 upon increased N supply to promote H3K27me3 modification that represses shoot branching inhibitory genes; DELLA proteins stabilize *NGR5* and sustain tiller promotion by competitively inhibiting gibberillin-driven destruction of *NGR5*.	5 haplotypes. Haplotype 2 contains a functional variant.		
28	*TCP19*	[83]	Represses tiller promoting gene *DLT*, the product of which can interact directly with *NGR5*, to negatively control cellular bud growth			
29	*NAC42*	[55]	Activate *NPF6.1*			
30	*NAC2-5A*	[84]	Regulate expression of NO_3^- transporter and *GS*			Increased uptake
31	*bZIP60*	[85]	Negative regulation on *NADH-GOGAT*			
32	*Ghd7*	[86,171]	Repress *ARE1* to positively regulate N utilization	At least 10 alleic variants. Alleic frequency correlates with N deposition rate.		
33	*ARE1*	[87,88]	Suppress *Fd-GOGAT*	3 haplotypes in promoter sequence. Under selection.		
34	*Nhd1*	[89,90]	Activate Hd3a for flowering time; Control negative feedback on N assimilation (loss-of-function increases Fd-GOGAT and LHT1 activities); Activate *AMT1;3, NRT2.4*	5 haplotypes. Similar between indica and japonica. Variation in promoter associated with nitrogen.		
35	*DEP1*	[91–93,172]	Interact to reduce heterotrimeric G-protein α-subunit 1 (*RGA1*) or enhance β-subunit 1 (*RGB1*) to inhibit N responses; Promote aerenchyma formation; Upregulate *GS/GOGAT*	Under selection during japonica domestication.		
36	*DNR1*	[94]	Negative regulator of auxin-regulated N metabolism; N supply lowers *DNR1*, thereby inducing Auxin Response Factors to upregulate *NPF6.5, NRT2.3a, NPF2.4,* and *NIA2*.	3 haplotypes. Haplotype A specific to indica is absent from japonica.		Increased uptake
37	*DREB1C*	[95]	Regulate NT2, NRT2.4, NPF6.5	3 haplotypes in promoter sequence. Haplotype 3 superior.		Increased uptake
38	*NFYA-B1*	[96]	Control root development and N, P usage			Increased uptake
39	*RDD1*	[97]	Upregulate *AMT1;3, GS1;1*; Uptake of N, P, K, Na, Mg, Cl, S, Ca	Highly conserved in wild rice relatives.	Increased uptake/accumulation	Increased uptake
40	*MIR396*	[98]	Only isoform e and f; Upregulate GRF4, GRF6, GRF8, NIR1, NIR2, GOGAT2, GS1.2, AAPs			

Nitrogen interacts more strongly with carbon assimilation as nitrogen supplies limit crop responses to enriched atmospheric CO_2 levels [173]. Meta-analyses demonstrate that nutritional quality of wheat and rice—especially protein and micronutrients such as iron and zinc—decline significantly under elevated CO_2 levels [174]. Among several alternative explanations for the declining crop protein at elevated CO_2 levels [175–178], direct inhibition of shoot nitrogen assimilation [179] is most consistent with observations under a wide range of experimental conditions [180–182].

Photorespiration provides energy for shoot NO_3^- assimilation in C_3 plants [3]. Photorespiration generates reductants when atmospheric CO_2, but not light levels, limits photosynthesis and enables C_3 plants to convert low energy nitrogen sources that most other organisms avoid like NO_3^- into organic nitrogen compounds. This confers an evolutionary advantage to C_3 plants, which remain dominant among plant species [3]. Under the current rapid surge in atmospheric CO_2 level, a condition which slows photorespiration, C_3 species using NO_3^- as a nitrogen source suffer most from decreased organic nitrogen production [179,183]. N2-fixing legumes and C4 plants with CO_2 concentrating mechanisms, are more resilient to changes in CO_2 [174] because their inorganic nitrogen acquisition does not depend on photorespiration.

The use of NH_4^+-based nitrogen fertilizer and breeding for genotypes with improved NH_4^+ assimilation and tolerance may offer a solution for sustaining plant protein levels under future CO_2-enriched atmospheres [184,185]. Biological Nitrification Inhibitors (BNI), which allow certain plant species to regulate their rhizosphere pools of inorganic nitrogen by releasing root exudates that specifically inhibit nitrifying bacteria that convert NH_4^+ into NO_3^-, may be beneficial [186]. Application of artificial BNI chemicals or incorporation of this trait into new cultivars may enhance crop growth under NH_4^+ nutrition [185,187].

Surprisingly, given the chemical differences between NO_3^- and NH_4^+, relatively little is known about how various nitrogen supplies shape crop adaptation and yield in a field setting (Table 3; see discussion below). The ability of the current germplasm to employ a specific nitrogen form as their predominant nitrogen source and maintain productivity at elevated CO_2 levels thus remains an open question.

5.3. Little Information about Natural Genetic Variation and Genome-Wide Interactions Limits Breeding Applications

Despite an increasing understanding of physiological adaptation of roots and shoot to nitrogen supply [122], less is known about genetic adaptations [188]. Recent advances in genetic approaches greatly facilitate the identification of genes responsible for specific physiological traits. Of particular interest are Genome Wide Association Studies (GWAS) that use extensive sets of molecular markers to explore genetic variation resulting from historical recombinant events and from adaptation to changes in environmental conditions over evolutionary time [189]. Genetic architecture of traits also strongly influences GWAS robustness such that traits with rare alleles are more difficult to identify [190,191].

To date, a combination of GWAS and linkage mapping have identified many loci that underlie nitrogen responses of agricultural crops [192,193]. Importantly, GWAS enables deeper understanding of how environments may have shaped crop adaptation [194]. As such, natural variation of functional alleles can help inform breeding applications to achieve a better match between genotype and location [195]. Haplotype analyses in global germplasm quantifies allelic frequency of different breeding and natural subpopulations [196] and can offer practical strategies in breeding programs [197].

Selection pressures that vary during the history of crop domestication or with local limiting growth factors [198] provide insights into crop evolution and adaptation. More commonly, studies focus on differences between major subpopulations and how selection drives divergence or convergence between them. For example, divergence between the indica and japonica subpopulations of rice can be accounted by variations in key nitrogen metabolism genes like *NPF6.5* [57] or *NR2* [72]. Around 8% of the rice genome covering major nitrogen metabolism genes appear to be under selection including *AMT1.1*, *NRT2.3*, *NAR2.2*, *NIR1*, *GS1;2*, and *GS1;3* [199]. Unfortunately, information is limited about the natural variation in candidate genes that enhance yield and the extent to which they have been under selection (Table 3). For instance, *OsNPF6.1*, which was identified through GWAS and functions under low nitrogen supply to increase NO_3^- uptake, is considered a rare allele, because it is present in less than 10% of cultivated varieties [55]. The absence or presence of a functional allele from diverse geographic regions may reflect adaptation to a particular soil nitrogen pool.

Apart from a few studies [83,86], we have limited information on the extent to which natural soil nitrogen availability shapes crop adaptation and, in turn, on subsequent responses to external nitrogen fertilizers in agricultural production systems. For example, *Ghd7* allelic variation also correlates with soil nitrogen deposition rates [86]. Likewise, rice *OsTCP19*, which was identified through a GWAS on tiller responsiveness to nitrogen availability, has a functional allele frequency that is correlated with soil nitrogen concentration, and the nitrogen-responsive genotypes are more common in regions with low nitrogen concentrations [83]. Extensive networks of genes interact to sense and signal perception of nitrogen, especially NO_3^- [106]. Interestingly, expression of *OsTCP19* follows changes in NO_3^-, but not NH_4^+ [83]. Overall, evidence is insufficient to conclude whether crops like wheat and rice, which have been exposed over the long term to certain nitrogen forms, show adaptation to a particular form. This information is vital for applying robust breeding strategies to improve future crops.

Genetic × environment interactions and expression patterns contingent upon growth conditions influence phenotypic plasticity [200,201], even when the same genes are being modified. Specifically, some genes may only be beneficial in certain environments or may even have detrimental pleiotropic effects in others. Field trials indicate that yield enhancement is highly dependent on growing conditions. For example, overexpression of *ASN1* enhanced rice grain yield in pot experiments under limited nitrogen supply, but had no observable effect under sufficient nitrogen supply in the field [73]. In sites with a longer growing season, *DREB1C* transgenic plants exhibited a much higher yield boost compared to wildtypes [95]. With more advanced molecular breeding and transgenic approaches, promoters inducible in specific tissues or by desirable environmental triggers could perhaps mitigate such issues [12,121]. Precision genome editing methods, like the CRISPR-Cas9 system, facilitate genetic modifications at multiple target tissues, developmental times, and traits all at once without the introduction of foreign genetic materials [202]. Furthermore, advanced GWAS pipelines allow more explicit consideration of environmental variations to quantify plasticity and predict phenotype in a particular environment [203]. Better understanding of crop genetics, yield components, and their responses to the environment should bridge the gap between improved nitrogen metabolism and yield improvement.

Epistatic interactions further complicate breeding for candidate genes in different genetic backgrounds [204]. Gene or trait stacking based on our current understanding of each individual gene, protein, or process have had limited success to date, perhaps because of too little understanding of the complex regulatory network [205]. For example, the introduction of the grain protein content *NAM-B1* transcription factor functional allele, which is generally absent from modern varieties, has only minimal influence on yield, but enhances grain nitrogen and protein content significantly across a wide range of environments [26,206,207]. A meta-analysis across 40 environments showed that 19% of bread wheat genotypes with *NAM-B1* functional alleles exhibit yield enhancement [207], suggesting that the global germplasm still has genetic yield potential. Furthermore, combining multiple nitrogen metabolism genes in the pathway, for example *NR2* and *NPF6.5* [72], or *AMT1;2* with *GOGAT* [69] offers greater chance of yield enhancement than modulating individual genes alone. These observations argue for manipulating either gene networks with multiple genes of relatively small effects or transcription factors that affect several genes and processes at the same time. Further understanding of system biology, especially underlying nitrogen metabolism, should prove useful in guiding such manipulations.

6. Concluding Remarks

Both yield and nitrogen metabolism pathways are complex traits with multiple layers of genetic control. While actual farm yield has increased in some regions of the world, increases in cereal potential yield—the scenario with no limitation on crop growth—have fallen down to below 1% annually [7]. We urgently need to apply new breeding strategies that accelerate genetic gains to meet the demands of our growing human population.

Here, we considered NUE on the basis of grain and total biomass production per unit of nitrogen applied or assimilated. Improvement of nitrogen acquisition, however, does not always translate into higher yields. For example, overexpression of transporter *NPF7.4* resulted in higher NO_3^- uptake, lower NO_3^- accumulation, but higher tissue amino acid concentration, indicating improved nitrogen assimilation; nevertheless, such enhanced nitrogen acquisition decreased biomass and grain production [58]. Knocking out *Lysine-Histidine-type Transporter 1* (*LHT1*), which transports amino acid, helped improve grain nutritional quality at maturity, but at the expense of vegetative biomass, grain weight, and germination rate [208,209]. Henceforth, defining and setting NUE as breeding targets to lower agricultural nitrogen inputs must take into account grain protein content [210,211]. These efforts are prime candidates for improving grain nutritional values. Therefore, if we define NUE as the amount of organic nitrogen that ends up in the consumable grains per nitrogen applied, these genes are worthy of consideration.

Breeding strategies that focus concurrently on both carbon and nitrogen assimilation also offer an opportunity to break the longstanding antagonistic relationship between grain biomass and protein concentration [212] that hampers genetic gains in yield over time. Genetic solutions are needed because management practices like applications of nitrogen fertilizers at booting stage to meet grain nitrogen demand can only partially alleviate this negative relationship at the field level [213]. Control of *NGR5* that uncouples yield components from nitrogen-dependent responses [82], or *GRF4* that breaks the tie between dwarfism-induced yield improvement and reduced nitrogen assimilation [42], establish the possibility of maximizing both yield and NUE at the same time. Genomic selection is theoretically feasible and genomic breeding tools are becoming readily available for breeders to target both sets of traits simultaneously [197,214].

7. Conclusions

This review highlights achievements in manipulating the genetics underlying nitrogen metabolism pathways to enhance yield of rice and wheat, focusing on relationships between yield components and crop nitrogen use during growth. Further fundamental understanding of ortholog genes between species, how different forms of nitrogen influence growth and development, and natural variation of desirable traits responsive to nitrogen should prove useful in achieving higher crop yields. Hopefully, continuous, albeit slow, progress on genetic gain in crop nitrogen assimilation and yield over time can fulfill the yield gap needed to feed our global community.

Author Contributions: Conceptualization, P.K. and A.J.B.; Methodology, P.K. and A.J.B.; Formal Analysis, P.K.; Investigation, P.K.; Data Curation, P.K.; Writing—Original Draft Preparation, P.K.; Writing—Review and Editing, P.K. and A.J.B.; Visualization, P.K.; Project Administration, A.J.B.; Funding Acquisition, P.K. and A.J.B. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported in part by USDA-IWYP-16-06702, NSF IOS 13-58675, NSF IOS 16-55810, and the John B. Orr Endowment to Arnold J. Bloom. Pornpipat Kasemsap was supported by Thailand—United States Educational Foundation (TUSEF/Fulbright Thailand), UC Davis Department of Plant Sciences Graduate Student Researcher fellowship, Henry A. Jastro research award, and Grad Innovator Fellowship from UC Davis Innovation Institute for Food and Health.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created in this study. Data sharing is not applicable to this article.

Acknowledgments: We thank Xiaoxiao Shi, Junli Zhang, and Carlos A. Robles-Zazueta for feedback on the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bloom, A.J. Energetics of Nitrogen Acquisition. In *Annual Plant Reviews Volume 42*; Wiley-Blackwell: Hoboken, NJ, USA, 2010; pp. 63–81; ISBN 978-1-4443-2860-8.
2. Ellis, R.J. The Most Abundant Protein in the World. *Trends Biochem. Sci.* **1979**, *4*, 241–244. [CrossRef]
3. Bloom, A.J. Photorespiration and Nitrate Assimilation: A Major Intersection between Plant Carbon and Nitrogen. *Photosynth. Res.* **2015**, *123*, 117–128. [CrossRef] [PubMed]
4. Xu, G.; Fan, X.; Miller, A.J. Plant Nitrogen Assimilation and Use Efficiency. *Annu. Rev. Plant Biol.* **2012**, *63*, 153–182. [CrossRef] [PubMed]
5. Yang, C.J.; Ladejobi, O.; Mott, R.; Powell, W.; Mackay, I. Analysis of Historical Selection in Winter Wheat. *Theor. Appl. Genet.* **2022**, *135*, 3005–3023. [CrossRef] [PubMed]
6. Chardon, F.; Noël, V.; Masclaux-Daubresse, C. Exploring NUE in Crops and in Arabidopsis Ideotypes to Improve Yield and Seed Quality. *J. Exp. Bot.* **2012**, *63*, 3401–3412. [CrossRef]
7. Fischer, R.A.T.; Edmeades, G.O. Breeding and Cereal Yield Progress. *Crop Sci.* **2010**, *50*, S-85–S-98. [CrossRef]
8. Ikeda, M.; Miura, K.; Aya, K.; Kitano, H.; Matsuoka, M. Genes Offering the Potential for Designing Yield-Related Traits in Rice. *Curr. Opin. Plant Biol.* **2013**, *16*, 213–220. [CrossRef]
9. Reynolds, M.; Foulkes, J.; Furbank, R.; Griffiths, S.; King, J.; Murchie, E.; Parry, M.; Slafer, G. Achieving Yield Gains in Wheat: Achieving Yield Gains in Wheat. *Plant Cell Environ.* **2012**, *35*, 1799–1823. [CrossRef]
10. Shearman, V.J.; Sylvester-Bradley, R.; Scott, R.K.; Foulkes, M.J. Physiological Processes Associated with Wheat Yield Progress in the UK. *Crop Sci.* **2005**, *45*, 175–185. [CrossRef]
11. Sreenivasulu, N.; Schnurbusch, T. A Genetic Playground for Enhancing Grain Number in Cereals. *Trends Plant Sci.* **2012**, *17*, 91–101. [CrossRef]
12. McAllister, C.H.; Beatty, P.H.; Good, A.G. Engineering Nitrogen Use Efficient Crop Plants: The Current Status: Engineering Nitrogen Use Efficient Crop Plants. *Plant Biotechnol. J.* **2012**, *10*, 1011–1025. [CrossRef]
13. Tegeder, M.; Masclaux-Daubresse, C. Source and Sink Mechanisms of Nitrogen Transport and Use. *New Phytol.* **2018**, *217*, 35–53. [CrossRef]
14. Britto, D.T.; Kronzucker, H.J. Ecological Significance and Complexity of N-Source Preference in Plants. *Ann. Bot.* **2013**, *112*, 957–963. [CrossRef]
15. Kirk, G.J.D.; Kronzucker, H.J. The Potential for Nitrification and Nitrate Uptake in the Rhizosphere of Wetland Plants: A Modelling Study. *Ann. Bot.* **2005**, *96*, 639–646. [CrossRef]
16. Jackson, L.E.; Schimel, J.P.; Firestone, M.K. Short-Term Partitioning of Ammonium and Nitrate between Plants and Microbes in an Annual Grassland. *Soil Biol. Biochem.* **1989**, *21*, 409–415. [CrossRef]
17. Subbarao, G.V.; Sahrawat, K.L.; Nakahara, K.; Rao, I.M.; Ishitani, M.; Hash, C.T.; Kishii, M.; Bonnett, D.G.; Berry, W.L.; Lata, J.C. A Paradigm Shift towards Low-Nitrifying Production Systems: The Role of Biological Nitrification Inhibition (BNI). *Ann. Bot.* **2013**, *112*, 297–316. [CrossRef]
18. Tischner, R. Nitrate Uptake and Reduction in Higher and Lower Plants. *Plant Cell Environ.* **2000**, *23*, 1005–1024. [CrossRef]
19. FAO. FAOSTAT: Food Balances (2010-). Available online: https://www.fao.org/faostat/en/#data/FBS (accessed on 13 December 2022).
20. Salse, J.; Bolot, S.; Throude, M.; Jouffe, V.; Piegu, B.; Quraishi, U.M.; Calcagno, T.; Cooke, R.; Delseny, M.; Feuillet, C. Identification and Characterization of Shared Duplications between Rice and Wheat Provide New Insight into Grass Genome Evolution. *Plant Cell* **2008**, *20*, 11–24. [CrossRef]
21. Makino, A. Photosynthesis, Grain Yield, and Nitrogen Utilization in Rice and Wheat. *Plant Physiol.* **2011**, *155*, 125–129. [CrossRef]
22. Valluru, R.; Reynolds, M.P.; Salse, J. Genetic and Molecular Bases of Yield-Associated Traits: A Translational Biology Approach between Rice and Wheat. *Theor. Appl. Genet.* **2014**, *127*, 1463–1489. [CrossRef]
23. Fageria, N.K. Yield Physiology of Rice. *J. Plant Nutr.* **2007**, *30*, 843–879. [CrossRef]
24. Sadras, V.O.; Slafer, G.A. Environmental Modulation of Yield Components in Cereals: Heritabilities Reveal a Hierarchy of Phenotypic Plasticities. *Field Crop. Res.* **2012**, *127*, 215–224. [CrossRef]
25. Munier, D.; Kearney, T.; Pettygrove, G.S.; Brittan, K.; Mathews, M.; Jackson, L. Fertilization of Small Grains. In *Small Grain Production Manual*; UC ANR, Ed.; UC ANR: Davis, CA, USA, 2006.
26. Uauy, C.; Distelfeld, A.; Fahima, T.; Blechl, A.; Dubcovsky, J. A NAC Gene Regulating Senescence Improves Grain Protein, Zinc, and Iron Content in Wheat. *Science* **2006**, *314*, 1298–1301. [CrossRef] [PubMed]
27. Teng, W.; He, X.; Tong, Y. Genetic Control of Efficient Nitrogen Use for High Yield and Grain Protein Concentration in Wheat: A Review. *Plants* **2022**, *11*, 492. [CrossRef] [PubMed]
28. Gaju, O.; Allard, V.; Martre, P.; Le Gouis, J.; Moreau, D.; Bogard, M.; Hubbart, S.; Foulkes, M.J. Nitrogen Partitioning and Remobilization in Relation to Leaf Senescence, Grain Yield and Grain Nitrogen Concentration in Wheat Cultivars. *Field Crop. Res.* **2014**, *155*, 213–223. [CrossRef]
29. Zhang, Z.; Gao, S.; Chu, C. Improvement of Nutrient Use Efficiency in Rice: Current Toolbox and Future Perspectives. *Theor. Appl. Genet.* **2020**, *133*, 1365–1384. [CrossRef]

30. Cormier, F.; Foulkes, J.; Hirel, B.; Gouache, D.; Moënne-Loccoz, Y.; Le Gouis, J. Breeding for Increased Nitrogen-Use Efficiency: A Review for Wheat (*T. Aestivum* L.). *Plant Breed.* **2016**, *135*, 255–278. [CrossRef]
31. Good, A.G.; Shrawat, A.K.; Muench, D.G. Can Less Yield More? Is Reducing Nutrient Input into the Environment Compatible with Maintaining Crop Production? *Trends Plant Sci.* **2004**, *9*, 597–605. [CrossRef]
32. Samonte, S.O.P.B.; Wilson, L.T.; Medley, J.C.; Pinson, S.R.M.; McClung, A.M.; Lales, J.S. Nitrogen Utilization Efficiency: Relationships with Grain Yield, Grain Protein, and Yield-Related Traits in Rice. *Agron. J.* **2006**, *98*, 168–176. [CrossRef]
33. The, S.V.; Snyder, R.; Tegeder, M. Targeting Nitrogen Metabolism and Transport Processes to Improve Plant Nitrogen Use Efficiency. *Front. Plant Sci.* **2021**, *11*, 628366. [CrossRef]
34. Sylvester-Bradley, R.; Kindred, D.R. Analysing Nitrogen Responses of Cereals to Prioritize Routes to the Improvement of Nitrogen Use Efficiency. *J. Exp. Bot.* **2009**, *60*, 1939–1951. [CrossRef]
35. Sakamoto, T.; Matsuoka, M. Generating High-Yielding Varieties by Genetic Manipulation of Plant Architecture. *Curr. Opin. Biotechnol.* **2004**, *15*, 144–147. [CrossRef]
36. Pearce, S. Towards the Replacement of Wheat 'Green Revolution' Genes. *J. Exp. Bot.* **2021**, *72*, 157–160. [CrossRef]
37. Pearce, S.; Saville, R.; Vaughan, S.P.; Chandler, P.M.; Wilhelm, E.P.; Sparks, C.A.; Al-Kaff, N.; Korolev, A.; Boulton, M.I.; Phillips, A.L.; et al. Molecular Characterization of *Rht-1* Dwarfing Genes in Hexaploid Wheat. *Plant Physiol.* **2011**, *157*, 1820–1831. [CrossRef]
38. Peng, J.; Richards, D.E.; Hartley, N.M.; Murphy, G.P.; Devos, K.M.; Flintham, J.E.; Beales, J.; Fish, L.J.; Worland, A.J.; Pelica, F.; et al. 'Green Revolution' Genes Encode Mutant Gibberellin Response Modulators. *Nature* **1999**, *400*, 256–261. [CrossRef]
39. Sasaki, A.; Ashikari, M.; Ueguchi-Tanaka, M.; Itoh, H.; Nishimura, A.; Swapan, D.; Ishiyama, K.; Saito, T.; Kobayashi, M.; Khush, G.S.; et al. A Mutant Gibberellin-Synthesis Gene in Rice. *Nature* **2002**, *416*, 701–702. [CrossRef]
40. Sinclair, T.R. Historical Changes in Harvest Index and Crop Nitrogen Accumulation. *Crop Sci.* **1998**, *38*, 638–643. [CrossRef]
41. Gooding, M.J.; Addisu, M.; Uppal, R.K.; Snape, J.W.; Jones, H.E. Effect of Wheat Dwarfing Genes on Nitrogen-Use Efficiency. *J. Agric. Sci.* **2012**, *150*, 3–22. [CrossRef]
42. Li, S.; Tian, Y.; Wu, K.; Ye, Y.; Yu, J.; Zhang, J.; Liu, Q.; Hu, M.; Li, H.; Tong, Y.; et al. Modulating Plant Growth–Metabolism Coordination for Sustainable Agriculture. *Nature* **2018**, *560*, 595–600. [CrossRef]
43. Kelly, S. The Quest for More Food. *Science* **2022**, *377*, 370–371. [CrossRef]
44. Reynolds, M.P.; Slafer, G.A.; Foulkes, J.M.; Griffiths, S.; Murchie, E.H.; Carmo-Silva, E.; Asseng, S.; Chapman, S.C.; Sawkins, M.; Gwyn, J.; et al. A Wiring Diagram to Integrate Physiological Traits of Wheat Yield Potential. *Nat. Food* **2022**, *3*, 318–324. [CrossRef]
45. Long, S.P.; Zhu, X.-G.; Naidu, S.L.; Ort, D.R. Can Improvement in Photosynthesis Increase Crop Yields? *Plant Cell Environ.* **2006**, *29*, 315–330. [CrossRef] [PubMed]
46. Sinclair, T.R.; Rufty, T.W.; Lewis, R.S. Increasing Photosynthesis: Unlikely Solution For World Food Problem. *Trends Plant Sci.* **2019**, *24*, 1032–1039. [CrossRef] [PubMed]
47. Yoon, D.-K.; Ishiyama, K.; Suganami, M.; Tazoe, Y.; Watanabe, M.; Imaruoka, S.; Ogura, M.; Ishida, H.; Suzuki, Y.; Obara, M.; et al. Transgenic Rice Overproducing Rubisco Exhibits Increased Yields with Improved Nitrogen-Use Efficiency in an Experimental Paddy Field. *Nat. Food* **2020**, *1*, 134–139. [CrossRef]
48. Araus, J.L.; Sanchez-Bragado, R.; Vicente, R. Improving Crop Yield and Resilience through Optimization of Photosynthesis: Panacea or Pipe Dream? *J. Exp. Bot.* **2021**, *72*, 3936–3955. [CrossRef]
49. Lee, S. Recent Advances on Nitrogen Use Efficiency in Rice. *Agronomy* **2021**, *11*, 753. [CrossRef]
50. Prelich, G. Gene Overexpression: Uses, Mechanisms, and Interpretation. *Genetics* **2012**, *190*, 841–854. [CrossRef]
51. Monroe, J.G.; Arciniegas, J.P.; Moreno, J.L.; Sánchez, F.; Sierra, S.; Valdes, S.; Torkamaneh, D.; Chavarriaga, P. The Lowest Hanging Fruit: Beneficial Gene Knockouts in Past, Present, and Future Crop Evolution. *Curr. Plant Biol.* **2020**, *24*, 100185. [CrossRef]
52. Ossowski, S.; Schwab, R.; Weigel, D. Gene Silencing in Plants Using Artificial MicroRNAs and Other Small RNAs: Engineering Small RNA-Mediated Gene Silencing. *Plant J.* **2008**, *53*, 674–690. [CrossRef]
53. Chen, K.; Wang, Y.; Zhang, R.; Zhang, H.; Gao, C. CRISPR/Cas Genome Editing and Precision Plant Breeding in Agriculture. *Annu. Rev. Plant Biol.* **2019**, *70*, 667–697. [CrossRef]
54. Fan, X.; Naz, M.; Fan, X.; Xuan, W.; Miller, A.J.; Xu, G. Plant Nitrate Transporters: From Gene Function to Application. *J. Exp. Bot.* **2017**, *68*, 2463–2475. [CrossRef]
55. Tang, W.; Ye, J.; Yao, X.; Zhao, P.; Xuan, W.; Tian, Y.; Zhang, Y.; Xu, S.; An, H.; Chen, G.; et al. Genome-Wide Associated Study Identifies NAC42-Activated Nitrate Transporter Conferring High Nitrogen Use Efficiency in Rice. *Nat. Commun.* **2019**, *10*, 5279. [CrossRef]
56. Wang, W.; Hu, B.; Yuan, D.; Liu, Y.; Che, R.; Hu, Y.; Ou, S.; Liu, Y.; Zhang, Z.; Wang, H.; et al. Expression of the Nitrate Transporter Gene *OsNRT1.1A/OsNPF6.3* Confers High Yield and Early Maturation in Rice. *Plant Cell* **2018**, *30*, 638–651. [CrossRef]
57. Hu, B.; Wang, W.; Ou, S.; Tang, J.; Li, H.; Che, R.; Zhang, Z.; Chai, X.; Wang, H.; Wang, Y.; et al. Variation in NRT1.1B Contributes to Nitrate-Use Divergence between Rice Subspecies. *Nat. Genet.* **2015**, *47*, 834–838. [CrossRef]
58. Huang, W.; Nie, H.; Feng, F.; Wang, J.; Lu, K.; Fang, Z. Altered Expression of OsNPF7.1 and OsNPF7.4 Differentially Regulates Tillering and Grain Yield in Rice. *Plant Sci.* **2019**, *283*, 23–31. [CrossRef]
59. Wang, J.; Lu, K.; Nie, H.; Zeng, Q.; Wu, B.; Qian, J.; Fang, Z. Rice Nitrate Transporter OsNPF7.2 Positively Regulates Tiller Number and Grain Yield. *Rice* **2018**, *11*, 12. [CrossRef]

60. Huang, W.; Bai, G.; Wang, J.; Zhu, W.; Zeng, Q.; Lu, K.; Sun, S.; Fang, Z. Two Splicing Variants of OsNPF7.7 Regulate Shoot Branching and Nitrogen Utilization Efficiency in Rice. *Front. Plant Sci.* **2018**, *9*, 300. [CrossRef]
61. Fang, Z.; Xia, K.; Yang, X.; Grotemeyer, M.S.; Meier, S.; Rentsch, D.; Xu, X.; Zhang, M. Altered Expression of the *PTR/NRT1* Homologue *OsPTR9* Affects Nitrogen Utilization Efficiency, Growth and Grain Yield in Rice. *Plant Biotechnol. J.* **2013**, *11*, 446–458. [CrossRef]
62. Luo, B.; Chen, J.; Zhu, L.; Liu, S.; Li, B.; Lu, H.; Ye, G.; Xu, G.; Fan, X. Overexpression of a High-Affinity Nitrate Transporter OsNRT2.1 Increases Yield and Manganese Accumulation in Rice Under Alternating Wet and Dry Condition. *Front. Plant Sci.* **2018**, *9*, 1192. [CrossRef]
63. Yan, M.; Fan, X.; Feng, H.; Miller, A.J.; Shen, Q.; Xu, G. Rice OsNAR2.1 Interacts with OsNRT2.1, OsNRT2.2 and OsNRT2.3a Nitrate Transporters to Provide Uptake over High and Low Concentration Ranges: Rice Two Component Nitrate Transport. *Plant Cell Environ.* **2011**, *34*, 1360–1372. [CrossRef]
64. Fan, X.; Tang, Z.; Tan, Y.; Zhang, Y.; Luo, B.; Yang, M.; Lian, X.; Shen, Q.; Miller, A.J.; Xu, G. Overexpression of a PH-Sensitive Nitrate Transporter in Rice Increases Crop Yields. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 7118–7123. [CrossRef] [PubMed]
65. Chen, J.; Liu, X.; Liu, S.; Fan, X.; Zhao, L.; Song, M.; Fan, X.; Xu, G. Co-Overexpression of OsNAR2.1 and OsNRT2.3a Increased Agronomic Nitrogen Use Efficiency in Transgenic Rice Plants. *Front. Plant Sci.* **2020**, *11*, 1245. [CrossRef] [PubMed]
66. Chen, J.; Qi, T.; Hu, Z.; Fan, X.; Zhu, L.; Iqbal, M.F.; Yin, X.; Xu, G.; Fan, X. OsNAR2.1 Positively Regulates Drought Tolerance and Grain Yield Under Drought Stress Conditions in Rice. *Front. Plant Sci.* **2019**, *10*, 197. [CrossRef] [PubMed]
67. Chen, J.; Fan, X.; Qian, K.; Zhang, Y.; Song, M.; Liu, Y.; Xu, G.; Fan, X. *POsNAR2.1:OsNAR2.1* Expression Enhances Nitrogen Uptake Efficiency and Grain Yield in Transgenic Rice Plants. *Plant Biotechnol. J.* **2017**, *15*, 1273–1283. [CrossRef] [PubMed]
68. Ranathunge, K.; El-kereamy, A.; Gidda, S.; Bi, Y.-M.; Rothstein, S.J. AMT1;1 Transgenic Rice Plants with Enhanced NH_4^+ Permeability Show Superior Growth and Higher Yield under Optimal and Suboptimal NH_4^+ Conditions. *J. Exp. Bot.* **2014**, *65*, 965–979. [CrossRef] [PubMed]
69. Lee, S.; Marmagne, A.; Park, J.; Fabien, C.; Yim, Y.; Kim, S.; Kim, T.; Lim, P.O.; Masclaux-Daubresse, C.; Nam, H.G. Concurrent Activation of *OsAMT1;2* and *OsGOGAT1* in Rice Leads to Enhanced Nitrogen Use Efficiency under Nitrogen Limitation. *Plant J.* **2020**, *103*, 7–20. [CrossRef]
70. James, D.; Borphukan, B.; Fartyal, D.; Ram, B.; Singh, J.; Manna, M.; Sheri, V.; Panditi, V.; Yadav, R.; Achary, V.M.M.; et al. Concurrent Overexpression of OsGS1;1 and OsGS2 Genes in Transgenic Rice (*Oryza Sativa* L.): Impact on Tolerance to Abiotic Stresses. *Front. Plant Sci.* **2018**, *9*, 786. [CrossRef]
71. Hu, M.; Zhao, X.; Liu, Q.; Hong, X.; Zhang, W.; Zhang, Y.; Sun, L.; Li, H.; Tong, Y. Transgenic Expression of Plastidic Glutamine Synthetase Increases Nitrogen Uptake and Yield in Wheat. *Plant Biotechnol. J.* **2018**, *16*, 1858–1867. [CrossRef]
72. Gao, Z.; Wang, Y.; Chen, G.; Zhang, A.; Yang, S.; Shang, L.; Wang, D.; Ruan, B.; Liu, C.; Jiang, H.; et al. The Indica Nitrate Reductase Gene OsNR2 Allele Enhances Rice Yield Potential and Nitrogen Use Efficiency. *Nat. Commun.* **2019**, *10*, 5207. [CrossRef]
73. Lee, S.; Park, J.; Lee, J.; Shin, D.; Marmagne, A.; Lim, P.O.; Masclaux-Daubresse, C.; An, G.; Nam, H.G. OsASN1 Overexpression in Rice Increases Grain Protein Content and Yield under Nitrogen-Limiting Conditions. *Plant Cell Physiol.* **2020**, *61*, 1309–1320. [CrossRef]
74. Ji, Y.; Huang, W.; Wu, B.; Fang, Z.; Wang, X. The Amino Acid Transporter AAP1 Mediates Growth and Grain Yield by Regulating Neutral Amino Acid Uptake and Reallocation in *Oryza Sativa*. *J. Exp. Bot.* **2020**, *71*, 4763–4777. [CrossRef]
75. Lu, K.; Wu, B.; Wang, J.; Zhu, W.; Nie, H.; Qian, J.; Huang, W.; Fang, Z. Blocking Amino Acid Transporter *Os* AAP 3 Improves Grain Yield by Promoting Outgrowth Buds and Increasing Tiller Number in Rice. *Plant Biotechnol. J.* **2018**, *16*, 1710–1722. [CrossRef]
76. Fang, Z.; Wu, B.; Ji, Y. The Amino Acid Transporter OsAAP4 Contributes to Rice Tillering and Grain Yield by Regulating Neutral Amino Acid Allocation through Two Splicing Variants. *Rice* **2021**, *14*, 2. [CrossRef]
77. Wang, J.; Wu, B.; Lu, K.; Wei, Q.; Qian, J.; Chen, Y.; Fang, Z. The Amino Acid Permease 5 (OsAAP5) Regulates Tiller Number and Grain Yield in Rice. *Plant Physiol.* **2019**, *180*, 1031–1045. [CrossRef]
78. Alfatih, A.; Wu, J.; Zhang, Z.-S.; Xia, J.-Q.; Jan, S.U.; Yu, L.-H.; Xiang, C.-B. Rice NIN-LIKE PROTEIN 1 Rapidly Responds to Nitrogen Deficiency and Improves Yield and Nitrogen Use Efficiency. *J. Exp. Bot.* **2020**, *71*, 6032–6042. [CrossRef]
79. Zhang, Z.; Xia, J.; Alfatih, A.; Song, Y.; Huang, Y.; Sun, L.; Wan, G.; Wang, S.; Wang, Y.; Hu, B.; et al. Rice *NIN-LIKE PROTEIN 3* Modulates Nitrogen Use Efficiency and Grain Yield under Nitrate-sufficient Conditions. *Plant Cell Environ.* **2022**, *45*, 1520–1536. [CrossRef]
80. Wu, J.; Zhang, Z.; Xia, J.; Alfatih, A.; Song, Y.; Huang, Y.; Wan, G.; Sun, L.; Tang, H.; Liu, Y.; et al. Rice NIN-LIKE PROTEIN 4 Plays a Pivotal Role in Nitrogen Use Efficiency. *Plant Biotechnol. J.* **2021**, *19*, 448–461. [CrossRef]
81. Yu, J.; Xuan, W.; Tian, Y.; Fan, L.; Sun, J.; Tang, W.; Chen, G.; Wang, B.; Liu, Y.; Wu, W.; et al. Enhanced OsNLP4-OsNiR Cascade Confers Nitrogen Use Efficiency by Promoting Tiller Number in Rice. *Plant Biotechnol. J.* **2021**, *19*, 167–176. [CrossRef]
82. Wu, K.; Wang, S.; Song, W.; Zhang, J.; Wang, Y.; Liu, Q.; Yu, J.; Ye, Y.; Li, S.; Chen, J.; et al. Enhanced Sustainable Green Revolution Yield via Nitrogen-Responsive Chromatin Modulation in Rice. *Science* **2020**, *367*, eaaz2046. [CrossRef]
83. Liu, Y.; Wang, H.; Jiang, Z.; Wang, W.; Xu, R.; Wang, Q.; Zhang, Z.; Li, A.; Liang, Y.; Ou, S.; et al. Genomic Basis of Geographical Adaptation to Soil Nitrogen in Rice. *Nature* **2021**, *590*, 600–605. [CrossRef]
84. He, X.; Qu, B.; Li, W.; Zhao, X.; Teng, W.; Ma, W.; Ren, Y.; Li, B.; Li, Z.; Tong, Y. The Nitrate Inducible NAC Transcription Factor TaNAC2-5A Controls Nitrate Response and Increases Wheat Yield. *Plant Physiol.* **2015**, *169*, 1991–2005. [CrossRef]

85. Yang, J.; Wang, M.; Li, W.; He, X.; Teng, W.; Ma, W.; Zhao, X.; Hu, M.; Li, H.; Zhang, Y.; et al. Reducing Expression of a Nitrate-responsive BZIP Transcription Factor Increases Grain Yield and N Use in Wheat. *Plant Biotechnol. J.* **2019**, *17*, 1823–1833. [CrossRef]
86. Wang, Q.; Su, Q.; Nian, J.; Zhang, J.; Guo, M.; Dong, G.; Hu, J.; Wang, R.; Wei, C.; Li, G.; et al. The Ghd7 Transcription Factor Represses ARE1 Expression to Enhance Nitrogen Utilization and Grain Yield in Rice. *Mol. Plant* **2021**, *14*, 1012–1023. [CrossRef] [PubMed]
87. Wang, Q.; Nian, J.; Xie, X.; Yu, H.; Zhang, J.; Bai, J.; Dong, G.; Hu, J.; Bai, B.; Chen, L.; et al. Genetic Variations in ARE1 Mediate Grain Yield by Modulating Nitrogen Utilization in Rice. *Nat. Commun.* **2018**, *9*, 735. [CrossRef] [PubMed]
88. Zhang, J.; Zhang, H.; Li, S.; Li, J.; Yan, L.; Xia, L. Increasing Yield Potential through Manipulating of an *ARE1* Ortholog Related to Nitrogen Use Efficiency in Wheat by CRISPR/Cas9. *J. Integr. Plant Biol.* **2021**, *63*, 1649–1663. [CrossRef] [PubMed]
89. Zhang, S.; Zhang, Y.; Li, K.; Yan, M.; Zhang, J.; Yu, M.; Tang, S.; Wang, L.; Qu, H.; Luo, L.; et al. Nitrogen Mediates Flowering Time and Nitrogen Use Efficiency via Floral Regulators in Rice. *Curr. Biol.* **2021**, *31*, 671–683.e5. [CrossRef]
90. Li, K.; Zhang, S.; Tang, S.; Zhang, J.; Dong, H.; Yang, S.; Qu, H.; Xuan, W.; Gu, M.; Xu, G. The Rice Transcription Factor Nhd1 Regulates Root Growth and Nitrogen Uptake by Activating Nitrogen Transporters. *Plant Physiol.* **2022**, *189*, 1608–1624. [CrossRef]
91. Sun, H.; Qian, Q.; Wu, K.; Luo, J.; Wang, S.; Zhang, C.; Ma, Y.; Liu, Q.; Huang, X.; Yuan, Q.; et al. Heterotrimeric G Proteins Regulate Nitrogen-Use Efficiency in Rice. *Nat. Genet.* **2014**, *46*, 652–656. [CrossRef]
92. Huang, X.; Qian, Q.; Liu, Z.; Sun, H.; He, S.; Luo, D.; Xia, G.; Chu, C.; Li, J.; Fu, X. Natural Variation at the DEP1 Locus Enhances Grain Yield in Rice. *Nat. Genet.* **2009**, *41*, 494–497. [CrossRef]
93. Zhao, M.; Zhao, M.; Gu, S.; Sun, J.; Ma, Z.; Wang, L.; Zheng, W.; Xu, Z. DEP1 Is Involved in Regulating the Carbon–Nitrogen Metabolic Balance to Affect Grain Yield and Quality in Rice (Oriza Sativa L.). *PLoS ONE* **2019**, *14*, e0213504. [CrossRef]
94. Zhang, S.; Zhu, L.; Shen, C.; Ji, Z.; Zhang, H.; Zhang, T.; Li, Y.; Yu, J.; Yang, N.; He, Y.; et al. Natural Allelic Variation in a Modulator of Auxin Homeostasis Improves Grain Yield and Nitrogen Use Efficiency in Rice. *Plant Cell* **2021**, *33*, 566–580. [CrossRef]
95. Wei, S.; Li, X.; Lu, Z.; Zhang, H.; Ye, X.; Zhou, Y.; Li, J.; Yan, Y.; Pei, H.; Duan, F.; et al. A Transcriptional Regulator That Boosts Grain Yields and Shortens the Growth Duration of Rice. *Science* **2022**, *377*, eabi8455. [CrossRef]
96. Qu, B.; He, X.; Wang, J.; Zhao, Y.; Teng, W.; Shao, A.; Zhao, X.; Ma, W.; Wang, J.; Li, B.; et al. A Wheat CCAAT Box-Binding Transcription Factor Increases the Grain Yield of Wheat with Less Fertilizer Input. *Plant Physiol.* **2015**, *167*, 411–423. [CrossRef]
97. Iwamoto, M.; Tagiri, A. Micro RNA -targeted Transcription Factor Gene RDD *1* Promotes Nutrient Ion Uptake and Accumulation in Rice. *Plant J.* **2016**, *85*, 466–477. [CrossRef]
98. Zhang, J.; Zhou, Z.; Bai, J.; Tao, X.; Wang, L.; Zhang, H.; Zhu, J.-K. Disruption of *MIR396e* and *MIR396f* Improves Rice Yield under Nitrogen-Deficient Conditions. *Natl. Sci. Rev.* **2020**, *7*, 102–112. [CrossRef]
99. Von Wittgenstein, N.J.; Le, C.H.; Hawkins, B.J.; Ehlting, J. Evolutionary Classification of Ammonium, Nitrate, and Peptide Transporters in Land Plants. *BMC Evol. Biol.* **2014**, *14*, 11. [CrossRef]
100. Li, H.; Hu, B.; Chu, C. Nitrogen Use Efficiency in Crops: Lessons from Arabidopsis and Rice. *J. Exp. Bot.* **2017**, *68*, 2477–2488. [CrossRef]
101. Buchner, P.; Hawkesford, M.J. Complex Phylogeny and Gene Expression Patterns of Members of the NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER Family (NPF) in Wheat. *J. Exp. Bot.* **2014**, *65*, 5697–5710. [CrossRef]
102. Kumar, A.; Sandhu, N.; Kumar, P.; Pruthi, G.; Singh, J.; Kaur, S.; Chhuneja, P. Genome-Wide Identification and in Silico Analysis of NPF, NRT2, CLC and SLAC1/SLAH Nitrate Transporters in Hexaploid Wheat (*Triticum aestivum*). *Sci. Rep.* **2022**, *12*, 11227. [CrossRef]
103. Britto, D.T.; Kronzucker, H.J. NH_4^+ Toxicity in Higher Plants: A Critical Review. *J. Plant Physiol.* **2002**, *159*, 567–584. [CrossRef]
104. Guo, N.; Zhang, S.; Gu, M.; Xu, G. Function, Transport, and Regulation of Amino Acids: What Is Missing in Rice? *Crop J.* **2021**, *9*, 530–542. [CrossRef]
105. Plett, D.C.; Holtham, L.R.; Okamoto, M.; Garnett, T.P. Nitrate Uptake and Its Regulation in Relation to Improving Nitrogen Use Efficiency in Cereals. *Semin. Cell Dev. Biol.* **2018**, *74*, 97–104. [CrossRef] [PubMed]
106. Liu, Q.; Wu, K.; Song, W.; Zhong, N.; Wu, Y.; Fu, X. Improving Crop Nitrogen Use Efficiency Toward Sustainable Green Revolution. *Annu. Rev. Plant Biol.* **2022**, *73*, 523–551. [CrossRef] [PubMed]
107. Brauer, E.K.; Rochon, A.; Bi, Y.-M.; Bozzo, G.G.; Rothstein, S.J.; Shelp, B.J. Reappraisal of Nitrogen Use Efficiency in Rice Overexpressing Glutamine Synthetase1. *Physiol. Plant.* **2011**, *141*, 361–372. [CrossRef] [PubMed]
108. Thomsen, H.C.; Eriksson, D.; Møller, I.S.; Schjoerring, J.K. Cytosolic Glutamine Synthetase: A Target for Improvement of Crop Nitrogen Use Efficiency? *Trends Plant Sci.* **2014**, *19*, 656–663. [CrossRef]
109. Zhang, Z.; Xiong, S.; Wei, Y.; Meng, X.; Wang, X.; Ma, X. The Role of Glutamine Synthetase Isozymes in Enhancing Nitrogen Use Efficiency of N-Efficient Winter Wheat. *Sci. Rep.* **2017**, *7*, 1000. [CrossRef]
110. Wei, Y.; Xiong, S.; Zhang, Z.; Meng, X.; Wang, L.; Zhang, X.; Yu, M.; Yu, H.; Wang, X.; Ma, X. Localization, Gene Expression, and Functions of Glutamine Synthetase Isozymes in Wheat Grain (*Triticum aestivum* L.). *Front. Plant Sci.* **2021**, *12*, 580405. [CrossRef]
111. Schulte, C.C.M.; Borah, K.; Wheatley, R.M.; Terpolilli, J.J.; Saalbach, G.; Crang, N.; de Groot, D.H.; Ratcliffe, R.G.; Kruger, N.J.; Papachristodoulou, A.; et al. Metabolic Control of Nitrogen Fixation in Rhizobium-Legume Symbioses. *Sci. Adv.* **2021**, *7*, eabh2433. [CrossRef]
112. Schauser, L.; Wieloch, W.; Stougaard, J. Evolution of NIN-Like Proteins in Arabidopsis, Rice, and Lotus Japonicus. *J. Mol. Evol.* **2005**, *60*, 229–237. [CrossRef]

113. Mu, X.; Luo, J. Evolutionary Analyses of NIN-like Proteins in Plants and Their Roles in Nitrate Signaling. *Cell. Mol. Life Sci.* **2019**, *76*, 3753–3764. [CrossRef]
114. Kumar, A.; Batra, R.; Gahlaut, V.; Gautam, T.; Kumar, S.; Sharma, M.; Tyagi, S.; Singh, K.P.; Balyan, H.S.; Pandey, R.; et al. Genome-Wide Identification and Characterization of Gene Family for RWP-RK Transcription Factors in Wheat (*Triticum aestivum* L.). *PLoS ONE* **2018**, *13*, e0208409. [CrossRef]
115. Liu, K.-H.; Liu, M.; Lin, Z.; Wang, Z.-F.; Chen, B.; Liu, C.; Guo, A.; Konishi, M.; Yanagisawa, S.; Wagner, G.; et al. NIN-like Protein 7 Transcription Factor Is a Plant Nitrate Sensor. *Science* **2022**, *377*, 1419–1425. [CrossRef]
116. Wang, M.; Hasegawa, T.; Beier, M.; Hayashi, M.; Ohmori, Y.; Yano, K.; Teramoto, S.; Kamiya, T.; Fujiwara, T. Growth and Nitrate Reductase Activity Are Impaired in Rice *Osnlp4* Mutants Supplied with Nitrate. *Plant Cell Physiol.* **2021**, *62*, 1156–1167. [CrossRef]
117. Mahmoud, D.; Pandey, R.; Sathee, L.; Dalal, M.; Singh, M.P.; Chinnusamy, V. Regulation of Expression of Genes Associated with Nitrate Response by Osmotic Stress and Combined Osmotic and Nitrogen Deficiency Stress in Bread Wheat (*Triticum aestivum* L.). *Plant Physiol. Rep.* **2020**, *25*, 200–215. [CrossRef]
118. Latchman, D.S. Transcription Factors: An Overview. *Int. J. Biochem. Cell Biol.* **1997**, *29*, 1305–1312. [CrossRef]
119. Gaudinier, A.; Rodriguez-Medina, J.; Zhang, L.; Olson, A.; Liseron-Monfils, C.; Bågman, A.-M.; Foret, J.; Abbitt, S.; Tang, M.; Li, B.; et al. Transcriptional Regulation of Nitrogen-Associated Metabolism and Growth. *Nature* **2018**, *563*, 259–264. [CrossRef]
120. Britto, D.T.; Kronzucker, H.J. Bioengineering Nitrogen Acquisition in Rice: Can Novel Initiatives in Rice Genomics and Physiology Contribute to Global Food Security? *BioEssays* **2004**, *26*, 683–692. [CrossRef]
121. Ueda, Y.; Yanagisawa, S. Transcription Factor-Based Genetic Engineering to Increase Nitrogen Use Efficiency. In *Engineering Nitrogen Utilization in Crop Plants*; Shrawat, A., Zayed, A., Lightfoot, D.A., Eds.; Springer International Publishing: Cham, Switzerland, 2018; pp. 37–55. ISBN 978-3-319-92957-6.
122. Luo, L.; Zhang, Y.; Xu, G. How Does Nitrogen Shape Plant Architecture? *J. Exp. Bot.* **2020**, *71*, 4415–4427. [CrossRef]
123. Sasaki, Y.; Ando, H.; Kakuda, K. Relationship between Ammonium Nitrogen in Soil Solution and Tiller Number at Early Growth Stage of Rice. *Soil Sci. Plant Nutr.* **2002**, *48*, 57–63. [CrossRef]
124. Allard, V.; Martre, P.; Le Gouis, J. Genetic Variability in Biomass Allocation to Roots in Wheat Is Mainly Related to Crop Tillering Dynamics and Nitrogen Status. *Eur. J. Agron.* **2013**, *46*, 68–76. [CrossRef]
125. Tian, G.; Gao, L.; Kong, Y.; Hu, X.; Xie, K.; Zhang, R.; Ling, N.; Shen, Q.; Guo, S. Improving Rice Population Productivity by Reducing Nitrogen Rate and Increasing Plant Density. *PLoS ONE* **2017**, *12*, e0182310. [CrossRef] [PubMed]
126. Yang, D.; Cai, T.; Luo, Y.; Wang, Z. Optimizing Plant Density and Nitrogen Application to Manipulate Tiller Growth and Increase Grain Yield and Nitrogen-Use Efficiency in Winter Wheat. *PeerJ* **2019**, *7*, e6484. [CrossRef] [PubMed]
127. Xu, J.; Zha, M.; Li, Y.; Ding, Y.; Chen, L.; Ding, C.; Wang, S. The Interaction between Nitrogen Availability and Auxin, Cytokinin, and Strigolactone in the Control of Shoot Branching in Rice (*Oryza sativa* L.). *Plant Cell Rep.* **2015**, *34*, 1647–1662. [CrossRef] [PubMed]
128. Li, X.; Xia, K.; Liang, Z.; Chen, K.; Gao, C.; Zhang, M. MicroRNA393 Is Involved in Nitrogen-Promoted Rice Tillering through Regulation of Auxin Signal Transduction in Axillary Buds. *Sci. Rep.* **2016**, *6*, 32158. [CrossRef] [PubMed]
129. Milner, M.J.; Swarbreck, S.M.; Craze, M.; Bowden, S.; Griffiths, H.; Bentley, A.R.; Wallington, E.J. Over-expression of TaDWF4 increases wheat productivity under low and sufficient nitrogen through enhanced carbon assimilation. *Commun Biol.* **2022**, *5*, 1–12. [CrossRef]
130. Lu, Z.; Yu, H.; Xiong, G.; Wang, J.; Jiao, Y.; Liu, G.; Jing, Y.; Meng, X.; Hu, X.; Qian, Q.; et al. Genome-Wide Binding Analysis of the Transcription Activator IDEAL PLANT ARCHITECTURE1 Reveals a Complex Network Regulating Rice Plant Architecture. *Plant Cell* **2013**, *25*, 3743–3759. [CrossRef]
131. Kazan, K.; Lyons, R. The Link between Flowering Time and Stress Tolerance. *J. Exp. Bot.* **2016**, *67*, 47–60. [CrossRef]
132. Gao, H.; Jin, M.; Zheng, X.-M.; Chen, J.; Yuan, D.; Xin, Y.; Wang, M.; Huang, D.; Zhang, Z.; Zhou, K.; et al. *Days to Heading 7*, a Major Quantitative Locus Determining Photoperiod Sensitivity and Regional Adaptation in Rice. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 16337–16342. [CrossRef]
133. Richards, R.A. Selectable Traits to Increase Crop Photosynthesis and Yield of Grain Crops. *J. Exp. Bot.* **2000**, *51*, 447–458. [CrossRef]
134. Weber, K.; Burow, M. Nitrogen–Essential Macronutrient and Signal Controlling Flowering Time. *Physiol. Plant.* **2018**, *162*, 251–260. [CrossRef]
135. Hall, A.J.; Savin, R.; Slafer, G.A. Is Time to Flowering in Wheat and Barley Influenced by Nitrogen?: A Critical Appraisal of Recent Published Reports. *Eur. J. Agron.* **2014**, *54*, 40–46. [CrossRef]
136. Hill, C.B.; Li, C. Genetic Architecture of Flowering Phenology in Cereals and Opportunities for Crop Improvement. *Front. Plant Sci.* **2016**, *7*, 1906. [CrossRef]
137. Quraishi, U.M.; Abrouk, M.; Murat, F.; Pont, C.; Foucrier, S.; Desmaizieres, G.; Confolent, C.; Rivière, N.; Charmet, G.; Paux, E.; et al. Cross-Genome Map Based Dissection of a Nitrogen Use Efficiency Ortho-MetaQTL in Bread Wheat Unravels Concerted Cereal Genome Evolution: Concerted Genome Evolution of a NUE MetaQTL in Cereals. *Plant J.* **2011**, *65*, 745–756. [CrossRef]
138. Xu, Q.; Saito, H.; Hirose, I.; Katsura, K.; Yoshitake, Y.; Yokoo, T.; Tsukiyama, T.; Teraishi, M.; Tanisaka, T.; Okumoto, Y. The Effects of the Photoperiod-Insensitive Alleles, Se13, Hd1 and Ghd7, on Yield Components in Rice. *Mol. Breed.* **2014**, *33*, 813–819. [CrossRef]
139. Cai, T.; Xu, H.; Peng, D.; Yin, Y.; Yang, W.; Ni, Y.; Chen, X.; Xu, C.; Yang, D.; Cui, Z.; et al. Exogenous Hormonal Application Improves Grain Yield of Wheat by Optimizing Tiller Productivity. *Field Crop. Res.* **2014**, *155*, 172–183. [CrossRef]

140. Wu, G.; Wilson, L.T.; McClung, A.M. Contribution of Rice Tillers to Dry Matter Accumulation and Yield. *Agron. J.* **1998**, *90*, 317–323. [CrossRef]
141. Forde, B.G.; Lea, P.J. Glutamate in Plants: Metabolism, Regulation, and Signalling. *J. Exp. Bot.* **2007**, *58*, 2339–2358. [CrossRef]
142. Bennetzen, J.L.; Ma, J. The Genetic Colinearity of Rice and Other Cereals on the Basis of Genomic Sequence Analysis. *Curr. Opin. Plant Biol.* **2003**, *6*, 128–133. [CrossRef]
143. Van Camp, W. Yield Enhancement Genes: Seeds for Growth. *Curr. Opin. Biotechnol.* **2005**, *16*, 147–153. [CrossRef]
144. Sorrells, M.E.; La Rota, M.; Bermudez-Kandianis, C.E.; Greene, R.A.; Kantety, R.; Munkvold, J.D.; Miftahudin; Mahmoud, A.; Ma, X.; Gustafson, P.J.; et al. Comparative DNA Sequence Analysis of Wheat and Rice Genomes. *Genome Res.* **2003**, *13*, 1818–1827. [CrossRef]
145. The International Wheat Genome Sequencing Consortium (IWGSC); Appels, R.; Eversole, K.; Stein, N.; Feuillet, C.; Keller, B.; Rogers, J.; Pozniak, C.J.; Choulet, F.; Distelfeld, A.; et al. Shifting the Limits in Wheat Research and Breeding Using a Fully Annotated Reference Genome. *Science* **2018**, *361*, eaar7191. [CrossRef]
146. Krasileva, K.V.; Vasquez-Gross, H.A.; Howell, T.; Bailey, P.; Paraiso, F.; Clissold, L.; Simmonds, J.; Ramirez-Gonzalez, R.H.; Wang, X.; Borrill, P. Uncovering Hidden Variation in Polyploid Wheat. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E913–E921. [CrossRef] [PubMed]
147. Debernardi, J.M.; Tricoli, D.M.; Ercoli, M.F.; Hayta, S.; Ronald, P.; Palatnik, J.F.; Dubcovsky, J. A GRF–GIF Chimeric Protein Improves the Regeneration Efficiency of Transgenic Plants. *Nat. Biotechnol.* **2020**, *38*, 1274–1279. [CrossRef] [PubMed]
148. Shan, Q.; Wang, Y.; Li, J.; Gao, C. Genome Editing in Rice and Wheat Using the CRISPR/Cas System. *Nat. Protoc.* **2014**, *9*, 2395–2410. [CrossRef]
149. Wu, D.; Li, Y.; Cao, Y.; Hu, R.; Wu, X.; Zhang, W.; Tao, W.; Xu, G.; Wang, X.; Zhang, Y. Increased Glutamine Synthetase by Overexpression of TaGS1 Improves Grain Yield and Nitrogen Use Efficiency in Rice. *Plant Physiol. Biochem.* **2021**, *169*, 259–268. [CrossRef] [PubMed]
150. Nadolska-Orczyk, A.; Rajchel, I.K.; Orczyk, W.; Gasparis, S. Major Genes Determining Yield-Related Traits in Wheat and Barley. *Theor. Appl. Genet.* **2017**, *130*, 1081–1098. [CrossRef]
151. Xue, W.; Xing, Y.; Weng, X.; Zhao, Y.; Tang, W.; Wang, L.; Zhou, H.; Yu, S.; Xu, C.; Li, X.; et al. Natural Variation in Ghd7 Is an Important Regulator of Heading Date and Yield Potential in Rice. *Nat. Genet.* **2008**, *40*, 761–767. [CrossRef]
152. Distelfeld, A.; Li, C.; Dubcovsky, J. Regulation of Flowering in Temperate Cereals. *Curr. Opin. Plant Biol.* **2009**, *12*, 178–184. [CrossRef]
153. Distelfeld, A.; Tranquilli, G.; Li, C.; Yan, L.; Dubcovsky, J. Genetic and Molecular Characterization of the *VRN2* Loci in Tetraploid Wheat. *Plant Physiol.* **2009**, *149*, 245–257. [CrossRef]
154. Rubio-Asensio, J.S.; Bloom, A.J. Inorganic Nitrogen Form: A Major Player in Wheat and Arabidopsis Responses to Elevated CO_2. *J. Exp. Bot.* **2016**, *68*, 2611–2625. [CrossRef]
155. Wood, C.C.; Porée, F.; Dreyer, I.; Koehler, G.J.; Udvardi, M.K. Mechanisms of Ammonium Transport, Accumulation, and Retention in Ooyctes and Yeast Cells Expressing Arabidopsis AtAMT1; 1. *FEBS Lett.* **2006**, *580*, 3931–3936. [CrossRef]
156. Wang, R.; Okamoto, M.; Xing, X.; Crawford, N.M. Microarray Analysis of the Nitrate Response in Arabidopsis Roots and Shoots Reveals over 1,000 Rapidly Responding Genes and New Linkages to Glucose, Trehalose-6-Phosphate, Iron, and Sulfate Metabolism. *Plant Physiol.* **2003**, *132*, 556–567. [CrossRef]
157. Bloom, A.J. The Increasing Importance of Distinguishing among Plant Nitrogen Sources. *Curr. Opin. Plant Biol.* **2015**, *25*, 10–16. [CrossRef]
158. Zhang, J.; Liu, Y.-X.; Zhang, N.; Hu, B.; Jin, T.; Xu, H.; Qin, Y.; Yan, P.; Zhang, X.; Guo, X.; et al. NRT1.1B Is Associated with Root Microbiota Composition and Nitrogen Use in Field-Grown Rice. *Nat. Biotechnol.* **2019**, *37*, 676–684. [CrossRef]
159. Katz, E.; Knapp, A.; Lensink, M.; Keller, C.K.; Stefani, J.; Li, J.J.; Shane, E.; Tuermer-Lee, K.; Bloom, A.J.; Kliebenstein, D.J. Genetic variation underlying differential ammonium and nitrate responses in Arabidopsis thaliana. *The Plant Cell.* **2022**, *34*, 4696–4713. [CrossRef]
160. Carlisle, E.; Myers, S.; Raboy, V.; Bloom, A. The Effects of Inorganic Nitrogen Form and CO_2 Concentration on Wheat Yield and Nutrient Accumulation and Distribution. *Front. Plant Sci.* **2012**, *3*, 195. [CrossRef]
161. Wang, W.; Hu, B.; Li, A.; Chu, C. NRT1.1s in Plants: Functions beyond Nitrate Transport. *J. Exp. Bot.* **2020**, *71*, 4373–4379. [CrossRef]
162. Kanstrup, C.; Nour-Eldin, H.H. The Emerging Role of the Nitrate and Peptide Transporter Family: NPF in Plant Specialized Metabolism. *Curr. Opin. Plant Biol.* **2022**, *68*, 102243. [CrossRef]
163. Hachiya, T.; Sakakibara, H. Interactions between Nitrate and Ammonium in Their Uptake, Allocation, Assimilation, and Signaling in Plants. *J. Exp. Bot.* **2016**, *68*, 2501–2512. [CrossRef]
164. Léran, S.; Varala, K.; Boyer, J.-C.; Chiurazzi, M.; Crawford, N.; Daniel-Vedele, F.; David, L.; Dickstein, R.; Fernandez, E.; Forde, B.; et al. A Unified Nomenclature of NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER Family Members in Plants. *Trends Plant Sci.* **2014**, *19*, 5–9. [CrossRef]
165. Feng, H.; Yan, M.; Fan, X.; Li, B.; Shen, Q.; Miller, A.J.; Xu, G. Spatial Expression and Regulation of Rice High-Affinity Nitrate Transporters by Nitrogen and Carbon Status. *J. Exp. Bot.* **2011**, *62*, 2319–2332. [CrossRef] [PubMed]
166. Araki, R.; Hasegawa, H. Expression of Rice (*Oryza sativa* L.) Genes Involved in High-Affinity Nitrate Transport during the Period of Nitrate Induction. *Breed. Sci.* **2006**, *56*, 295–302. [CrossRef]

167. Konishi, N.; Ma, J.F. Three Polarly Localized Ammonium Transporter 1 Members Are Cooperatively Responsible for Ammonium Uptake in Rice under Low Ammonium Condition. *New Phytol.* **2021**, *232*, 1778–1792. [CrossRef] [PubMed]
168. Li, C.; Tang, Z.; Wei, J.; Qu, H.; Xie, Y.; Xu, G. The OsAMT1.1 Gene Functions in Ammonium Uptake and Ammonium–Potassium Homeostasis over Low and High Ammonium Concentration Ranges. *J. Genet. Genom.* **2016**, *43*, 639–649. [CrossRef] [PubMed]
169. Yamaya, T.; Kusano, M. Evidence Supporting Distinct Functions of Three Cytosolic Glutamine Synthetases and Two NADH-Glutamate Synthases in Rice. *J. Exp. Bot.* **2014**, *65*, 5519–5525. [CrossRef]
170. Pereira, E.G.; Bucher, C.P.C.; Bucher, C.A.; Santos, L.A.; Lerin, J.; Catarina, C.S.; Fernandes, M.S. The Amino Acid Transporter OsAAP1 Regulates the Fertility of Spikelets and the Efficient Use of N in Rice. *Plant Soil* **2022**, *480*, 507–521. [CrossRef]
171. Yang, M.; Lu, K.; Zhao, F.-J.; Xie, W.; Ramakrishna, P.; Wang, G.; Du, Q.; Liang, L.; Sun, C.; Zhao, H.; et al. Genome-Wide Association Studies Reveal the Genetic Basis of Ionomic Variation in Rice. *Plant Cell* **2018**, *30*, 2720–2740. [CrossRef]
172. Chen, Y.; Chen, Y.; Zhang, Y.; Zhang, D.; Li, G.; Wei, J.; Hua, X.; Lv, B.; Liu, L. Heterotrimeric G Protein γ Subunit DEP1 Is Involved in Hydrogen Peroxide Signaling and Promotes Aerenchyma Formation in Rice Roots. *Plant Signal. Behav.* **2021**, *16*, 1889251. [CrossRef]
173. Ainsworth, E.A.; Long, S.P. 30 Years of Free-air Carbon Dioxide Enrichment (FACE): What Have We Learned about Future Crop Productivity and Its Potential for Adaptation? *Glob. Chang. Biol.* **2020**, *27*, 27–49. [CrossRef]
174. Myers, S.S.; Zanobetti, A.; Kloog, I.; Huybers, P.; Leakey, A.D.B.; Bloom, A.J.; Carlisle, E.; Dietterich, L.H.; Fitzgerald, G.; Hasegawa, T.; et al. Increasing CO_2 Threatens Human Nutrition. *Nature* **2014**, *510*, 139–142. [CrossRef]
175. Jauregui, I.; Aroca, R.; Garnica, M.; Zamarreño, Á.M.; García-Mina, J.M.; Serret, M.D.; Parry, M.; Irigoyen, J.J.; Aranjuelo, I. Nitrogen Assimilation and Transpiration: Key Processes Conditioning Responsiveness of Wheat to Elevated [CO_2] and Temperature. *Physiol. Plant.* **2015**, *155*, 338–354. [CrossRef]
176. Long, S.P.; Ainsworth, E.A.; Rogers, A.; Ort, D.R. RISING ATMOSPHERIC CARBON DIOXIDE: Plants FACE the Future. *Annu. Rev. Plant Biol.* **2004**, *55*, 591–628. [CrossRef]
177. Luo, Y.; Su, B.O.; Currie, W.S.; Dukes, J.S.; Finzi, A.; Hartwig, U.; Hungate, B.; Mc Murtrie, R.E.; Oren, R.A.M.; Parton, W.J. Progressive Nitrogen Limitation of Ecosystem Responses to Rising Atmospheric Carbon Dioxide. *BioScience* **2004**, *54*, 731–739. [CrossRef]
178. Taub, D.R.; Wang, X. Why Are Nitrogen Concentrations in Plant Tissues Lower under Elevated CO_2? A Critical Examination of the Hypotheses. *J. Integr. Plant Biol.* **2008**, *50*, 1365–1374. [CrossRef]
179. Bloom, A.J.; Smart, D.R.; Nguyen, D.T.; Searles, P.S. Nitrogen Assimilation and Growth of Wheat under Elevated Carbon Dioxide. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 1730–1735. [CrossRef]
180. Gojon, A.; Cassan, O.; Bach, L.; Lejay, L.; Martin, A. The Decline of Plant Mineral Nutrition under Rising CO_2: Physiological and Molecular Aspects of a Bad Deal. *Trends Plant Sci.* 2022, *in press*. [CrossRef]
181. Wang, L.; Feng, Z.; Schjoerring, J.K. Effects of Elevated Atmospheric CO 2 on Physiology and Yield of Wheat (*Triticum aestivum* L.): A Meta-Analytic Test of Current Hypotheses. *Agric. Ecosyst. Environ.* **2013**, *178*, 57–63. [CrossRef]
182. Pleijel, H.; Uddling, J. Yield vs. Quality Trade-offs for Wheat in Response to Carbon Dioxide and Ozone. *Glob. Chang. Biol.* **2012**, *18*, 596–605. [CrossRef]
183. Bloom, A.J.; Burger, M.; Asensio, J.S.R.; Cousins, A.B. Carbon Dioxide Enrichment Inhibits Nitrate Assimilation in Wheat and Arabidopsis. *Science* **2010**, *328*, 899–903. [CrossRef]
184. Bloom, A.J.; Burger, M.; Kimball, B.A.; Pinter, J., Jr. Nitrate assimilation is inhibited by elevated CO_2 in field-grown wheat. *Nat. Clim. Chang.* **2014**, *4*, 477–480. [CrossRef]
185. Subbarao, G.V.; Searchinger, T.D. Opinion: A "More Ammonium Solution" to Mitigate Nitrogen Pollution and Boost Crop Yields. *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2107576118. [CrossRef] [PubMed]
186. Subbarao, G.V.; Rondon, M.; Ito, O.; Ishikawa, T.; Rao, I.M.; Nakahara, K.; Lascano, C.; Berry, W.L. Biological Nitrification Inhibition (BNI)—Is It a Widespread Phenomenon? *Plant Soil* **2007**, *294*, 5–18. [CrossRef]
187. Subbarao, G.V.; Kishii, M.; Bozal-Leorri, A.; Ortiz-Monasterio, I.; Gao, X.; Ibba, M.I.; Karwat, H.; Gonzalez-Moro, M.B.; Gonzalez-Murua, C.; Yoshihashi, T.; et al. Enlisting Wild Grass Genes to Combat Nitrification in Wheat Farming: A Nature-Based Solution. *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2106595118. [CrossRef] [PubMed]
188. Masclaux-Daubresse, C.; Daniel-Vedele, F.; Dechorgnat, J.; Chardon, F.; Gaufichon, L.; Suzuki, A. Nitrogen Uptake, Assimilation and Remobilization in Plants: Challenges for Sustainable and Productive Agriculture. *Ann. Bot.* **2010**, *105*, 1141–1157. [CrossRef] [PubMed]
189. Atwell, S.; Huang, Y.S.; Vilhjálmsson, B.J.; Willems, G.; Horton, M.; Li, Y.; Meng, D.; Platt, A.; Tarone, A.M.; Hu, T.T. Genome-Wide Association Study of 107 Phenotypes in Arabidopsis Thaliana Inbred Lines. *Nature* **2010**, *465*, 627–631. [CrossRef]
190. Nordborg, M.; Weigel, D. Next-Generation Genetics in Plants. *Nature* **2008**, *456*, 720–723. [CrossRef]
191. Lopez-Arboleda, W.A.; Reinert, S.; Nordborg, M.; Korte, A. Global Genetic Heterogeneity in Adaptive Traits. *Mol. Biol. Evol.* **2021**, *38*, 4822–4831. [CrossRef]
192. Sandhu, N.; Sethi, M.; Kumar, A.; Dang, D.; Singh, J.; Chhuneja, P. Biochemical and Genetic Approaches Improving Nitrogen Use Efficiency in Cereal Crops: A Review. *Front. Plant Sci.* **2021**, *12*, 657629. [CrossRef]
193. Wang, Q.; Tang, J.; Han, B.; Huang, X. Advances in Genome-Wide Association Studies of Complex Traits in Rice. *Theor. Appl. Genet.* **2020**, *133*, 1415–1425. [CrossRef]

194. Brachi, B.; Morris, G.P.; Borevitz, J.O. Genome-Wide Association Studies in Plants: The Missing Heritability Is in the Field. *Genome Biol.* **2011**, *12*, 232. [CrossRef]
195. Alonso-Blanco, C.; Aarts, M.G.M.; Bentsink, L.; Keurentjes, J.J.B.; Reymond, M.; Vreugdenhil, D.; Koornneef, M. What Has Natural Variation Taught Us about Plant Development, Physiology, and Adaptation? *Plant Cell* **2009**, *21*, 1877–1896. [CrossRef]
196. Bhat, J.A.; Yu, D.; Bohra, A.; Ganie, S.A.; Varshney, R.K. Features and Applications of Haplotypes in Crop Breeding. *Commun. Biol.* **2021**, *4*, 1266. [CrossRef]
197. Varshney, R.K.; Bohra, A.; Yu, J.; Graner, A.; Zhang, Q.; Sorrells, M.E. Designing Future Crops: Genomics-Assisted Breeding Comes of Age. *Trends Plant Sci.* **2021**, *26*, 631–649. [CrossRef]
198. Wadgymar, S.M.; DeMarche, M.L.; Josephs, E.B.; Sheth, S.N.; Anderson, J.T. Local Adaptation: Causal Agents of Selection and Adaptive Trait Divergence. *Annu. Rev. Ecol. Evol. Syst.* **2022**, *53*, 87–111. [CrossRef]
199. Xie, W.; Wang, G.; Yuan, M.; Yao, W.; Lyu, K.; Zhao, H.; Yang, M.; Li, P.; Zhang, X.; Yuan, J.; et al. Breeding Signatures of Rice Improvement Revealed by a Genomic Variation Map from a Large Germplasm Collection. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E5411–E5419. [CrossRef]
200. Nicotra, A.B.; Atkin, O.K.; Bonser, S.P.; Davidson, A.M.; Finnegan, E.J.; Mathesius, U.; Poot, P.; Purugganan, M.D.; Richards, C.L.; Valladares, F.; et al. Plant Phenotypic Plasticity in a Changing Climate. *Trends Plant Sci.* **2010**, *15*, 684–692. [CrossRef]
201. Hawkesford, M.J.; Riche, A.B. Impacts of G x E x M on Nitrogen Use Efficiency in Wheat and Future Prospects. *Front. Plant Sci.* **2020**, *11*, 1157. [CrossRef]
202. Gao, C. Genome Engineering for Crop Improvement and Future Agriculture. *Cell* **2021**, *184*, 1621–1635. [CrossRef]
203. Li, X.; Guo, T.; Wang, J.; Bekele, W.A.; Sukumaran, S.; Vanous, A.E.; McNellie, J.P.; Tibbs-Cortes, L.E.; Lopes, M.S.; Lamkey, K.R.; et al. An Integrated Framework Reinstating the Environmental Dimension for GWAS and Genomic Selection in Crops. *Mol. Plant* **2021**, *14*, 874–887. [CrossRef]
204. Soyk, S.; Benoit, M.; Lippman, Z.B. New Horizons for Dissecting Epistasis in Crop Quantitative Trait Variation. *Annu. Rev. Genet.* **2020**, *54*, 287–307. [CrossRef]
205. Wei, X.; Qiu, J.; Yong, K.; Fan, J.; Zhang, Q.; Hua, H.; Liu, J.; Wang, Q.; Olsen, K.M.; Han, B.; et al. A Quantitative Genomics Map of Rice Provides Genetic Insights and Guides Breeding. *Nat. Genet.* **2021**, *53*, 243–253. [CrossRef] [PubMed]
206. Brevis, J.C.; Dubcovsky, J. Effects of the Chromosome Region Including the *Gpc-B1* Locus on Wheat Grain and Protein Yield. *Crop Sci.* **2010**, *50*, 93–104. [CrossRef]
207. Tabbita, F.; Pearce, S.; Barneix, A.J. Breeding for Increased Grain Protein and Micronutrient Content in Wheat: Ten Years of the GPC-B1 Gene. *J. Cereal Sci.* **2017**, *73*, 183–191. [CrossRef]
208. Wang, X.; Yang, G.; Shi, M.; Hao, D.; Wei, Q.; Wang, Z.; Fu, S.; Su, Y.; Xia, J. Disruption of an Amino Acid Transporter LHT1 Leads to Growth Inhibition and Low Yields in Rice. *BMC Plant Biol.* **2019**, *19*, 268. [CrossRef] [PubMed]
209. Guo, N.; Gu, M.; Hu, J.; Qu, H.; Xu, G. Rice OsLHT1 Functions in Leaf-to-Panicle Nitrogen Allocation for Grain Yield and Quality. *Front. Plant Sci.* **2020**, *11*, 1150. [CrossRef]
210. Swarbreck, S.M.; Wang, M.; Wang, Y.; Kindred, D.; Sylvester-Bradley, R.; Shi, W.; Varinderpal-Singh; Bentley, A.R.; Griffiths, H. A Roadmap for Lowering Crop Nitrogen Requirement. *Trends Plant Sci.* **2019**, *24*, 892–904. [CrossRef]
211. Hawkesford, M.J.; Araus, J.; Park, R.; Calderini, D.; Miralles, D.; Shen, T.; Zhang, J.; Parry, M.A.J. Prospects of Doubling Global Wheat Yields. *Food Energy Secur.* **2013**, *2*, 34–48. [CrossRef]
212. Simmonds, N.W. The Relation between Yield and Protein in Cereal Grain. *J. Sci. Food Agric.* **1995**, *67*, 309–315. [CrossRef]
213. Wu, W.; Wang, Y.; Wang, L.; Xu, H.; Zörb, C.; Geilfus, C.-M.; Xue, C.; Sun, Z.; Ma, W. Booting Stage Is the Key Timing for Split Nitrogen Application in Improving Grain Yield and Quality of Wheat–A Global Meta-Analysis. *Field Crop. Res.* **2022**, *287*, 108665. [CrossRef]
214. Michel, S.; Löschenberger, F.; Ametz, C.; Pachler, B.; Sparry, E.; Bürstmayr, H. Simultaneous Selection for Grain Yield and Protein Content in Genomics-Assisted Wheat Breeding. *Theor. Appl. Genet.* **2019**, *132*, 1745–1760. [CrossRef]

Chapter 2

Genome-wide Association Study of Rice Vegetative Biomass under Different Inorganic Nitrogen Forms — Ammonium or Nitrate

Submitted for publication and deposited on *biorxiv* (https://doi.org/10.1101/2024.08.12.607622)

Genome-wide Association Study of Rice Vegetative Biomass under Different Inorganic Nitrogen Forms — Ammonium or Nitrate

Pornpipat Kasemsap[ab]†*, Itay Cohen[a]†, Arnold J. Bloom[a]

[a] Department of Plant Sciences, University of California at Davis, Mailstop 3, Davis, CA 95616 USA

[b] Graduate Group Horticulture and Agronomy, University of California at Davis, Davis, CA 95616 USA

† These authors contributed equally to this work.

* Corresponding author, e-mail pkasemsap@ucdavis.edu

Abstract

Rice is the most important source of daily calories in human diets and second only to wheat as the most important protein source. Rice is generally exposed to high ammonium (NH_4^+) levels in the rhizosphere but may employ both NH_4^+ and nitrification-derived nitrate (NO_3^-) as major sources of nitrogen. However, the genetic basis underlying rice adaptation to different nitrogen forms remains poorly characterized. Here, we assessed biomass under either NH_4^+ or NO_3^- as a sole nitrogen source in 390 accessions from the USDA Rice Diversity Panel 1. Rice effectively used either form of nitrogen to support early growth. Tolerance to a high-NH_4^+ exposure was correlated with biomass under NO_3^- and lower NH_4^+ levels. Both genotype and nitrogen source strongly influenced biomass accumulation and partitioning between shoot and root. Root showed the greatest biomass variability and sensitivity to nitrogen source. Genome-wide analyses identified 176 single nucleotide polymorphism (SNP) markers associated with biomass across the full diversity panel and individual populations. The majority of the associations were unique to the individual nitrogen source. We compiled a list of candidate genes, including putative genes involved in nitrogen metabolism, located within 150 kb of 112 most significant SNPs, each with at least 3 adjacent markers detected under the same combination of population and nitrogen source. A flexible consumer, rice may employ distinct genetic mechanisms to use different nitrogen sources, making the species more resilient to fluctuations in soil nitrogen. These insights can guide matching rice genotypes with fertilizer management to improve nitrogen-use efficiency.

Keywords

Nitrogen use efficiency, *Oryza sativa*, genetic, nutrient, fertilizer, indica, japonica

Introduction

Rice (*Oryza sativa* L.) supplies the highest portion of calories in the human diet as well as the second highest portion of protein of any crop (FAOSTAT, 2022). Rice production depends heavily on nitrogen fertilization (Fageria & Baligar, 2003), consuming 16% of the global production of nitrogen fertilizer derived from the Haber-Bosch process (Ladha et al., 2016). Nitrogen fertilization powered the Green Revolution that boosted yields and accelerated human population growth, yet excessive use of fertilizer is responsible for major environmental problems (Melillo, 2012). Meeting the demands of projected human population growth by 2050 will require more than a doubling of crop yield, despite increasing resource scarcity and changing climate conditions (Tilman et al., 2011). Improved cultivars combined with optimized nitrogen management will be crucial for enhancing crop productivity to fulfill global food demands.

Plants assimilate the inorganic nitrogen forms, ammonium (NH_4^+) or nitrate (NO_3^-), that they absorb from the soil. NH_4^+ tends to be the prevalent inorganic form under flooded and acidic soils where most rice is cultivated (Tabuchi et al., 2007). NH_4^+ assimilation may be more favorable than NO_3^- assimilation for multiple reasons. Assimilation of NH_4^+ into amino acids requires less metabolic energy than that of NO_3^- (Bloom, 2015). Plants generally can absorb NH_4^+ faster than NO_3^- when provided either as a sole nitrogen source (Näsholm et al., 2009): specifically, nitrogen deficient genotypes take up and accumulate ^{15}N-labeled NH_4^+ 2.7 to 6.9 times faster than ^{15}N-NO_3^- (Ueda et al., 2020). With equal access to both NH_4^+ and NO_3^-, rice roots still absorbed NH_4^+ during the first 10 minutes to 1 hr of exposure without any lag, while NO_3^- uptake showed a 1-hr lag prior to a rapid absorption phase (Sasakawa & Yamamoto, 1978). Rice roots may further manipulate rhizosphere NH_4^+ level by releasing biological nitrification inhibitors, to counteract an increase in aeration and microbial nitrifiers in the root zone (X. Zhang et al., 2019). These lines of evidence suggest that rice depends on NH_4^+ as its major nitrogen source.

Exposure to high concentrations of NH_4^+, however, may have detrimental consequences; NH_4^+ becomes toxic to plants when it accumulates in tissues at high quantities because it disrupts energy metabolism and active uptake of mineral nutrients (Britto & Kronzucker, 2002; Esteban et al., 2016). Despite being well adapted to NH_4^+-rich rhizospheres, rice may accumulate NH_4^+ in its tissues to far above the external concentration (Britto & Kronzucker, 2002). Upon exposure to high NH_4^+concentrations, rice suffers from biomass losses, leaf chlorosis, stunted root growth, and reduced concentrations of other essential mineral nutrients (Britto & Kronzucker, 2002). NH_4^+ nutrition may inhibit rice root elongation by causing an asymmetric auxin distribution through NH_4^+ uptake-induced acidity (Jia et al., 2020) and may promote root branching through interactions of multiple NH_4^+

transporters (Luo et al., 2022). NH_4^+-tolerant rice cultivars by maintaining high root auxin accumulation may withstand surplus NH_4^+ and sustain root growth (Di et al., 2018). To alleviate NH_4^+ toxicity, plants also rapidly convert the majority of root-absorbed NH_4^+ into organic nitrogen compounds before exporting it to other organs (Britto & Kronzucker, 2002; Tabuchi et al., 2007). Nonetheless, the exact mechanisms underlying rice NH_4^+ utilization and tolerance remain poorly understood.

Microbial nitrification converts NH_4^+ into NO_3^- (Britto & Kronzucker, 2013), another inorganic form that may serve as a significant nitrogen source for rice (Kirk & Kronzucker, 2005). The rate of nitrification depends on soil water status (Araus et al., 2020; Gonzalez-Dugo et al., 2010), O_2 level (Fageria & Baligar, 2003), and pH (Y. Yang et al., 2016). As a consequence, soil NO_3^- concentration can vary over ten folds throughout the season following changes in water availability, as NO_3^- accumulates and readily leaches out, or is lost to the atmosphere via denitrification after a rainfall (George et al., 1993). Nitrification can operate in as low as 2% root zone oxygen concentrations to convert nearly all inorganic nitrogen forms into NO_3^- within 24 hr (Y. Yang et al., 2016). Formation of aerenchyma tissues in rice root promotes nitrifying microbe populations and nitrification activities in the rhizosphere, which leads to increased NO_3^- availability for immediate absorption (Ghosh & Kashyap, 2003; Y. L. Li, Fan, et al., 2007). As such, the pool of NO_3^- generally increases with O_2 levels in the root zone as rice paddies dry up (Fageria & Baligar, 2003).

Rice may have already evolved several adaptations to NO_3^- nutrition. In contrast to NH_4^+, excessive tissue accumulation of NO_3^- does not cause significant physiological distress (Devienne-Barret, 2000). When provided with NO_3^- rather than NH_4^+, rice exhibits (1) higher influx of NO_3^- than NH_4^+ from 2.5 – 500 µM external concentration range, (2) higher proportion of nitrogen available for shoot nitrogen assimilation into amino acids, (3) lower proportion of root nitrogen efflux, (4) higher cytoplasmic nitrogen concentration, (5) faster transport induction time, an (6) higher transporter/substrate affinity for NO_3^-(Kronzucker et al., 2000). Yet, the genetic basis underlying rice adaptation to different nitrogen sources remains obscure.

Genome-wide association studies (GWAS) use natural variation derived from recombination throughout the evolution of a crop to identify genomic signatures of adaptation and potential causal relationships between genotypic variants and phenotypic traits of interest (X. Huang & Han, 2014). The mixed linear model approach, which includes random effects of kinship estimates derived from genetic markers, better accounts for relatedness among individual members that may otherwise lead to more false positive signals in populations with high family relatedness (Yu et al., 2006). However, complex mixed models require significant additional computational time (Z. Zhang et al., 2010). Recent developments in GWAS introduce multiple large effect candidate loci as covariates

(Segura et al., 2012) and further alleviate any confounding effects between markers and genetic structures by separately addressing fixed and random effects simultaneously (X. Liu et al., 2016). A recent iteration of mixed linear model approach that replaces the random effect model component with Bayesian information criteria, proves to be most time-efficient and statistically powerful (M. Huang et al., 2019). To date, GWAS have successfully identified several key genes underlying nitrogen metabolism that may improve yields of food crops (Kasemsap & Bloom, 2022). Selecting appropriate phenotypes to characterize nutrient responses, however, remains challenging, and so only a limited number of genes regulating nutrient response have been validated to date (Z. Zhang et al., 2020).

Most previous studies focus on responses to varying total nitrogen levels, but fail to distinguish between forms of inorganic nitrogen (Kasemsap & Bloom, 2022). Inorganic nitrogen forms strongly influence crop carbon acquisition and subsequently grain yield and nutritional quality (Bloom, 2015). Exposure to elevated atmospheric CO_2 levels inhibit NO_3^- assimilation into protein and results in lower grain protein content, whereas NH_4^+ assimilation is resilient to changes in CO_2 levels (Asensio et al., 2015). Expression of many identified nitrogen transporter genes such as rice NO_3^- transporter (*Nitrate transporter 1/Peptide transporter Family 6.3*; *NPF6.3*) are responsive to either NO_3^- or NH_4^+, (W. Wang et al., 2018, p. 3). Scientists have long noticed significant variations of Nitrogen Use Efficiency (NUE) among different rice subpopulations (Z. Zhang & Chu, 2020). For example, enhanced NO_3^- assimilation primarily underlies higher nitrogen responsiveness of INDICA than JAPONICA rice varietal groups (Z. Zhang & Chu, 2020). To date, however, only a few nitrogen responsive alleles that diverge between INDICA and JAPONICA varietal groups have been identified: *High-affinity nitrate transporter 2.1* (*NRT1.1B*) (Gao et al., 2019; J. Zhang et al., 2019), *Abnormal cytokinin response1 REpressor 1* (*ARE1*) (Q. Wang et al., 2018), *Nitrate reductase 2* (*NR2*) (Gao et al., 2019), *Amino acid permease 5* (*AAP5*) (J. Wang et al., 2019), *Amino acid permease 3* (*AAP3*) (Lu et al., 2018), and multiple quantitative trait loci (QTL) with small effects (B. Li et al., 2016). Attempts to manipulate regulatory elements of nitrogen pathways for grain yield improvement therefore have had only limited success (Kasemsap & Bloom, 2022).

An understanding of how different nitrogen forms interact with diverse genetic backgrounds in a crop is needed to breed improved genotypes that meet growing food demands. Here, we explored the genetic basis of vegetative growth in response to the inorganic nitrogen source using a panel of diverse rice accessions, developed specifically for GWAS (Eizenga et al., 2014). We evaluated biomass production as an indicator of growth and conducted genome-wide analyses with several different models to understand the extent to which plant materials in the global germplasm can tolerate low and high concentrations of NH_4^+, and to identify the genetic basis of biomass production and partitioning in response to NH_4^+ versus NO_3^-. The genetic loci identified in this study may help maximize rice genetic potentials under different nitrogen management approaches in a broad range of environments.

Methods

Plant Materials

The USDA Rice Diversity Panel 1 (RDP1) from the USDA/ARS Genetics Stocks-*Oryza* (GSOR) collection is comprised of 434 *Oryza sativa* L. accessions, representing 5 major subpopulations within global germplasm (Eizenga et al., 2014; McCouch et al., 2016). This panel was genotyped with a high-density rice array of 700,000 SNP (single nucleotide polymorphism) markers, which greatly improved the resolution of genetic analyses (McCouch et al., 2016). Previous studies have identified associations of SNPs with a wide range of traits in multiple environments for both the whole panel and individual subpopulations (McCouch et al., 2016; Shakiba et al., 2017; Zhao et al., 2011).

Our experimental panel of 390 accessions includes two varietal groups, INDICA and JAPONICA, and 5 subpopulations and admixtures within the main varietal groups: INDICA/aus (60 accessions), INDICA/indica (84), INDICA/admixed (7), JAPONICA/aromatic (14), JAPONICA/temperate (95), JAPONICA/tropical (90), JAPONICA/admixed (31), and admixed accessions (9) (Data S1). Admixtures are defined as accessions that have no more than 70% of their ancestry shared with any particular subpopulation (McCouch et al., 2016). We excluded 44 accessions from the full panel because we lacked sufficient seeds for adequate biological replication.

Growth conditions

We evaluated growth of rice seedlings under NO_3^- versus NH_4^+ nutrition, and under different concentrations of NH_4^+ as sole nitrogen sources. We germinated seeds on a rolled germination paper soaked with 1 mM $CaSO_4$ and placed the rolls in a controlled environment chamber at 28°/20°C light/dark, under 450 $\mu mol \cdot s^{-1} \cdot m^{-2}$ photosynthetic photon flux density at plant height during the 12 hr light period. At 7 d post germination, when seedlings had one true leaf, we transferred seedlings into 20 dm^3 opaque, aerated hydroponic polyethylene containers, each filled with 16 dm^3 of nutrient solution. Each tub contained 48 seedlings of different genotypes. The nutrient solution was composed of 1 mM K_2HPO_4, 1 mM KH_2PO_4, 2 mM $CaCl_2$, 2 mM $MgSO_4$, and 0.06 g L^{-1} Fe-NaDTPA, and micronutrients according to a modified Hoagland solution (Epstein & Bloom, 2005). Nitrogen treatments included 3 concentrations of $(NH_4)_2SO_4$ (0.3, 3, and 10 mM) and a single concentration of KNO_3 (3 mM). We adjusted the solution pH with H_2SO_4 to 5.95 and replaced the solution every few days when pH dropped below 5.30. We arranged the containers in a randomized block design with 4 biological plant replicates of each genotype. We repeated the experiment twice in a greenhouse with day/night temperatures approximately 28°/24°C under natural sunlight in Davis, California (38°32'20.2"N 121°46'52.5"W): the first replicate examined accessions ID# 0001 – 0220 (last 4 digits of the USDA Genetic Stocks Oryza; GSOR ID) on December 10, 2018 – January 3, 2019 and accessions ID#0220 – 2020 on January 10 – February 3, 2019); the second replicate examined accessions ID#0001 – 0220 on March 14 – April 7, 2019 and accessions ID#0220 – 2020 on April 12 – May 4, 2019). We harvested shoot and root separately at three weeks after transplanting, dried them at 65°C and then weighed them with Sartorius BL120S balance (Sartorius AG, Göttingen, Germany).

Genome-wide Association Analyses

We performed all analyses in R (R Core Team, 2023) version 4.0.3 (GWAS) and 4.1.2 (other analyses), and reported a list of packages used for each analysis in an integrated development environment Rstudio (RStudio Team, 2020) with the analysis codes. Primarily, we processed data with R/tidyverse version 2.0 (Wickham et al., 2019) and R/data.table version 1.14.8 (Dowle & Srinivasan, 2023). We visualized numerical data with R/ggplot2 version 3.4.1 (Wickham, 2016), a correlation matrix with R/corrplot version 0.92 (Wei & Simko, 2021), and manhattan and quantile-quantile (Q-Q) plots with R/rMVP version 1.0.6 (Yin et al., 2020).

For each genotype, we calculated mean dry mass of root and shoot under each nitrogen treatment for the two replicated experiments, each with 4 biological plant replicates. We further used the mean across biological replicates to calculate ratios of dry mass of each nitrogen treatment divided by the dry mass of the moderate NH_4^+ concentration (3 mM NH_4^+). For each biomass trait, we conducted an analysis of variance (ANOVA) with a linear model [trait ~ nitrogen treatment + genotype + (nitrogen treatment x genotype) + experiment replicate + experiment replicate/group within each replicate + block] and calculated Type II sums of squares (Langsrud, 2003) using R/car version 3.1 (Fox & Weisberg, 2019). We conducted post-hoc mean comparisons with Tukey’s Honest Significant Difference (Tukey’s HSD) method with R/emmeans version 1.8.6 (Lenth, 2023) and R/multcomp version 1.4-23 (Hothorn et al., 2008). Broad-sense heritability was estimated as the proportion of variation as Type II sums of squares accounted by genotype within the total variance.

Not all the residuals of the biomass models were normally distributed. With our large sample size (n= 9878 individual plants), violations to the normality assumption should not have significant impacts on the mean comparisons and derived conclusions (Ghasemi & Zahediasl, 2012; Lumley et al., 2002). To alleviate deviations from the normal distribution and retain only the variations caused by nitrogen treatments and genetic components for further GWAS, we fitted new linear models for shoot, root and whole plants with only experiment replicates and sub-experiment groups as regressors: (trait ~ experiment replicate + experiment replicate/group within each replicate), and quantile normalized the residuals from these new models (McCaw et al., 2020; Pirinen, 2023). The mean quantile normalized residuals across 4 blocks of shoot, root, and whole plant for each individual nitrogen treatment served as inputs for GWAS with 700k SNP markers (McCouch et al., 2016) (Data S2).

We performed GWAS for each biomass trait with R/GAPIT version 3.0 (J. Wang & Zhang, 2020) with Model.selection = FALSE. We compared GWAS outputs from four different linear models: General Linear Model (GLM) (Price et al., 2006); Mixed Linear Model (MLM) (Yu et al., 2006), Fixed and random model Circulating Probability Unification (FarmCPU) (X. Liu et al., 2016), and Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway (BLINK) (M. Huang et al., 2019). We accounted for any existing population structure by including principal components (PC) as covariates and selected a number of PCs based on scree plots of eigenvalues from principal component analyses and previous studies (McCouch et al., 2016; Shakiba et al., 2017). Kinship matrices based on only markers of genotypes for each individual group within 390 accessions were generated by R/GAPIT for each genome-wide association analysis. GLM and BLINK addressed only fixed effects and do not require kinship matrices. For each individual population, we filtered and conducted GWAS using only SNPs with Minor Allele Frequency (MAF) higher than 0.05.

Candidate QTL and genes

We identified SNPs that were both significant marker-wise ($P < 0.0001$) and experiment-wise ($P < 1.43 \times 10^{-7}$ given a Bonferroni correction method (α = 0.1) to account for the total number of SNPs tested). Because the number of SNP with MAF >0.05 vary across populations, we used the total SNP number (700,000) to set the same stringent experiment-wise threshold for analyses. We examined Q-Q plots of p-values from each model and only included results from models with at least one experiment-wise significant marker for further analyses. For each individual combination of population and nitrogen treatment, we defined a QTL as any peak experiment-wise significant SNP with more than 3 marker-wise significant SNPs within 150 kb of the peak SNP position. Previous studies have also used similar criteria based on linkage disequilibrium decay to lower the number of false positive signals (McCouch et al., 2016; Shakiba et al., 2017).

We searched for candidate genes within the range of the peak significant SNP with a local deployment of the UCSC Genome Browser software (Kent et al., 2002) at http://genome-mirror.cshl.edu/cgi-bin/hgGateway?clade=poaceae&org=O.+sativa&db=orySat2 by the Rice Diversity Project. We retrieved gene annotations based on the reference genome of Nipponbare, and putative functions from the MSUv7, orySat2 genome assembly in Rice Genome Annotation Project Database (Kawahara et al., 2013) at http://rice.uga.edu/downloads_gad.shtml. We further analyzed Gene Ontology (GO) enrichment for all candidate genes against the default 24075 annotated numbers as references from MSU 7.0 gene ID of *Oryza sativa japonica* via the Singular Enrichment Analysis method in agriGO (Tian et al., 2017) using Fisher's test with Bonferroni correction method ($\alpha = 0.05$).

Results

Genetic backgrounds and inorganic nitrogen sources shaped biomass accumulation

To dissect the genetic basis of responses to inorganic nitrogen source, we evaluated biomass accumulation of diverse rice accessions after three weeks of growth under NO_3^- or NH_4^+ at varying concentrations. Biomass traits were low to moderately heritable with a broad-sense heritability of 0.13 for fraction biomass partitioned to root, 0.22 for root biomass, 0.32 for whole plant biomass, and 0.32 for shoot biomass (Figure 1). In our replicated greenhouse experiments that differed in day lengths and natural sunlight levels, time of year and experiment block structure influenced whole plant, shoot, and root biomass (Table S1).

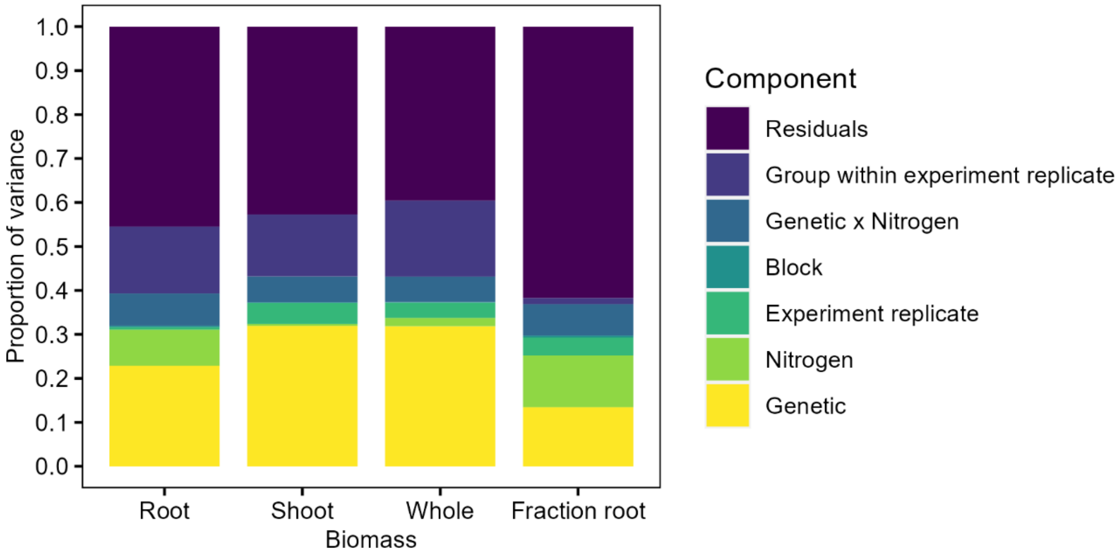


Figure 1 Genetic differences account for the largest proportion of biomass variation.

Broad-sense heritability and percentage of variance explained by each variable: Genetic, Nitrogen treatment, Experiment replicate x Nitrogen treatment interaction, Group within experiment replicate, and Residuals for each biomass trait, calculated as a proportion of total sums of squares (type II) from a linear model in ANOVA for (from left) root dry weight, shoot dry weight, total dry weight, fraction of biomass partitioned to root.

We tested the influence of genetic backgrounds with respect to individual genotypes, subpopulations, and varietal groups. Seedling growth under the different nitrogen treatments demonstrated that genetic background and nitrogen source generally influenced biomass accumulation and partitioning independently (Table S1). Tukey's HSD results for all pairwise comparisons are provided in Table S2. High level of NH_4^+ (10 mM) decreased growth of all organs (Figure 2A, B, C, Table S2). Seedlings accumulated similar whole plant biomass under the other nitrogen treatments, 0.3 mM NH_4^+, 3 mM NH_4^+ and 3 mM NO_3^-. On the basis of subpopulation, shoot biomass was the highest under 3 mM NH_4^+, followed by similar biomass under 0.3 mM NH_4^+ and 3 mM NO_3^-. The opposite was observed in root; root grew under 0.3 mM NH_4^+ and 3 mM NO_3^-, followed by moderate growth under 3 mM NH_4^+. In other words, nitrogen source influenced growth of individual organs at the same, non-toxic concentration, but plants still maintained similar whole plant biomass. Both whole plant and individual organ biomass production were positively correlated among nitrogen treatments (Figure S1) with all Pearson's correlation coefficients being significant at $P < 0.05$. Overall, whole plant biomass followed changes in shoot biomass more strongly than those of root.

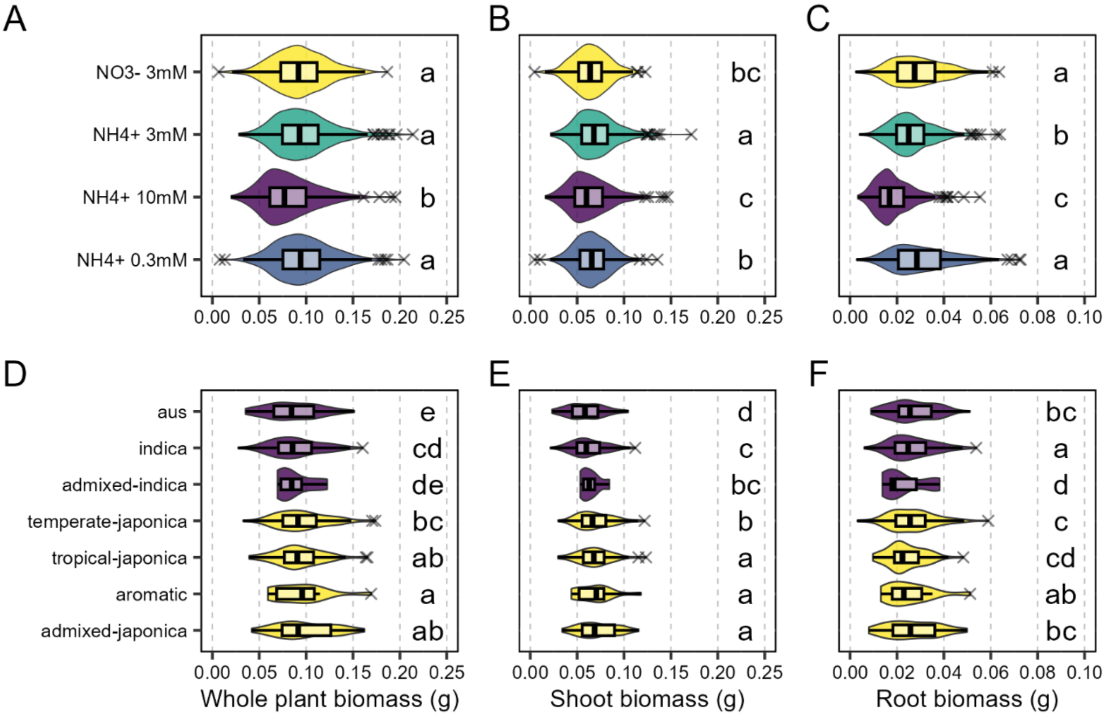


Figure 2 Genetic backgrounds and inorganic nitrogen sources independently shaped biomass accumulation.

Distribution of whole plant (A, D), shoot (B, E), root (C, F) biomass at 21 d after germination of 390 *Oryza sativa* accessions as influenced independently by subpopulation (top panel) or by 4 nitrogen treatments: NH_4^+ (0.3, 3, 10 mM) or NO_3^- (3 mM) (bottom panel). Admixed accessions (n=9 accessions) are not shown. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima. Each dot represents means of individual accessions (n=390; number of accessions listed in the bracket) across 4 nitrogen treatments, 4 biological replicates and 2 experiment replicates for the top panel and across 4 biological replicates and 2 experiment replicates for the bottom panel. Outliers are labelled with (X). Groups based on individual plants with similar letters are not statistically different according to Tukey's HSD tests ($p<0.05$).

Shoot, root, and total plant biomass varied between the two varietal groups, INDICA vs. JAPONICA, as well as among the embedded five subpopulations and three admixed groups, indicating strong genetic effect on biomass production (Figure 2D, E, F, Table S2). Although we observed significant interactions of genetic and nitrogen treatment for shoot biomass on the basis of varietal groups and root biomass on the basis of individual genotypes when we included admixed groups in the comparison, the INDICA varietal group generally accumulated smaller whole plant and shoot biomass than the JAPONICA varietal group under all nitrogen treatments. By contrast, the JAPONICA varietal group, except for the aromatic subpopulation, generally had smaller root biomass than the INDICA varietal group across all nitrogen treatments.

Biomass partitioning in response to different inorganic nitrogen sources depends on the genetic background

The contrasting biomass pattern of individual organs suggested interactions between genetics and nitrogen treatments on biomass partitioning. Both genetic background and nitrogen source interacted to shape the biomass allocation on the basis of subpopulations and varietal groups (Figure 3, Table S1). In general, the INDICA varietal group partitioned more biomass toward root than the JAPONICA varietal group (Figure 3A, B, C, D). We further compared subpopulations within each varietal group and observed a large range of values suggesting diverse partitioning strategies within the global germplasm (Figure 3E, F, G, H, Table S2). While most genotypes showed less biomass partitioning to root under the high NH_4^+ concentration, the long-tails (Figure 3B, F) indicates that some accessions, especially from the INDICA varietal group, still partitioned ~75% of biomass to root under high NH_4^+ stress.

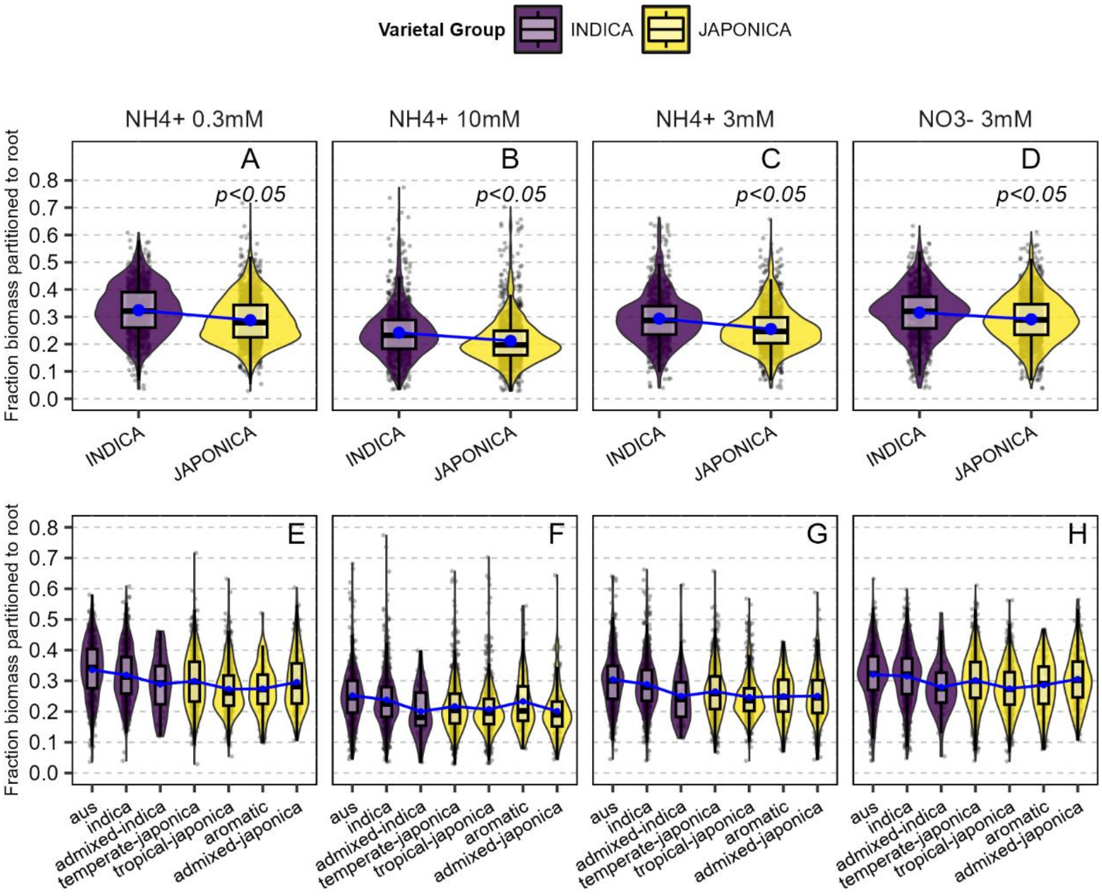


Figure 3 Biomass partitioning to root in response to different inorganic nitrogen sources depends on genetic background.

Distribution of fraction biomass partitioned to root from whole plant biomass of 390 *Oryza sativa* accessions from two varietal groups (A to D) and 7 subpopulations within each group (E to H): INDICA (aus, indica, admixed indica), JAPONICA (temperate japonica, tropical japonica, aromatic, admixed japonica), under 4 nitrogen treatment: 0.3 mM NH_4^+ (A, E), 10 mM NH_4^+ (B, F), 3 mM NH_4^+ (C, G) and 3 mM NO_3^- (D, H) as a sole nitrogen source at 21 d after germination. Admixed accessions (n=9 accessions) are not shown. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima. For comparison, we added lines (blue) between boxplots to connect means (white closed circle) of different populations within the same nitrogen treatment. Each black dot adjacent to the violin plots represents biological plant replicates (n=9878). Tukey's HSD ($p<0.05$) mean separations are in Table S2.

Root responsiveness to nitrogen consistently differed across subpopulations

To compare nitrogen responsiveness, we considered 3 mM NH_4^+ as a reference nitrogen treatment. Supplies of 3 mM inorganic nitrogen, particularly NH_4^+, which were replenished regularly, were neither high enough to be toxic nor low enough to limit growth (Chen et al., 2013). Using means across the four biological replicates from independent blocks, we calculated for each accession the biomass ratio of each organ in comparison to the reference biomass of each respective accession grown at 3 mM NH_4^+ (Figure 4). Comparisons of biomasses in the reference treatment with those in 3 mM NO_3^-, 10 mM NH_4^+ and 0.3 mM NH_4^+ were taken, respectively, as indications of conserved or diverged nitrogen source preference, high NH_4^+ tolerance, and low NH_4^+ tolerance.

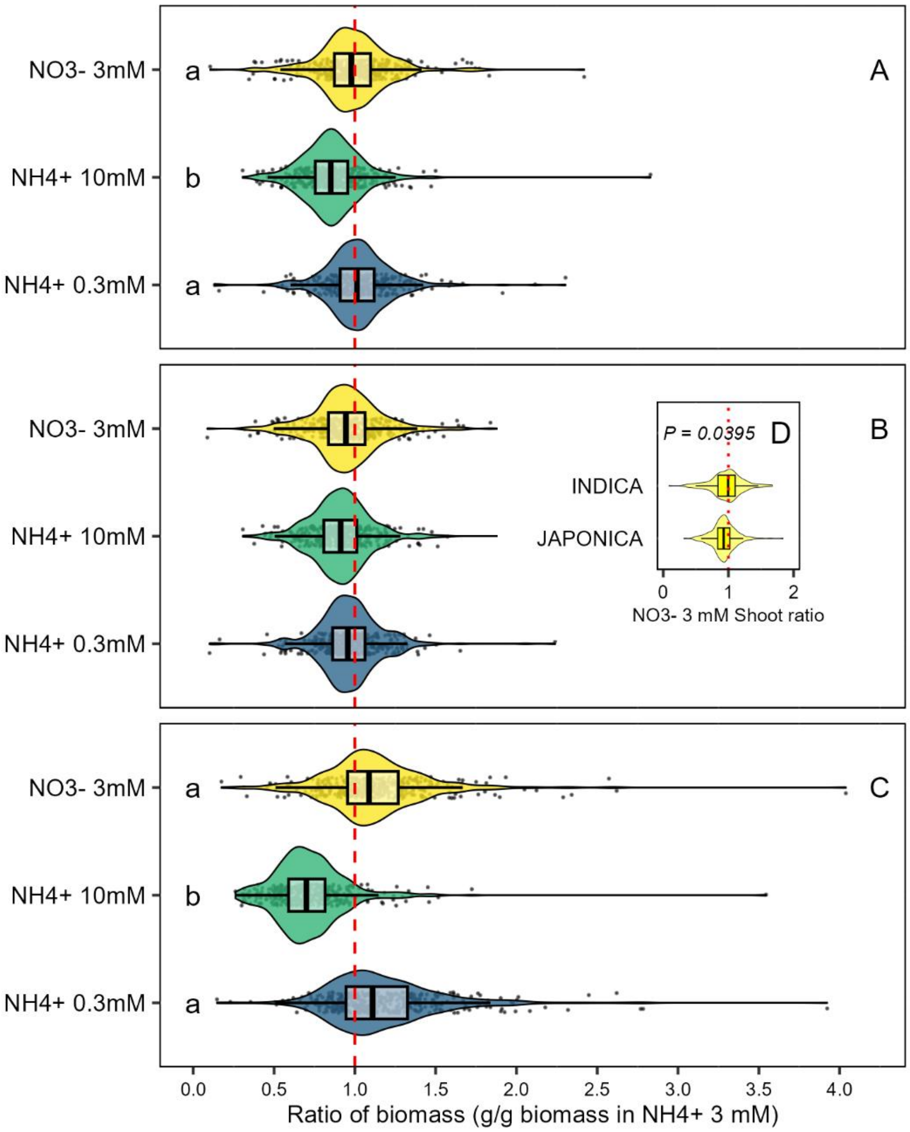


Figure 4 Root primarily underlies whole plant responsiveness to nitrogen form and concentrations.

Distribution of biomass ratio by (A) whole plant, (B) shoot, or (C) root of biomass at different nitrogen treatments to that of biomass at 3 mM NH4+ of each *Oryza sativa* accession at 21 d after germination. An inset plot (D) distinguishes 3 mM NO_3^- : 3 mM NH_4^+ shoot biomass ratio of INDICA and JAPONICA varietal groups. Red dashed line represents a ratio of 1:1. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima. Each black dot adjacent to the violin plots represents means of each accession across 2 experiment replicates (n=390). Groups with similar letters are not statistically different according to Tukey's HSD tests (p<0.05).

Nitrogen treatment explained most of the variation in the ratio of whole plant, shoot, or root biomass ratio to the reference treatment (Figure 4, Table S1). Roots were more responsive than shoots to the nitrogen source and more sensitive to high NH_4^+ levels. Biomass of root showed the strongest decrease in response to 10 mM NH_4^+ and the greatest increase at 0.3 mM NH_4^+ or when receiving NO_3^- (Figure 4C). Root nitrogen responsiveness was also consistent between the two experimental replicates (Table S1). The long tails in root biomass ratios demonstrate a larger range of plasticity in root growth, especially under NO_3^- and low NH_4^+.

Because we pooled biomass of the same accession across blocks to calculate the ratio, we only examined the influence of genetic components as varietal groups or subpopulations. Nitrogen responsiveness was generally comparable across populations. A shoot biomass ratio centered close to 1.0 implied that shoot growth under NO_3^- nutrition for most accessions was similar to their NH_4^+ counterparts (Figure 4B, Figure S2). However, when we contrasted the varietal groups, the INDICA varietal group shoots had relatively larger NO_3^-: NH_4^+ growth ratio than the JAPONICA varietal group (Figure 4D). This derived primarily from the JAPONICA varietal group having slower shoot growth under NO_3^-, while shoots of the INDICA varietal group growing at relatively the same rate under both nitrogen sources (Figure S2).

GWAS demonstrates polygenic control of biomass production

To understand the genetic basis underlying different responses to nitrogen forms, we performed genome-wide analyses to identify associations between biomass traits and genetic markers across the rice genome. Accounting for subpopulation-specific variation, we were able to capture signals that might have been lost when looking just at the full panel alone. We examined outputs from both single locus and multiple locus models, and Q-Q plots of p-values (Data S3). The significant SNPs identified by multi-locus models, FarmCPU (X. Liu et al., 2016) and BLINK (M. Huang et al., 2019) had relatively lower p-values among different model outputs. By including major significant markers as covariates, minor loci with significant contribution to the phenotypes can be identified (McCouch et al., 2016). All models in our experiment identified partially overlapping sets of significant SNPs. We compiled model outputs with at least one experiment-wise significant marker for further analysis.

Overall, we identified 176 unique SNP markers from 236 associations with the seedling biomass at different nitrogen levels and sources (Figure 5, Figure 6, Table S3). Most associations (145; 61%) were identified in the full panel (Figure 5A). We examined SNPs that were significant in a specific condition or across multiple conditions. Within each population, we dissected associations by individual component of variations (nitrogen treatment, organ, model) to examine their contributions (Figure 5B, Figure S3). Associations were mostly (92%) unique to the individual nitrogen treatment (Figure 5B, C). Within the same model, population, experiment replicate, and organ, we found 93, 85, 15, and 4 SNPs associated with 0.3 mM NH_4^+, 3 mM NO_3^-, 10 mM NH_4^+, and 3 mM NH_4^+, respectively. Only 19 associations (8%) were found in multiple nitrogen treatments. For other components of variations, contributions of individual factors varied. For plant organ, shoots accounted for the majority of associations in all population groups, except for the aus subpopulation and the JAPONICA varietal group in which significant associations were from the whole plant and root biomass, respectively (Figure S3B). Analyzing different organs separately may allowed us to identify a genetic background-specific plant part that was most responsive to nitrogen supplies. Regarding the models, either BLINK and FarmCPU identified most associations across most populations, except for the full panel where GLM accounted for the majority of the significant associations (Figure S3D).

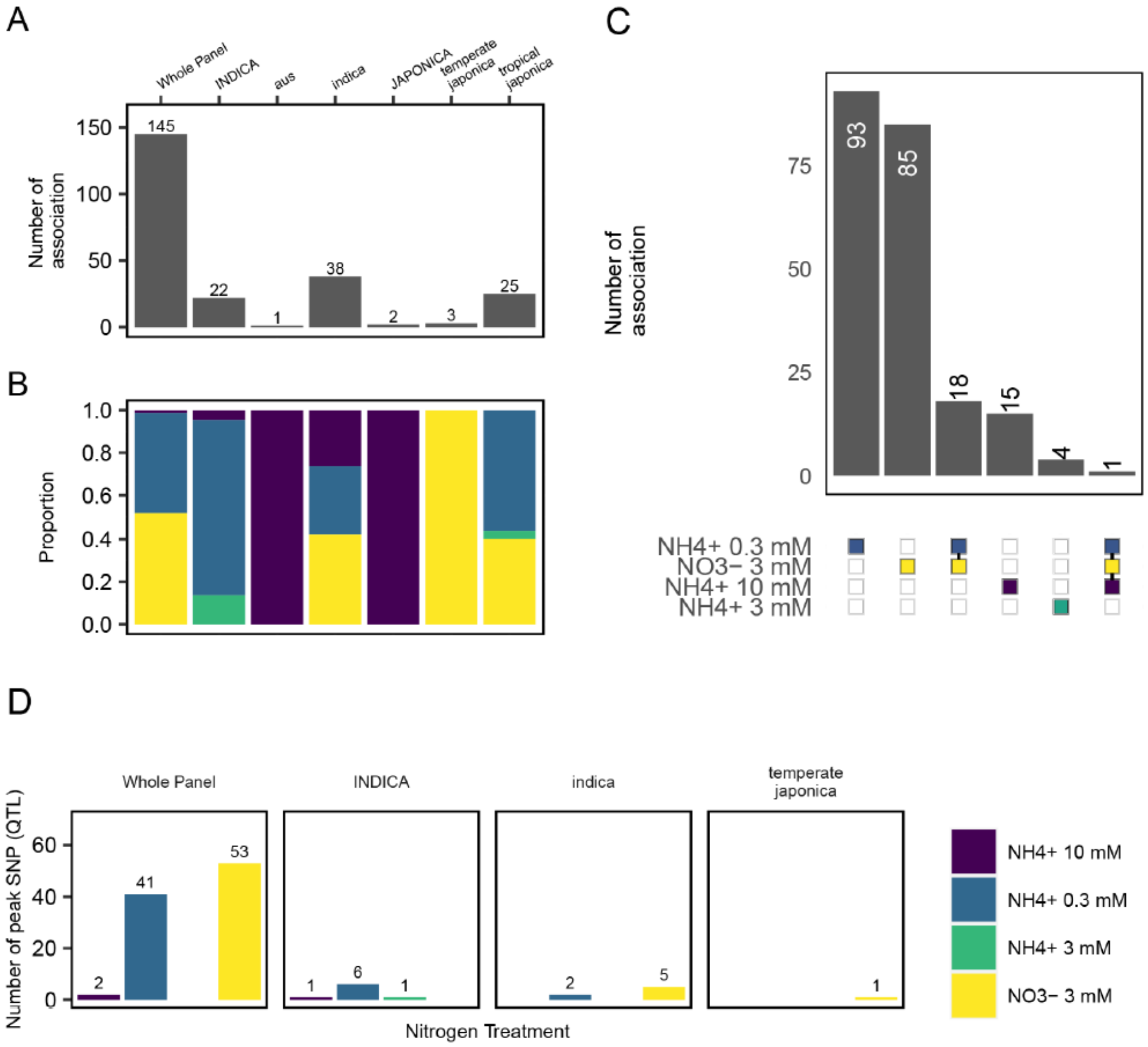


Figure 5 The majority of significant marker-trait associations are unique to individual nitrogen treatments.

(A-C) Number of experiment-wise significant ($P < 1.43 \times 10^{-7}$) SNP marker-trait associations and (D) number of peak SNP (QTL of interest) in each population and nitrogen treatment combination. (A) Total number of experiment-wise significant association by population and (B) breakdown into proportion by nitrogen treatments within each population. Labels on the top of each bar indicates the number of associations. (C) Color boxes beneath the bar chart indicate sets displayed in the bar charts above. Overlaps between significant associations across sets of nitrogen treatments are connected with black lines. (D) Within each population and nitrogen treatment combination, we selected peak experiment-wise significant SNP with > 3 adjacent SNP ($P<0.001$) within 150kb also being significant and defined them as QTL of interest. (B-D) Bar and box colors distinguish nitrogen treatment: 10 mM NH_4^+ (purple), 0.3 mM NH_4^+ (turquoise), 3 mM NH_4^+ (green), and 3 mM NO_3^- (yellow)

Within the same population and nitrogen treatment combination, we pooled significant associations across models and organs, and examined all adjacent SNPs clustering around the experiment-wise significant SNP with the lowest p-values. We narrowed down candidate regions by identifying peak SNPs with at least 3 marker-wise significant SNP within 150 kb of the peak SNP and designated them as QTL of interest (Figure 5D, Figure

6B, Table S4). As a result, we retained 96 (66%), 8 (21%), 7 (32%), and 1 (33%) peak SNPs of the full panel, INDICA varietal group, indica subpopulation, and temperate japonica subpopulation from all experiment-wise significant associations. In total, we reported 112 QTL from 98 unique combinations of populations and nitrogen treatments (Figure 6B, Table S4), with most QTL associated with 3 mM NO_3^- (37%) and 0.3 mM NH_4^+ (47%) in the full panel (Figure 5D).

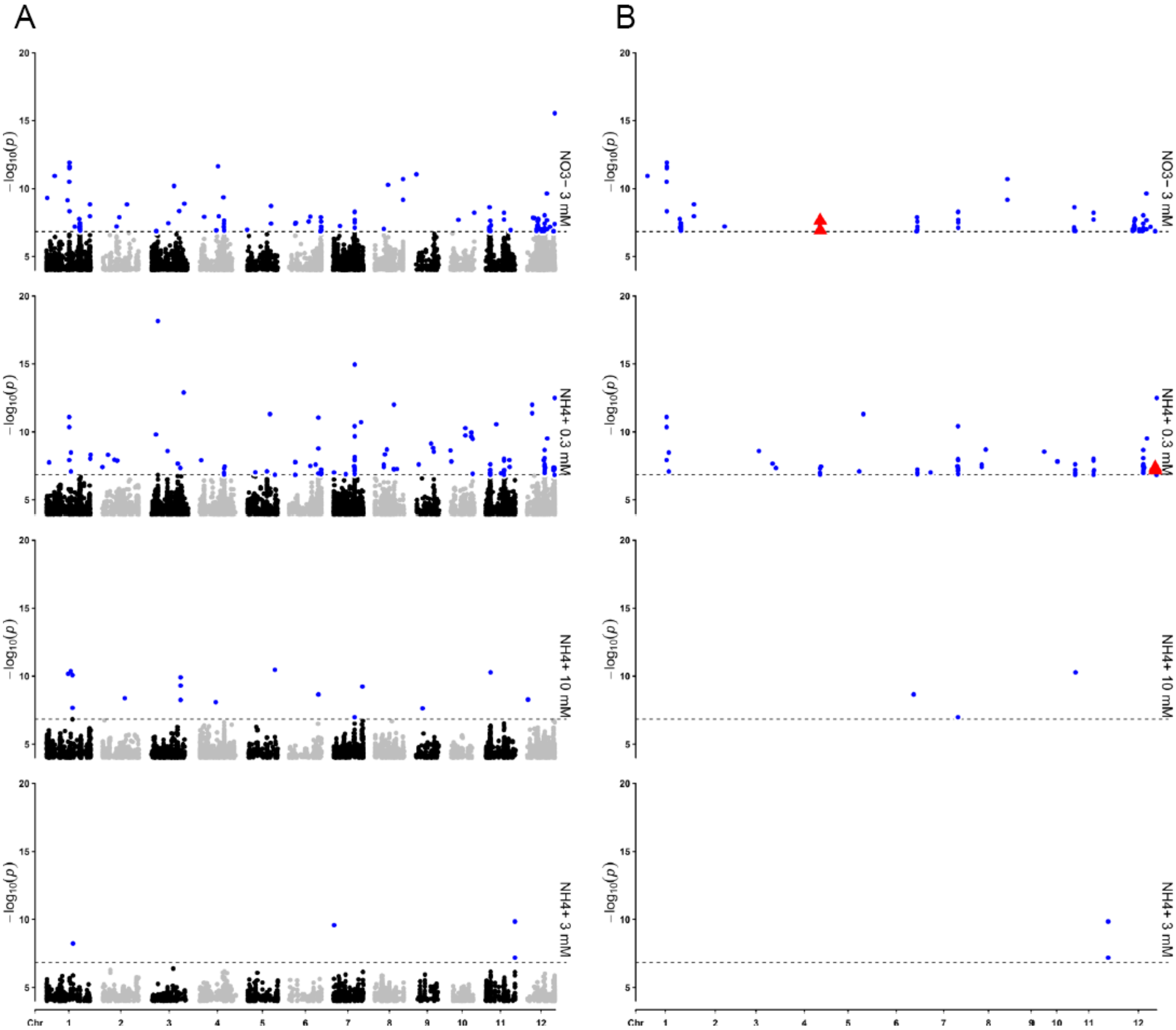


Figure 6 Complex genetic architecture underlying inorganic nitrogen responses involves multiple QTL distributed across the genome.

Marker-trait associations of biomass under 3 mM NO_3^-, 0.3 mM NH_4^+, 10 mM NH_4^+, and 3 mM NH_4^+ where each dot represents SNP markers significant above the marker-wise threshold ($P < 0.0001$). (A) 236 experiment-wise significant markers (blue circle). (B) A subset of 138 experiment-wise significant markers with at least 3 marker-wise significant SNPs within 150 kb of the peak SNP, designated as QTL. Horizontal dashed lines represent p-value threshold at the experiment-wise ($P < 1.43 \times 10^{-7}$) level. Significant associations included in this figure were pooled across populations and plant organs. Putative loci are re-marked with red triangles for *NAR2.1* and *AAP4* on chromosome 4 and 12 respectively.

We then queried the candidate genes within the range of each clustered SNPs. We identified 1133 unique candidate genes adjacent to the peak SNPs of interest (Table S5). Gene ontology enrichment analysis of all candidate genes (Table S6) suggests the prevalence of biological processes related to carbohydrate metabolism and the controlled release of a substance by cells (Figure S4A), and molecular functions related to transmembrane transporter activity, hydrolase activity, and aspartic-type peptidase activity (Figure S4B).

The genetic basis underlying general responses to nitrogen inputs can be as important as nitrogen form-specific responses. We examined QTL that were unique to individual nitrogen treatment as well as shared across multiple nitrogen treatments, with a focus on candidate genes with functions related to nitrogen metabolism (Figure 6B). Several candidate genes identified from the full panel include putative genes involved in nitrogen assimilation and transport. Putative candidate gene for high affinity nitrate transporter (LOC_Os04g40410.1) was identified in both 3 mM NO_3^- and 0.3 mM NH_4^+ on chromosome 4. LOC_Os04g40410.1 encodes a plasma membrane transporter orthologous to Poaceae relatives including rice *NAR2.1* (Yan et al., 2011). LOC_Os12g41890.1 that encodes for an amino acid permease family protein, orthologous to maize and sorghum *AAP4* (Alexandrov et al., 2009; Paterson et al., 2009), which was adjacent to 3 co-located QTL on chromosome 12. Further characterization and validations are needed to narrow down the list and confirm the functions of these genes.

Discussion

Management of nitrogen to maximize crop productivity and minimize excessive applications is a major challenge for agriculture worldwide. Because soil nitrogen dynamics fluctuate greatly in space and time (George et al., 1993), matching soil nitrogen supplies with varying crop nitrogen demand at different growth stages is key to maximizing crop productivity (Fageria, 2007). Crop preference for a particular nitrogen form can further shape growth and ecosystem responses to environmental changes (Britto & Kronzucker, 2013). Our findings suggested that rice may be able to take advantage of fluctuating soil nitrogen pool comprised of different nitrogen forms better than previously assumed.

The global germplasm features substantial variations in population-specific nitrogen responses at an early growth stage

Our study essentially demonstrates the diversity of early vigor in response to nitrogen supplies within the global germplasm. Seedling vigor correlates positively with higher yield potential and may serve as an effective selection criterion for grain yield (Kumar et al., 2009). Early biomass accumulation is also vital for outcompeting weeds (Namuco et al., 2009). Although the nitrogen demand may have not reached the maximum level at this early vegetative stage, we already observed a large variation in biomass and nitrogen responsiveness within the global germplasm.

Segregation of alleles in different genetic backgrounds requires examining each subpopulation individually for population-specific genetic heterogeneity (Zhao et al., 2011). As such, performing genome-wide analyses with individual subpopulations increased the power to identify associations that might otherwise be masked in the whole panel (Zhao et al., 2011). We found that subpopulation-specific genome-wide analyses detected additional sets of candidate loci from analysis performed on the full diversity panel. Multiple studies have demonstrated the increased power to detect associations when analyzing individual subpopulations separately (Crowell et al., 2016; Magwa et al., 2016; McCouch et al., 2016; Shakiba et al., 2017; F. Xu et al., 2016). Several putative loci associated with the candidate markers from both the full panel and subpopulation-specific genome-wide analyses were close to or within the genes that may be involved in nitrogen metabolism. Characterization and validation of candidate loci and genes, including those in linkage disequilibrium with the significant markers in our study, are needed to confirm the roles of these genetic regulatory elements in nitrogen responses.

Natural variation in rice tolerance to NH_4^+ can guide breeding for improved nitrogen use efficiency

Given the prevalence of NH_4^+ in a flooded root environment, can rice suffer under high NH_4^+ concentrations? Our study confirmed that most rice accessions exhibit slower growth at a high NH_4^+ concentrations (Figure 2, Figure 4). Although rice is often considered to be NH_4^+-adapted crop because of its capacity to tolerate excessive NH_4^+ applications (Britto & Kronzucker, 2002), NH_4^+ toxicity symptoms were already documented over a century ago (Esteban et al., 2016). As global applications of nitrogen fertilizer increased about 730% during the past 50 years from 15 to 110 Tg-N, and the fraction of NH_4^+-based fertilizer applied increased from 65% in 1961 to 91% of total nitrogen fertilizer inputs in 2010 (Nishina et al., 2017). NH_4^+-based compounds such as anhydrous ammonia, ammonium sulfate, and urea are common fertilizers used in rice production (Fageria et al., 2011). High field nitrogen applications may result in a zone of 1 to 5 cm with high transient soil NH_4^+ concentrations that are likely detrimental to the plants (Pan et al., 2016). In particular, root growth of rice seedlings in the field can be vulnerable to toxicity from excessive NH_4^+inputs (Qi et al., 2012). In our experiment, root biomass was also most sensitive to changes in NH_4^+ concentrations (Figure 4). Therefore, NH_4^+-based fertilizers require careful application to minimize toxicity.

Natural variation in rice tolerance to NH_4^+ serve as a tool for improving nitrogen use efficiency and productivity. NH_4^+-intolerant cultivars expend additional energy to avoid NH_4^+ accumulation (Chen et al., 2013). Accessions with higher tolerance to high NH_4^+ concentrations may therefore have higher nitrogen use efficiency. Some genotypes in our study displayed faster growth at 10 mM NH_4^+ than at lower concentrations (Figure S2B).

Our findings implied that tolerance to high NH_4^+ or preference for a certain nitrogen form does not seem to come at a cost of lower nitrogen use efficiency for other forms. The positive correlations among biomass traits across nitrogen forms and concentrations in our study suggested that many of these traits may be helpful for identifying genotypes with superior nitrogen utilization (Figure S1).

Coordination of carbon and nitrogen assimilation helps rice adapt to environmental changes

Our large diversity panel provided insights into strategies that rice employs to use different inorganic nitrogen sources. These strategies may reflect local adaptation of accessions with different genetic backgrounds, for example, by varying biomass partitioning between different organs. Increased nitrogen availability generally enhances shoot carbon assimilation and subsequent partitioning into the soil (Xiao et al., 2019). Our findings confirmed that rice varied root biomass partitioning in response to different nitrogen sources. Particularly, the INDICA varietal group exhibited great variations in whole plant biomass (Figure 2A-C), with generally higher proportions of biomass allocated to root (Figure 3). Greater relative root growth may underlie the superior nitrogen responsiveness of INDICA.

Shifting of carbon into the soil may help rice regulate nitrogen assimilation through interactions with microorganisms. Actively shaping and interacting with microbiota surrounding the root zone throughout different growth stages (J. Edwards et al., 2019; J. A. Edwards et al., 2018), rice leverages its capacity to nurture soil nitrifying bacteria populations by increasing root biomass and porosity (Ghosh & Kashyap, 2003). As such, INDICA's variant of *NRT1.1B,* a nitrate transporter gene, recruits more ammonification microbes than that of JAPONICA varieties (J. Zhang et al., 2019). Although root NH_4^+ uptake appears to be similar in INDICA and JAPONICA (Sun et al., 2016), divergence of *NRT1.1B* allows INDICA to better absorb and use NO_3^- than JAPONICA (Hu et al., 2015, p. 1). As a result, rice makes use of the more ubiquitous NH_4^+ and regulates the balance between NH_4^+ and NO_3^- through control of the local microbial population (Ghosh & Kashyap, 2003; Y. L. Li, Fan, et al., 2007; Y. L. Li, Zhang, et al., 2007). Indeed, soil nitrogen mineralization, nitrification rate, and nitrifier abundance seem to be positively correlated with grain yields (Ghosh & Kashyap, 2003). Better understanding of underlying mechanisms through which rice controls and responds to rhizosphere nitrogen forms should facilitate breeding for improved nitrogen use efficiency.

At elevated atmospheric CO_2 levels expected in the near future, solving the complexity of crop carbon and nitrogen coordination in response to nitrogen availability becomes even more critical. Up to 40% of rice total nitrogen acquisition may in fact be supplied by NO_3^- (Kirk & Kronzucker, 2005). Under the current atmospheric CO_2 concentration, fields with more nitrifying microbes also had higher yields (Zhong et al., 2020). But, reliance on NO_3^- as a major nitrogen source may make food crops vulnerable to future conditions, because rising atmospheric CO_2 levels inhibit NO_3^- assimilation into protein in the shoots (Bloom, 2015). Recently, *Teosinte branched1, Cycloidea, Proliferating cell factor 19* (*TCP19*) was identified as a modulator targeting the tiller-promoting *DWARF AND LOW-TILLERING* (*DLT*) gene responsive to soil nitrogen concentration (Y. Liu et al., 2021). Specifically, gene expression assay of *OsTCP19* suggested that rice geographical adaptation likely followed changes in soil NO_3^-, rather than NH_4^+ concentration (Y. Liu et al., 2021). Our study suggested that rice may be well-adapted to both nitrogen forms, despite the prevalence of NH_4^+ in the growth environments. Nevertheless, higher shoot biomass ratio implies that the INDICA varietal group may have adapted to using NO_3^- better than NH_4^+ when compared to the JAPONICA varietal group (Figure 4D). Contribution of different forms to the total plant nitrogen pool requires further investigations to help us understand how vulnerable different genetic backgrounds may be to the anticipated climate changes.

Nitrogen plasticity—rice can use both ammonium and nitrate through distinct genetic mechanisms

Plants are likely exposed simultaneously to multiple nitrogen forms in nature and field conditions. Crop management and water availability generally influence variations in soil NO_3^-, but less so for variations in NH_4^+ (George et al., 1993). Because NH_4^+ as a cation binds to most soil particles, which are generally negatively charged, it is usually less mobile in the rhizosphere than NO_3^-, NH_4^+ concentration declines with distance from the root, whereas NO_3^- concentration remains relatively consistent (Y. L. Li, Fan, et al., 2007; Y. L. Li, Zhang, et al., 2007). Although the relative availability of various nitrogen sources can change precipitously, most tropical plants develop the ability to take advantage of the most available form of nitrogen in their environment (Houlton et al., 2007). We found that most rice accessions accumulated comparable whole plant biomass when provided with a non-toxic level of either NH_4^+ or NO_3^- (Figure 2). Rather than specializing on a certain form of nitrogen, plants may benefit more from being a ‘flexitarian’, a user of all nitrogen forms, allowing them to more readily cope with environmental changes (Houlton et al., 2007). Such plasticity in nitrogen form utilization may help rice stay resilient to environmental changes by readily shifting their major nitrogen source.

Our study identified mostly unique sets of loci that are involved with rice carbon assimilation in response to different inorganic nitrogen forms and concentrations (Figure 5C, Table S3-S5). Distinct uptake mechanisms for NH_4^+ and NO_3^- at different concentrations (Sasakawa & Yamamoto, 1978) should help rice adjust to the highly variable nature of soil nitrogen balance. A previous GWAS with a large collection of 1135 *Arabidopsis thaliana* accessions also identified primarily unique, rather than shared, sets of genes for each specific nitrogen form (Katz et al., 2022). Recent rice root transcriptomic analyses that examined combined effects of both NO_3^- and NH_4^+

revealed that expression of key NO_3^--responsive genes were selectively induced 5.2 to 65.8 fold (H.-C. Yang et al., 2017). Most previous studies, however, focused primarily on a few candidate genes with large effects, rather than exploring a potentially complex genetic regulatory network that underpins nitrogen responses (Kasemsap & Bloom, 2022; Katz et al., 2022). Therefore, it is important to examine and cross-validate significant loci across nitrogen forms.

Conclusions

In summary, our genome-wide association analyses demonstrated substantial natural variations in population-specific biomass responses to nitrogen at an early growth stage within the global rice germplasm and identified candidate genes putatively involved in nitrogen form-dependent growth. Most rice accessions are well-adapted to using either form of inorganic nitrogen, which may involve distinct genetic mechanisms. Plasticity in nitrogen utilization may help rice better adapt to environmental changes. These efforts to find the best match that maximize interactions between crop genetic potentials and nitrogen inputs remain the key goals in breeding for climate-resilient crops.

List of abbreviations

- *Abnormal cytokinin response1 REpressor 1* (*ARE1*)
- *Amino acid permease 3* (*AAP3*)
- *Amino acid permease 4* (*AAP4*)
- *Amino acid permease 5* (*AAP5*)
- Ammonium (NH_4^+)
- Analysis of variance (ANOVA)
- Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway (BLINK)
- *DWARF AND LOW-TILLERING* (*DLT*)
- Fixed and random model Circulating Probability Unification (FarmCPU)
- Gene Ontology (GO)
- Genome-wide association studies (GWAS)
- General Linear Model (GLM)
- *High-affinity nitrate transporter 2.1* (*NRT1.1B*)
- *High-affinity nitrate transporter-activating protein 2.1* (*NAR2.1*)
- Minor Allele Frequency (MAF)

- Mixed Linear Model (MLM)
- Nitrate (NO_3^-),
- *Nitrate reductase 2* (*NR2*)
- Nitrogen Use Efficiency (NUE)
- Principal components (PC)
- Quantile-quantile (Q-Q)
- Quantitative trait loci (QTL)
- Single nucleotide polymorphism (SNP)
- *Teosinte branched1, Cycloidea, Proliferating cell factor 19* (*TCP19*)
- Tukey's honestly significant difference (Tukey's HSD)
- USDA Rice Diversity Panel 1 (RDP1)

Acknowledgments

We acknowledge the land on which we conducted the experiments. For thousands of years, this land has been the home of Patwin people. We thank Ella Katz for a discussion on result visualization (Figure S2), Junli Zhang for a discussion on GWAS analyses, Kreingkrai Nonkum for help with remote workstations, Daniel Kliebenstein for a discussion on rice genetic variations, Jorge Dubcovsky and David J. Mackill for feedback on the manuscript, and Thomas H. Tai for feedback on the manuscript and for multiplying seeds from the original USDA collection. We greatly appreciate the assistance of Joshua J. Claxton, Dana R. Lawrence, Jenna O'Kelley, Sydney Koelbel, James K. Schmidt, Guilherme M. Silva, Jordan A. Stefani, Samuel Doolittle and Timothy Congleton on plant maintenance and biomass measurements in this study.

Data statement

All main findings generated and analyzed during this study are included in this published article, and its Supplementary Materials. The biomass dataset was deposited at https://doi.org/10.25338/B8JP8C. Analysis R codes are available at https://www.github.com/paulkasemsap/RDP1_Nform.

Competing Interests

The authors declare that they have no competing interests.

Funding

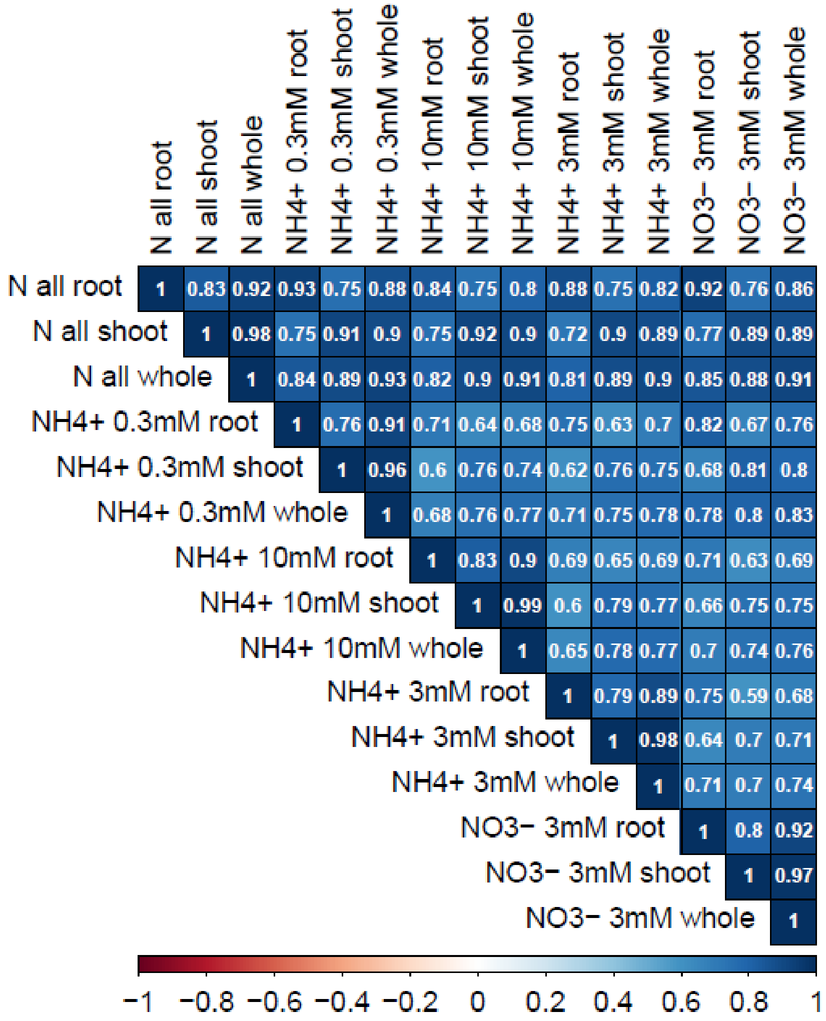
This work was supported in part by the John B. Orr Endowment and NSF IOS-16-55810 (to Arnold J. Bloom), Thailand – United States Educational Foundation/Fulbright Thailand, UC Davis Department of Plant Sciences Graduate Student Researcher fellowship, Henry A. Jastro research award, Innovation Institute for Food and Health Graduate Innovator Fellowship (to Pornpipat Kasemsap), and United States-Israel Binational Agricultural Research and Development Fund (to Itay Cohen).

Authors’ contributions

PK performed all analyses and wrote the first draft of the manuscript. IC planned and conducted biomass experiments. AJB conceived the project and obtained funding. All authors reviewed and edited the manuscript.

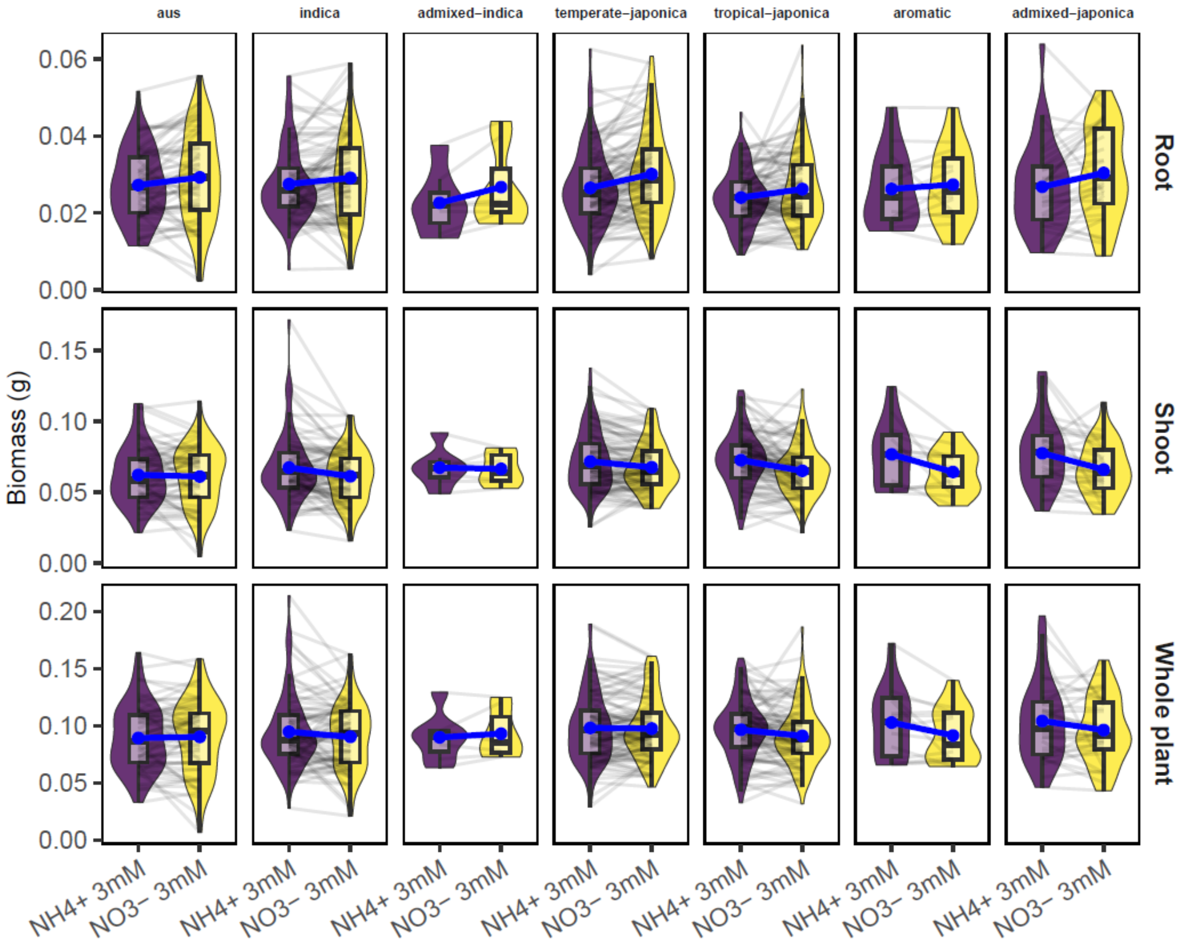
Supplementary Materials

Supplementary Figure 1 Correlation on biomass

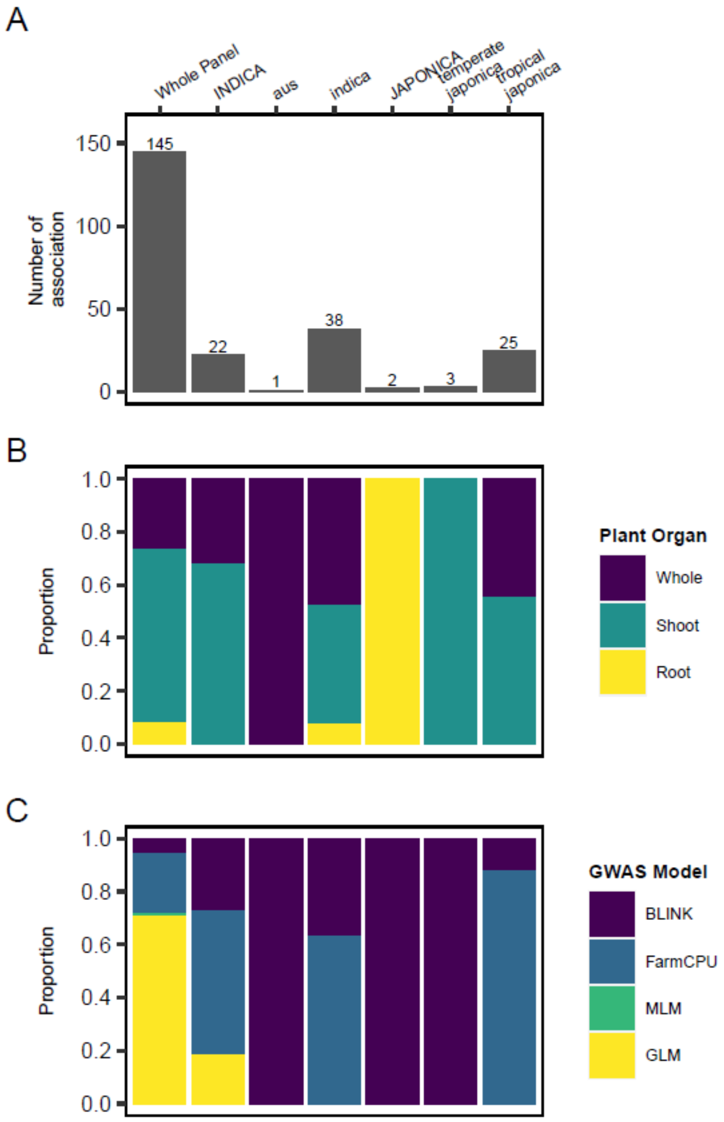


	N all root	N all shoot	N all whole	NH4+ 0.3mM root	NH4+ 0.3mM shoot	NH4+ 0.3mM whole	NH4+ 10mM root	NH4+ 10mM shoot	NH4+ 10mM whole	NH4+ 3mM root	NH4+ 3mM shoot	NH4+ 3mM whole	NO3− 3mM root	NO3− 3mM shoot	NO3− 3mM whole
N all root	1	0.83	0.92	0.93	0.75	0.88	0.84	0.75	0.8	0.88	0.75	0.82	0.92	0.76	0.86
N all shoot		1	0.98	0.75	0.91	0.9	0.75	0.92	0.9	0.72	0.9	0.89	0.77	0.89	0.89
N all whole			1	0.84	0.89	0.93	0.82	0.9	0.91	0.81	0.89	0.9	0.85	0.88	0.91
NH4+ 0.3mM root				1	0.76	0.91	0.71	0.64	0.68	0.75	0.63	0.7	0.82	0.67	0.76
NH4+ 0.3mM shoot					1	0.96	0.6	0.76	0.74	0.62	0.76	0.75	0.68	0.81	0.8
NH4+ 0.3mM whole						1	0.68	0.76	0.77	0.71	0.75	0.78	0.78	0.8	0.83
NH4+ 10mM root							1	0.83	0.9	0.69	0.65	0.69	0.71	0.63	0.69
NH4+ 10mM shoot								1	0.99	0.6	0.79	0.77	0.66	0.75	0.75
NH4+ 10mM whole									1	0.65	0.78	0.77	0.7	0.74	0.76
NH4+ 3mM root										1	0.79	0.89	0.75	0.59	0.68
NH4+ 3mM shoot											1	0.98	0.64	0.7	0.71
NH4+ 3mM whole												1	0.71	0.7	0.74
NO3− 3mM root													1	0.8	0.92
NO3− 3mM shoot														1	0.97
NO3− 3mM whole															1

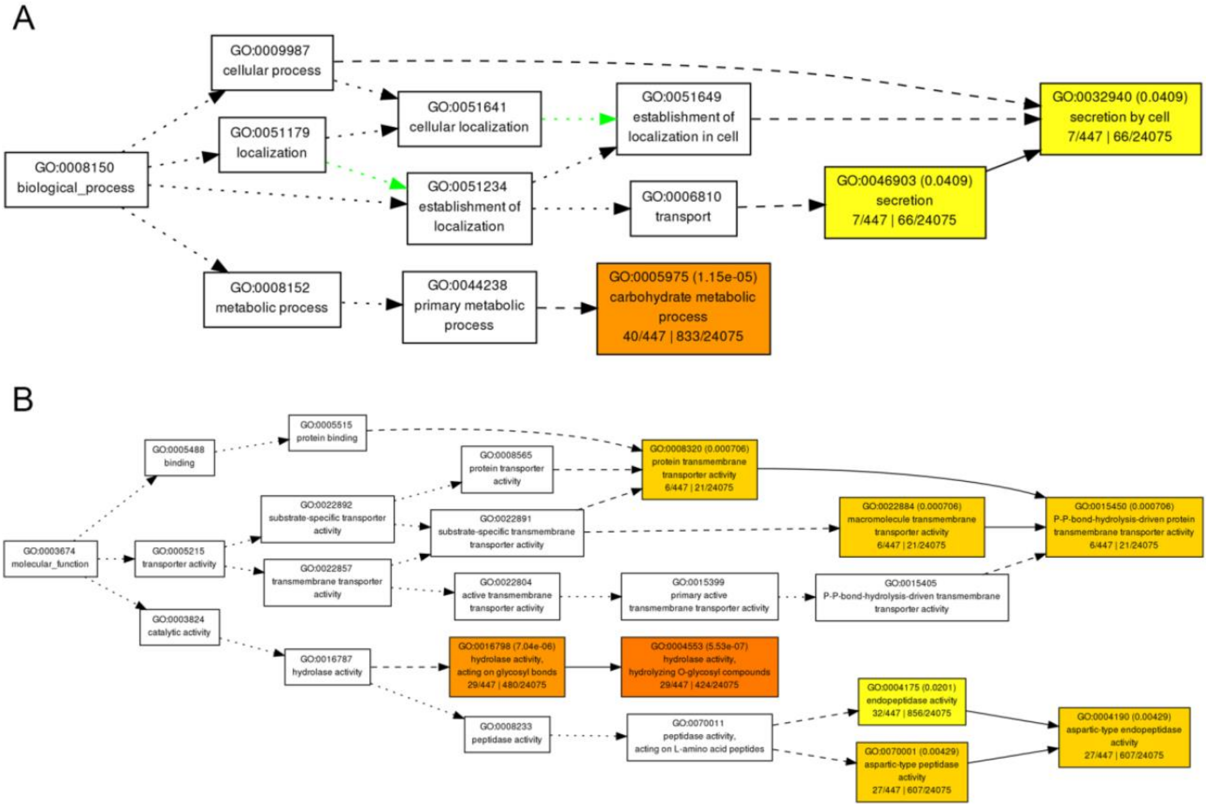
Supplementary Figure 2 Biomass distribution by organ and subpopulation compared to the reference nitrogen



Supplementary Figure 3 Proportion of significant markers by organ and model



Supplementary Figure 4 Gene ontology enrichment



The following materials are available as supplementary files and online at https://doi.org/10.1101/2024.08.12.607622

Supplementary Data 1 Accession list

Supplementary Data 2 Quantile normalized residuals for GWAS

Supplementary Data 3 Number of significant markers

Supplementary Table 1 P-values from ANOVA

Supplementary Table 2 Mean separations from Tukey’s HSD tests

Supplementary Table 3 Significant SNPs

Supplementary Table 4 Experiment-wise significant SNPs with at least 3 adjacent markers

Supplementary Table 5 Candidate genes

Supplementary Table 6 Gene ontology enrichment analysis

References

Alexandrov, N. N., Brover, V. V., Freidin, S., Troukhan, M. E., Tatarinova, T. V., Zhang, H., Swaller, T. J., Lu, Y.-P., Bouck, J., Flavell, R. B., & Feldmann, K. A. (2009). Insights into corn genes derived from large-scale cDNA sequencing. *Plant Molecular Biology*, *69*(1–2), 179–194. https://doi.org/10.1007/s11103-008-9415-4

Andrews, M., Raven, J. A., & Lea, P. J. (2013). Do plants need nitrate? The mechanisms by which nitrogen form affects plants: Do plants need nitrate? *Annals of Applied Biology*, *163*(2), 174–199. https://doi.org/10.1111/aab.12045

Araus, V., Swift, J., Alvarez, J. M., Henry, A., & Coruzzi, G. M. (2020). A balancing act: How plants integrate nitrogen and water signals. *Journal of Experimental Botany*, *71*(15), 4442–4451. https://doi.org/10.1093/jxb/eraa054

Asensio, J. S. R., Rachmilevitch, S., & Bloom, A. J. (2015). Responses of Arabidopsis and Wheat to Rising CO $_2$ Depend on Nitrogen Source and Nighttime CO $_2$ Levels. *Plant Physiology*, *168*(1), 156–163. https://doi.org/10.1104/pp.15.00110

Bloom. (2015). The increasing importance of distinguishing among plant nitrogen sources. *Current Opinion in Plant Biology*, *25*, 10–16. http://dx.doi.org/10.1016/j.pbi.2015.03.002

Britto, D. T., & Kronzucker, H. J. (2002). NH4+ toxicity in higher plants: A critical review. *Journal of Plant Physiology*, *159*(6), 567–584. http://dx.doi.org/10.1078/0176-1617-0774

Britto, D. T., & Kronzucker, H. J. (2013). Ecological significance and complexity of N-source preference in plants. *Annals of Botany*, *112*(6), 957–963. https://doi.org/10.1093/aob/mct157

Burger, M., & Jackson, L. E. (2003). Microbial immobilization of ammonium and nitrate in relation to ammonification and nitrification rates in organic and conventional cropping systems. *Soil Biology and Biochemistry*, *35*(1), 29–36. https://doi.org/10.1016/S0038-0717(02)00233-X

Chen, G., Guo, S., Kronzucker, H. J., & Shi, W. (2013). Nitrogen use efficiency (NUE) in rice links to NH4 + toxicity and futile NH4 + cycling in roots. *Plant and Soil*, *369*(1), 351–363. https://doi.org/10.1007/s11104-012-1575-y

Crowell, S., Korniliev, P., Falcão, A., Ismail, A., Gregorio, G., Mezey, J., & McCouch, S. (2016). Genome-wide association and high-resolution phenotyping link Oryza sativa panicle traits to numerous trait-specific QTL clusters. *Nature Communications*, *7*(1), 10527. https://doi.org/10.1038/ncomms10527

Devienne-Barret, F. (2000). Integrated Control of Nitrate Uptake by Crop Growth Rate and Soil Nitrate Availability under Field Conditions. *Annals of Botany*, *86*(5), 995–1005. https://doi.org/10.1006/anbo.2000.1264

Di, D.-W., Sun, L., Zhang, X., Li, G., Kronzucker, H. J., & Shi, W. (2018). Involvement of auxin in the regulation of ammonium tolerance in rice (Oryza sativa L.). *Plant and Soil*, *432*(1–2), 373–387. https://doi.org/10.1007/s11104-018-3813-4

Dowle, M., & Srinivasan, A. (2023). *data.table: Extension of `data.frame`*. https://r-datatable.com, https://Rdatatable.gitlab.io/data.table, https://github.com/Rdatatable/data.table

Edwards, J. A., Santos-Medellín, C. M., Liechty, Z. S., Nguyen, B., Lurie, E., Eason, S., Phillips, G., & Sundaresan, V. (2018). Compositional shifts in root-associated bacterial and archaeal microbiota track the plant life cycle in field-grown rice. *PLOS Biology*, *16*(2), e2003862. https://doi.org/10.1371/journal.pbio.2003862

Edwards, J., Santos-Medellín, C., Nguyen, B., Kilmer, J., Liechty, Z., Veliz, E., Ni, J., Phillips, G., & Sundaresan, V. (2019). Soil domestication by rice cultivation results in plant-soil feedback through shifts in soil microbiota. *Genome Biology*, *20*(1), 221. https://doi.org/10.1186/s13059-019-1825-x

Eizenga, G. C., Ali, Md. L., Bryant, R. J., Yeater, K. M., McClung, A. M., & McCouch, S. R. (2014). Registration of the Rice Diversity Panel 1 for Genomewide Association Studies. *Journal of Plant Registrations*, *8*(1), 109–116. https://doi.org/10.3198/jpr2013.03.0013crmp

Epstein, E., & Bloom, A. J. (2005). *Mineral Nutrition of Plants: Principles and Perspectives*. Sinauer Associates, Incorporated.

Esteban, R., Ariz, I., Cruz, C., & Moran, J. F. (2016). Review: Mechanisms of ammonium toxicity and the quest for tolerance. *Plant Science*, *248*, 92–101. https://doi.org/10.1016/j.plantsci.2016.04.008

Fageria, N. K. (2007). Yield Physiology of Rice. *Journal of Plant Nutrition*, *30*(6), 843–879. https://doi.org/10.1080/15226510701374831

Fageria, N. K., & Baligar, V. C. (2003). Methodology for Evaluation of Lowland Rice Genotypes for Nitrogen Use Efficiency. *Journal of Plant Nutrition*, *26*(6), 1315–1333. https://doi.org/10.1081/PLN-120020373

Fageria, N. K., Moreira, A., & Coelho, A. M. (2011). YIELD AND YIELD COMPONENTS OF UPLAND RICE AS INFLUENCED BY NITROGEN SOURCES. *Journal of Plant Nutrition*, *34*(3), 361–370. https://doi.org/10.1080/01904167.2011.536878

FAOSTAT. (2022). *Food Supply: Crops Primary Equivalent.*

Fox, J., & Weisberg, S. (2019). *An R companion to applied regression* (Third edition). SAGE.

Gao, Z., Wang, Y., Chen, G., Zhang, A., Yang, S., Shang, L., Wang, D., Ruan, B., Liu, C., Jiang, H., Dong, G., Zhu, L., Hu, J., Zhang, G., Zeng, D., Guo, L., Xu, G., Teng, S., Harberd, N. P., & Qian, Q. (2019). The indica nitrate reductase gene OsNR2 allele enhances rice yield potential and nitrogen use efficiency. *Nature Communications*, *10*(1), 5207. https://doi.org/10.1038/s41467-019-13110-8

George, T., Ladha, J. K., Buresh, R. J., & Garrity, D. P. (1993). Nitrate Dynamics during the Aerobic Soil Phase in Lowland Rice-Based Cropping Systems. *Soil Science Society of America Journal*, *57*(6), 1526–1532. https://doi.org/10.2136/sssaj1993.03615995005700060022x

Ghasemi, A., & Zahediasl, S. (2012). Normality Tests for Statistical Analysis: A Guide for Non-Statisticians. *International Journal of Endocrinology and Metabolism*, *10*(2), 486–489. https://doi.org/10.5812/ijem.3505

Ghosh, P., & Kashyap, A. K. (2003). Effect of rice cultivars on rate of N-mineralization, nitrification and nitrifier population size in an irrigated rice ecosystem. *Applied Soil Ecology*, *24*(1), 27–41. https://doi.org/10.1016/S0929-1393(03)00068-4

Gonzalez-Dugo, V., Durand, J.-L., & Gastal, F. (2010). Water deficit and nitrogen nutrition of crops. A review. *Agronomy for Sustainable Development*, *30*(3), 529–544. https://doi.org/10.1051/agro/2009059

Hothorn, T., Bretz, F., & Westfall, P. (2008). *Simultaneous Inference in General Parametric Models*. *50*(3), 346–363.

Houlton, B. Z., Sigman, D. M., Schuur, E. A. G., & Hedin, L. O. (2007). A climate-driven switch in plant nitrogen acquisition within tropical forest communities. *Proceedings of the National Academy of Sciences*, *104*(21), 8902–8906. https://doi.org/10.1073/pnas.0609935104

Hu, B., Wang, W., Ou, S., Tang, J., Li, H., Che, R., Zhang, Z., Chai, X., Wang, H., Wang, Y., Liang, C., Liu, L., Piao, Z., Deng, Q., Deng, K., Xu, C., Liang, Y., Zhang, L., Li, L., & Chu, C. (2015). Variation in NRT1.1B contributes to nitrate-use divergence between rice subspecies. *Nature Genetics*, *47*(7), 834–838. https://doi.org/10.1038/ng.3337

Huang, M., Liu, X., Zhou, Y., Summers, R. M., & Zhang, Z. (2019). BLINK: A package for the next level of genome-wide association studies with both individuals and markers in the millions. *GigaScience*, *8*(2). https://doi.org/10.1093/gigascience/giy154

Huang, X., & Han, B. (2014). Natural Variations and Genome-Wide Association Studies in Crop Plants. *Annual Review of Plant Biology*, *65*(1), 531–551. https://doi.org/10.1146/annurev-arplant-050213-035715

Jia, L., Xie, Y., Wang, Z., Luo, L., Zhang, C., Pélissier, P., Parizot, B., Qi, W., Zhang, J., Hu, Z., Motte, H., Luo, L., Xu, G., Beeckman, T., & Xuan, W. (2020). Rice plants respond to ammonium stress by adopting a helical root growth pattern. *The Plant Journal*, *104*(4), 1023–1037. https://doi.org/10.1111/tpj.14978

Kasemsap, P., & Bloom, A. J. (2022). Breeding for Higher Yields of Wheat and Rice through Modifying Nitrogen Metabolism. *Plants*, *12*(1), 85. https://doi.org/10.3390/plants12010085

Katz, E., Knapp, A., Lensink, M., Keller, C. K., Stefani, J., Li, J.-J., Shane, E., Tuermer-Lee, K., Bloom, A. J., & Kliebenstein, D. J. (2022). Genetic variation underlying differential ammonium and nitrate responses in *Arabidopsis thaliana*. *The Plant Cell*, *34*(12), 4696–4713. https://doi.org/10.1093/plcell/koac279

Kawahara, Y., de la Bastide, M., Hamilton, J. P., Kanamori, H., McCombie, W. R., Ouyang, S., Schwartz, D. C., Tanaka, T., Wu, J., Zhou, S., Childs, K. L., Davidson, R. M., Lin, H., Quesada-Ocampo, L., Vaillancourt, B., Sakai, H., Lee, S. S., Kim, J., Numa, H., … Matsumoto, T. (2013). Improvement of the Oryza sativa Nipponbare reference genome using next generation sequence and optical map data. *Rice*, *6*(1), 4. https://doi.org/10.1186/1939-8433-6-4

Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M., & Haussler, and D. (2002). The Human Genome Browser at UCSC. *Genome Research*, *12*(6), 996–1006. https://doi.org/10.1101/gr.229102

Kirk, G. J. D., & Kronzucker, H. J. (2005). The Potential for Nitrification and Nitrate Uptake in the Rhizosphere of Wetland Plants: A Modelling Study. *Annals of Botany*, *96*(4), 639–646. https://doi.org/10.1093/aob/mci216

Kronzucker, H. J., Glass, A. D. M., Siddiqi, M. Y., & Kirk, G. J. D. (2000). Comparative kinetic analysis of ammonium and nitrate acquisition by tropical lowland rice: Implications for rice cultivation and yield potential: Nitrate acquisition by rice. *New Phytologist*, *145*(3), 471–476. https://doi.org/10.1046/j.1469-8137.2000.00606.x

Kumar, A., Verulkar, S., Dixit, S., Chauhan, B., Bernier, J., Venuprasad, R., Zhao, D., & Shrivastava, M. N. (2009). Yield and yield-attributing traits of rice (Oryza sativa L.) under lowland drought and suitability of early vigor as a selection criterion. *Field Crops Research*, *114*(1), 99–107. https://doi.org/10.1016/j.fcr.2009.07.010

Kuzyakov, Y., & Xu, X. (2013). Competition between roots and microorganisms for nitrogen: Mechanisms and ecological relevance. *New Phytologist*, *198*(3), 656–669. https://doi.org/10.1111/nph.12235

Ladha, J. K., Tirol-Padre, A., Reddy, C. K., Cassman, K. G., Verma, S., Powlson, D. S., van Kessel, C., de B. Richter, D., Chakraborty, D., & Pathak, H. (2016). Global nitrogen budgets in cereals: A 50-year assessment for maize, rice and wheat production systems. *Scientific Reports*, *6*(1), 19355. https://doi.org/10.1038/srep19355

Langsrud, Ø. (2003). ANOVA for unbalanced data: Use Type II instead of Type III sums of squares. *Statistics and Computing*, *13*(2), 163–167. https://doi.org/10.1023/A:1023260610025

Lemaire, G., & Ciampitti, I. (2020). Crop Mass and N Status as Prerequisite Covariables for Unraveling Nitrogen Use Efficiency across Genotype-by-Environment-by-Management Scenarios: A Review. *Plants*, *9*(10), 1309. https://doi.org/10.3390/plants9101309

Lemaire, G., Oosterom, E. van, Sheehy, J., Jeuffroy, M. H., Massignam, A., & Rossato, L. (2007). Is crop N demand more closely related to dry matter accumulation or leaf area expansion during vegetative growth? *Field Crops Research*, *100*(1), 91–106. https://doi.org/10.1016/j.fcr.2006.05.009

Lenth, R. V. (2023). *emmeans: Estimated Marginal Means, aka Least-Squares Means* (R package version 1.8.6). https://CRAN.R-project.org/package=emmeans

Li, B., Zhang, Y., Mohammadi, S. A., Huai, D., Zhou, Y., & Kliebenstein, D. J. (2016). An Integrative Genetic Study of Rice Metabolism, Growth and Stochastic Variation Reveals Potential C/N Partitioning Loci. *Scientific Reports*, *6*(1), 30143. https://doi.org/10.1038/srep30143

Li, Y. L., Fan, X. R., & Shen, Q. R. (2007). The relationship between rhizosphere nitrification and nitrogen-use efficiency in rice plants. *Plant, Cell & Environment*, *0*(0), 071018035446001-??? https://doi.org/10.1111/j.1365-3040.2007.01737.x

Li, Y. L., Zhang, Y. L., Hu, J., & Shen, Q. R. (2007). Contribution of nitrification happened in rhizospheric soil growing with different rice cultivars to N nutrition. *Biology and Fertility of Soils*, *43*(4), 417–425. https://doi.org/10.1007/s00374-006-0119-0

Liu, X., Huang, M., Fan, B., Buckler, E. S., & Zhang, Z. (2016). Iterative Usage of Fixed and Random Effect Models for Powerful and Efficient Genome-Wide Association Studies. *PLOS Genetics*, *12*(2), e1005767. https://doi.org/10.1371/journal.pgen.1005767

Liu, Y., Wang, H., Jiang, Z., Wang, W., Xu, R., Wang, Q., Zhang, Z., Li, A., Liang, Y., Ou, S., Liu, X., Cao, S., Tong, H., Wang, Y., Zhou, F., Liao, H., Hu, B., & Chu, C. (2021). Genomic basis of geographical adaptation to soil nitrogen in rice. *Nature*. https://doi.org/10.1038/s41586-020-03091-w

Lu, K., Wu, B., Wang, J., Zhu, W., Nie, H., Qian, J., Huang, W., & Fang, Z. (2018). Blocking amino acid transporter *Os AAP 3* improves grain yield by promoting outgrowth buds and increasing tiller number in rice. *Plant Biotechnology Journal*, *16*(10), 1710–1722. https://doi.org/10.1111/pbi.12907

Lumley, T., Diehr, P., Emerson, S., & Chen, L. (2002). The Importance of the Normality Assumption in Large Public Health Data Sets. *Annual Review of Public Health*, *23*(1), 151–169. https://doi.org/10.1146/annurev.publhealth.23.100901.140546

Luo, L., Zhu, M., Jia, L., Xie, Y., Wang, Z., & Xuan, W. (2022). Ammonium transporters cooperatively regulate rice crown root formation responding to ammonium nitrogen. *Journal of Experimental Botany*, erac059. https://doi.org/10.1093/jxb/erac059

Magwa, R. A., Zhao, H., & Xing, Y. (2016). Genome-wide association mapping revealed a diverse genetic basis of seed dormancy across subpopulations in rice (Oryza sativa L.). *BMC Genetics*, *17*(1), 28. https://doi.org/10.1186/s12863-016-0340-2

McCaw, Z. R., Lane, J. M., Saxena, R., Redline, S., & Lin, X. (2020). Operating characteristics of the rank-based inverse normal transformation for quantitative trait analysis in genome-wide association studies. *Biometrics*, *76*(4), 1262–1272. https://doi.org/10.1111/biom.13214

McCouch, S. R., Wright, M. H., Tung, C.-W., Maron, L. G., McNally, K. L., Fitzgerald, M., Singh, N., DeClerck, G., Agosto-Perez, F., Korniliev, P., Greenberg, A. J., Naredo, Ma. E. B., Mercado, S. M. Q., Harrington, S. E., Shi, Y., Branchini, D. A., Kuser-Falcão, P. R., Leung, H., Ebana, K., … Mezey, J. (2016). Open access resources for genome-wide association mapping in rice. *Nature Communications*, *7*(1), 10532. https://doi.org/10.1038/ncomms10532

Melillo, E. D. (2012). The First Green Revolution: Debt Peonage and the Making of the Nitrogen Fertilizer Trade, 1840–1930. *The American Historical Review*, *117*(4), 1028–1060. https://doi.org/10.1093/ahr/117.4.1028

Namuco, O. S., Cairns, J. E., & Johnson, D. E. (2009). Investigating early vigour in upland rice (Oryza sativa L.): Part I. Seedling growth and grain yield in competition with weeds. *Field Crops Research*, *113*(3), 197–206. https://doi.org/10.1016/j.fcr.2009.05.008

Näsholm, T., Kielland, K., & Ganeteg, U. (2009). Uptake of organic nitrogen by plants: Tansley review. *New Phytologist*, *182*(1), 31–48. https://doi.org/10.1111/j.1469-8137.2008.02751.x

Nishina, K., Ito, A., Hanasaki, N., & Hayashi, S. (2017). Reconstruction of spatially detailed global map of NH<sub>4</sub><sup>+</sup> and

NO<sub>3</sub><sup>−</sup> application in synthetic nitrogen fertilizer. *Earth System Science Data*, *9*(1), 149–162. https://doi.org/10.5194/essd-9-149-2017

Pan, W. L., Madsen, I. J., Bolton, R. P., Graves, L., & Sistrunk, T. (2016). Ammonia/Ammonium Toxicity Root Symptoms Induced by Inorganic and Organic Fertilizers and Placement. *Agronomy Journal*, *108*(6), 2485–2492. https://doi.org/10.2134/agronj2016.02.0122

Paterson, A. H., Bowers, J. E., Bruggmann, R., Dubchak, I., Grimwood, J., Gundlach, H., Haberer, G., Hellsten, U., Mitros, T., Poliakov, A., Schmutz, J., Spannagl, M., Tang, H., Wang, X., Wicker, T., Bharti, A. K., Chapman, J., Feltus, F. A., Gowik, U., … Rokhsar, D. S. (2009). The Sorghum bicolor genome and the diversification of grasses. *Nature*, *457*(7229), 551–556. https://doi.org/10.1038/nature07723

Pirinen, M. (2023, March 2). *GWAS 1: What is a GWAS?* GWAS 1: What Is a GWAS? https://www.mv.helsinki.fi/home/mjxpirin/GWAS_course/material/GWAS1.html

Price, A. L., Patterson, N. J., Plenge, R. M., Weinblatt, M. E., Shadick, N. A., & Reich, D. (2006). Principal components analysis corrects for stratification in genome-wide association studies. *Nature Genetics*, *38*(8), 904–909. https://doi.org/10.1038/ng1847

Qi, X., Wu, W., Shah, F., Peng, S., Huang, J., Cui, K., Liu, H., & Nie, L. (2012). Ammonia Volatilization from Urea-Application Influenced Germination and Early Seedling Growth of Dry Direct-Seeded Rice. *The Scientific World Journal*, *2012*, 1–7. https://doi.org/10.1100/2012/857472

R Core Team,. (2023). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. https://www.R-project.org/

RStudio Team. (2020). *RStudio: Integrated Development for R*. http://www.rstudio.com/.

Sadras, V. O., & Lemaire, G. (2014). Quantifying crop nitrogen status for comparisons of agronomic practices and genotypes. *Field Crops Research*, *164*, 54–64. https://doi.org/10.1016/j.fcr.2014.05.006

Sasakawa, H., & Yamamoto, Y. (1978). Comparison of the Uptake of Nitrate and Ammonium by Rice Seedlings: Influences of Light, Temperature, Oxygen Concentration, Exogenous Sucrose, and Metabolic Inhibitors. *Plant Physiology*, *62*(4), 665–669. https://doi.org/10.1104/pp.62.4.665

Segura, V., Vilhjálmsson, B. J., Platt, A., Korte, A., Seren, Ü., Long, Q., & Nordborg, M. (2012). An efficient multi-locus mixed-model approach for genome-wide association studies in structured populations. *Nature Genetics*, *44*(7), 825–830. https://doi.org/10.1038/ng.2314

Shakiba, E., Edwards, J. D., Jodari, F., Duke, S. E., Baldo, A. M., Korniliev, P., McCouch, S. R., & Eizenga, G. C. (2017). Genetic architecture of cold tolerance in rice (Oryza sativa) determined through high resolution genome-wide analysis. *PLOS ONE*, *12*(3), e0172133. https://doi.org/10.1371/journal.pone.0172133

Sun, L., Lu, Y., Yu, F., Kronzucker, H. J., & Shi, W. (2016). Biological nitrification inhibition by rice root exudates and its relationship with nitrogen-use efficiency. *New Phytologist*, *212*(3), 646–656. https://doi.org/10.1111/nph.14057

Tabuchi, M., Abiko, T., & Yamaya, T. (2007). Assimilation of ammonium ions and reutilization of nitrogen in rice (Oryza sativa L.). *Journal of Experimental Botany*, *58*(9), 2319–2327. https://doi.org/10.1093/jxb/erm016

Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z., Xu, W., & Su, Z. (2017). agriGO v2.0: A GO analysis toolkit for the agricultural community, 2017 update. *Nucleic Acids Research*, *45*(W1), W122–W129. https://doi.org/10.1093/nar/gkx382

Tilman, D., Balzer, C., Hill, J., & Befort, B. L. (2011). Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences*, *108*(50), 20260–20264. https://doi.org/10.1073/pnas.1116437108

Ueda, Y., Ohtsuki, N., Kadota, K., Tezuka, A., Nagano, A. J., Kadowaki, T., Kim, Y., Miyao, M., & Yanagisawa, S. (2020). Gene regulatory network and its constituent transcription factors that control nitrogen-deficiency responses in rice. *New Phytologist*, *227*(5), 1434–1452. https://doi.org/10.1111/nph.16627

Wang, J., Wu, B., Lu, K., Wei, Q., Qian, J., Chen, Y., & Fang, Z. (2019). The Amino Acid Permease 5 (OsAAP5) Regulates Tiller Number and Grain Yield in Rice. *Plant Physiology*, *180*(2), 1031–1045. https://doi.org/10.1104/pp.19.00034

Wang, J., & Zhang, Z. (2020). *GAPIT Version 3: Boosting Power and Accuracy for Genomic Association and Prediction* [Preprint]. Bioinformatics. https://doi.org/10.1101/2020.11.29.403170

Wang, Q., Nian, J., Xie, X., Yu, H., Zhang, J., Bai, J., Dong, G., Hu, J., Bai, B., Chen, L., Xie, Q., Feng, J., Yang, X., Peng, J., Chen, F., Qian, Q., Li, J., & Zuo, J. (2018). Genetic variations in ARE1 mediate grain yield by modulating nitrogen utilization in rice. *Nature Communications*, *9*(1), 735. https://doi.org/10.1038/s41467-017-02781-w

Wang, W., Hu, B., Yuan, D., Liu, Y., Che, R., Hu, Y., Ou, S., Liu, Y., Zhang, Z., Wang, H., Li, H., Jiang, Z., Zhang, Z., Gao, X., Qiu, Y., Meng, X., Liu, Y., Bai, Y., Liang, Y., … Chu, C. (2018). Expression of the Nitrate Transporter Gene *OsNRT1.1A/OsNPF6.3* Confers High Yield and Early Maturation in Rice. *The Plant Cell*, *30*(3), 638–651. https://doi.org/10.1105/tpc.17.00809

Wei, T., & Simko, V. (2021). *R package "corrplot": Visualization of a Correlation* (Version 0.92). https://github.com/taiyun/corrplot

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis* (2nd ed. 2016). Springer International Publishing : Imprint: Springer. https://doi.org/10.1007/978-3-319-24277-4

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T., Miller, E., Bache, S., Müller, K., Ooms, J., Robinson, D., Seidel, D., Spinu, V., … Yutani, H. (2019). Welcome to the Tidyverse. *Journal of Open Source Software*, *4*(43), 1686. https://doi.org/10.21105/joss.01686

Xiao, M., Zang, H., Liu, S., Ye, R., Zhu, Z., Su, Y., Wu, J., & Ge, T. (2019). Nitrogen fertilization alters the distribution and fates of photosynthesized carbon in rice–soil systems: A 13C-CO2 pulse labeling study. *Plant and Soil*, *445*(1–2), 101–112. https://doi.org/10.1007/s11104-019-04030-z

Xu, F., Bao, J., He, Q., & Park, Y.-J. (2016). Genome-wide association study of eating and cooking qualities in different subpopulations of rice (Oryza sativa L.). *BMC Genomics*, *17*(1), 663. https://doi.org/10.1186/s12864-016-3000-z

Xu, G., Fan, X., & Miller, A. J. (2012). Plant nitrogen assimilation and use efficiency. *Annual Review of Plant Biology*, *63*, 153–182.

Yan, M., Fan, X., Feng, H., Miller, A. J., Shen, Q., & Xu, G. (2011). Rice OsNAR2.1 interacts with OsNRT2.1, OsNRT2.2 and OsNRT2.3a nitrate transporters to provide uptake over high and low concentration ranges: Rice two component nitrate transport. *Plant, Cell & Environment*, *34*(8), 1360–1372. https://doi.org/10.1111/j.1365-3040.2011.02335.x

Yang, H.-C., Kan, C.-C., Hung, T.-H., Hsieh, P.-H., Wang, S.-Y., Hsieh, W.-Y., & Hsieh, M.-H. (2017). Identification of early ammonium nitrate-responsive genes in rice roots. *Scientific Reports*, *7*(1), 16885. https://doi.org/10.1038/s41598-017-17173-9

Yang, Y., Zhang, J., & Cai, Z. (2016). Nitrification activities and N mineralization in paddy soils are insensitive to oxygen concentration. *Acta Agriculturae Scandinavica, Section B — Soil & Plant Science*, *66*(3), 272–281. https://doi.org/10.1080/09064710.2015.1093653

Yin, L., Zhang, H., Tang, Z., Xu, J., Yin, D., Zhang, Z., Yuan, X., Zhu, M., Zhao, S., Li, X., & Liu, X. (2020). *rMVP: A Memory-efficient, Visualization-enhanced, and Parallel-accelerated tool for Genome-Wide Association Study* [Preprint]. Genetics. https://doi.org/10.1101/2020.08.20.258491

Yu, J., Pressoir, G., Briggs, W. H., Vroh Bi, I., Yamasaki, M., Doebley, J. F., McMullen, M. D., Gaut, B. S., Nielsen, D. M., Holland, J. B., Kresovich, S., & Buckler, E. S. (2006). A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. *Nature Genetics*, *38*(2), 203–208. https://doi.org/10.1038/ng1702

Zhang, J., Liu, Y.-X., Zhang, N., Hu, B., Jin, T., Xu, H., Qin, Y., Yan, P., Zhang, X., Guo, X., Hui, J., Cao, S., Wang, X., Wang, C., Wang, H., Qu, B., Fan, G., Yuan, L., Garrido-Oter, R., … Bai, Y. (2019). NRT1.1B is associated with root microbiota composition and nitrogen use in field-grown rice. *Nature Biotechnology*, *37*(6), 676–684. https://doi.org/10.1038/s41587-019-0104-4

Zhang, X., Lu, Y., Yang, T., Kronzucker, H. J., & Shi, W. (2019). Factors influencing the release of the biological nitrification inhibitor 1,9-decanediol from rice (Oryza sativa L.) roots. *Plant and Soil*, *436*(1–2), 253–265. https://doi.org/10.1007/s11104-019-03933-1

Zhang, Z., & Chu, C. (2020). Nitrogen-Use Divergence Between Indica and Japonica Rice: Variation at Nitrate Assimilation. *Molecular Plant*, *13*(1), 6–7. https://doi.org/10.1016/j.molp.2019.11.011

Zhang, Z., Ersoz, E., Lai, C.-Q., Todhunter, R. J., Tiwari, H. K., Gore, M. A., Bradbury, P. J., Yu, J., Arnett, D. K., Ordovas, J. M., & Buckler, E. S. (2010). Mixed linear model approach adapted for genome-wide association studies. *Nature Genetics*, *42*(4), 355–360. https://doi.org/10.1038/ng.546

Zhang, Z., Gao, S., & Chu, C. (2020). Improvement of nutrient use efficiency in rice: Current toolbox and future perspectives. *Theoretical and Applied Genetics*, *133*(5), 1365–1384. https://doi.org/10.1007/s00122-019-03527-6

Zhao, K., Tung, C.-W., Eizenga, G. C., Wright, M. H., Ali, M. L., Price, A. H., Norton, G. J., Islam, M. R., Reynolds, A., Mezey, J., McClung, A. M., Bustamante, C. D., & McCouch, S. R. (2011). Genome-wide association mapping reveals a rich genetic architecture of complex traits in Oryza sativa. *Nature Communications*, *2*(1), 467. https://doi.org/10.1038/ncomms1467

Zhong, Y., Hu, J., Xia, Q., Zhang, S., Li, X., Pan, X., Zhao, R., Wang, R., Yan, W., Shangguan, Z., Hu, F., Yang, C., & Wang, W. (2020). Soil microbial mechanisms promoting ultrahigh rice yield. *Soil Biology and Biochemistry*, *143*, 107741. https://doi.org/10.1016/j.soilbio.2020.107741

Chapter 3

Natural Variations of Spring Wheat Nitrogen and Carbon Assimilation under Different Inorganic Nitrogen Forms and CO_2 levels

Natural Variations of Spring Wheat Nitrogen and Carbon Assimilation under Different Inorganic Nitrogen Forms and CO_2 levels

Pornpipat Kasemsap[a]*, Junli Zhang[a], Daniel J. Kliebenstein[ab], Arnold J. Bloom[a]

[a]University of California at Davis, Department of Plant Sciences, Davis, CA 95616 USA

[b]DynaMo Center of Excellence, University of Copenhagen, Thorvaldsensvej 40, DK-1871, Frederiksberg C, Denmark

*Corresponding author, e-mail pkasemsap@ucdavis.edu

Abstract

Wheat (*Triticum aestivum* L.) production depends upon nitrogen fertilization and may be threatened by the climate conditions anticipated in the next few decades. The natural variation in the ability of wheat to assimilate carbon and different inorganic nitrogen forms, nitrate (NO_3^-) and ammonium (NH_4^+), into vegetative growth remains unexplored. Here, we evaluated growth under either NO_3^- or NH_4^+ as a sole nitrogen source for 875 spring hexaploid wheat accessions that represent the genetic diversity within the global germplasm. These accessions varied over 8-fold in vegetative biomass but grew similarly under moderate levels of either nitrogen form. At high, potentially toxic concentrations of NH_4^+, however, they lost approximately 20% of their biomass. We characterized the influence of changing CO_2 levels in bi-parental Nested Association Mapping populations. Genetic backgrounds determined wheat biomass responses to CO_2 enrichment and nitrogen form. Genome-Wide Association and linkage mapping identified certain loci as consistently associated with biomass accumulation under different nitrogen forms and CO_2 levels. These results will assist breeding efforts to develop food crops that are resilient to the world's climate changes.

Keywords

breeding, cereal, climate change, genes, GWAS, mapping, nitrogen, nitrogen use efficiency, wheat, yield

Introduction

Nitrogen, as an essential element for crop growth and food quality, is a major factor in global food security. Nitrogen use efficiency, however, the proportion of harvestable nitrogen in the final food product, remains below 50% (X. Zhang et al., 2021) and may decline under the climate conditions anticipated in the next few decades (Ren et al., 2023). Nitrogen fertilization itself is responsible for about 5% of the anthropogenic greenhouse gas emissions that drive global climate change (Gao & Cabrera Serrenho, 2023). Production of food crops like wheat (*Triticum aestivum* L.), the major plant source of protein and calories in the human diet, relies heavily on nitrogen fertilization (Hawkesford, 2014). Successful deployment of wheat cultivars with improved nitrogen use efficiency may simultaneously mitigate climate change and ensure global food security.

Plants convert the major soil inorganic nitrogen forms, ammonium (NH_4^+), or nitrate (NO_3^-), into organic forms such as proteins and nucleic acids necessary for life (Xu et al., 2012). Biomass production and nitrogen use efficiency are complex polygenic traits involving multiple genetic and physiological interactions that remain unresolved (Lammerts Van Bueren & Struik, 2017). To date, only a handful of genes that improve yield and nitrogen use efficiency have been incorporated into breeding programs; these involve vegetative tiller development, flowering time, and ammonium assimilation (Kasemsap & Bloom, 2022). Because up to 90% of grain nitrogen is relocated from growing tissues prior to anthesis (Kong et al., 2016), vegetative biomass accumulation is critical to both grain yield and protein concentration. We lack information about the genetic architecture of biomass production under different inorganic nitrogen sources, although such information would facilitate the breeding of crops with improved nitrogen use efficiency.

NH_4^+ nutrition may be advantageous over NO_3^- nutrition for multiple reasons. First, plants require less energy to assimilate NH_4^+ than NO_3^- into amino acids (Bloom, 2010; Cox & Reisenauer, 1973). Second, NH_4^+ binds to soil particles in temperate soils because they are usually negatively charged; in contrast, NO_3^- remains mobile in the soil solution and may leach away from the root zone before being absorbed by a crop (J. Norton & Ouyang, 2019). Third, as a result of the lower soil mobility of NH_4^+, NH_4^+-based crop production systems emit less NO_2, a highly potent greenhouse gas, to the atmosphere. Fourth, elite wheat varieties that were modified to sustain NH_4^+ in the root zone through biological nitrification inhibition yielded more grain without sacrificing grain quality (Subbarao et al., 2021). Lastly, wheat under the elevated atmospheric CO_2 levels anticipated in the near future maintained grain protein and nutritional qualities when it received NH_4^+, but not when it received NO_3^- (Rubio-Asensio & Bloom, 2016).

Nevertheless, plants generally cannot tolerate high concentrations of NH_4^+ (Britto & Kronzucker, 2002). Although NH_4^+-based fertilizers are widely used globally (Nishina et al., 2017), rapid microbial nitrification of NH_4^+ into NO_3^- in aerobic agricultural soils typically results in NO_3^- being the prevalent form available to crops (J. M. Norton, 2015). Most previous genetic studies on nitrogen use efficiency primarily focused on varying nitrogen levels (Cormier et al., 2016). A few studies reported contrasting responses to different nitrogen forms based on results from limited genetic materials (Cox & Reisenauer, 1973; Fuertes-Mendizábal et al., 2013; C. O'Sullivan et al., 2016; Sun et al., 2013). The extent to which the current wheat germplasm utilizes either form of inorganic nitrogen or tolerates high NH_4^+ levels essentially remains unexplored.

Association mapping enables examination of the regulatory genetic elements underlying phenotypic changes. Genome-wide association (GWA) mapping overcomes the limited recombination events and genetic variation of a traditional linkage mapping (Korte & Farlow, 2013). From 2009 to 2020, wheat GWA studies cumulatively reported ~47,000 putative loci for a wide range of traits (Saini et al., 2022). Yet GWA analysis alone remains insufficient to identify the causal loci (McCarthy & Hirschhorn, 2008), especially for traits with complex genetic architecture, even those with high heritability (Korte & Farlow, 2013). Additional high-resolution mappings can provide further validation and functional characterization. For example, combining the quantitative trait loci (QTL) information from a GWA panel with a linkage mapping improves the design of wheat breeding pipelines (Wang et al., 2023). Rather than deploying genome-wide mapping as a sole approach, successful endeavors initiated GWA as an exploratory tool followed by cross-validation with linkage mapping of different plant materials such as nested-association mapping (NAM) populations (J. Zhang et al., 2018). NAM populations (Blake et al., 2019; McMullen et al., 2009; Yu et al., 2008) provide a high mapping resolution from the recombinant inbred lines as well as rich allelic variations from multiple founder parents (Saini et al., 2022).

Here, we employed diverse genetic materials to characterize the genetic basis of wheat carbon and nitrogen assimilation under ammonium versus nitrate as a sole nitrogen source. First, we explored the vast diversity within the global germplasm and mapped loci associated with biomass production under ambient CO_2 environments via a GWA analysis. Second, we used bi-parental nested association mapping populations to validate and narrow down candidate loci and to evaluate further how CO_2 enrichment alters responses to different nitrogen sources. Our study established the extent to which wheat utilizes ammonium or nitrate as a major nitrogen source, including potentially toxic concentrations of NH_4^+. We identified candidate loci that can guide breeding food crops that are resilient to future climate conditions.

Materials and Methods

National Small Grains Core Collection at ambient CO_2

We grew a panel of 875 spring hexaploid wheat (*Triticum aestivum* L.) accessions from the USDA-ARS National Small Grains Core Collection (NSGCC) that included 154 landraces, 261 registered cultivars, and 460 actively cultivated materials, which represent the germplasm in 89 countries worldwide (Maccaferri et al., 2015). We disinfected mature seeds collected from field and greenhouse experiments in Davis, CA with 10% v/v $NaHClO_3$ for 10 minutes, rinsed them multiple times with deionized water, and placed them on a germination paper soaked with deionized water. We kept rolls of the germination paper at 4°C for at least 48 hr and later moved them to room temperature for 4 d until transplanting. We transplanted seedlings into a 20 dm^3 tub filled with a modified Hoagland solution (Epstein & Bloom, 2005) containing either NH_4^+ (0.5 mM or 20 mM) or NO_3^- (0.5 mM) as a sole nitrogen source. The solution pH was initially adjusted to 6.0, and the solution was replenished twice in the first week, and every 1 to 2 days during the second week of growth. We grew seedlings at ambient CO_2 concentrations in an evaporative cooled, ventilated greenhouse at University of California, Davis (38.5365 N, 121.7470 E) and harvested the whole plant at the tillering stage (Zadoks 21-25 or Feekes 2-3) 15 d after transplanting, when growth differences due to nutrient treatments became clearly visible.

Our experiment consists of 6 replicates: 3 replicates grown consecutively during summer months from June to August of 2016 and again in 2017. In each replication, we randomly divided the whole panel into 5 groups; each group was germinated and transplanted into the same greenhouse consecutively one day after the other for 5 sequential days. We retrieved weather records from a local CIMIS station (38.5358 N, 121.7764 E) located 2.55 km from the greenhouse (https://cimis.water.ca.gov/). Seasonal solar radiation levels and temperatures characterized each summer month (June 2016, July 2016, August 2016, June 2017, July 2017, and August 2017) as a distinct environment, resulting in 6 growth environments.

Genome-Wide Association mapping

We calculated BLUP (Best linear unbiased prediction) for each genotype in a mixed linear model using the function lmer in R/lme4 (Bates et al., 2015) where we specified year, replicate, germination group, and blocks within each replicate as random effects. The mixed linear model was as followed for each nitrogen treatment: mass ~ genotype × nitrogen + (1|year) + (1|year: replication) + (1|year: replication: germination group) + (1|year: replication: germination group: block). We then used the fitted biomass under each nutrient treatment, corrected by the biomass of the reference genotype Veery-10 in each germination group, to calculate the dry mass ratio of interest. To determine a broad sense heritability and the proportion of variance explained by each component, we ran a linear model with all factors as fixed effects and calculated type II sums of squares (Langsrud, 2003) using R/car version 3.1 (Fox & Weisberg, 2019) from an analysis of variance (ANOVA).

As inputs for Genome Wide Association (GWA) mapping with 4,585 SNP markers (Maccaferri et al., 2015), we used the mean of total plant dry mass under NH_4^+ and NO_3^-, and the ratio of the dry mass of plants under different concentrations and forms of nitrogen, across 3 replicates in each year and 6 replicates over the 2 years. We performed GWA of each biomass trait with the K (kinship matrix) in a mixed linear model (MLM) in R/GAPIT (Lipka et al., 2012), as described previously to be most the suitable for this mapping population (Maccaferri et al., 2015). MLM corrected with population structure (Q) and principal components (10), as well as BLINK (Huang et al., 2019) without any correction besides K resulted in generally the same set of significant QTL (data not shown) under our stringent threshold. For each trait, we identified SNPs that were significant ($P < 0.05$, marker-wise) in at least one replicates in any year and with at least one replicate, or the means across replicates or years, being highly significant overall ($P < 9.65 \times 10^{-5}$, experiment-wise), given $\alpha = 0.1$ for the Bonferoni correction accounting for the number of tag-SNPs (1036 SNPs). The 50% LD decay rate is 1 cM in this diversity panel when genotyped with the 9K SNP array because of the rich diversity and high level of historical recombination (Maccaferri et al., 2015). We set the confidence intervals for the QTL-harboring regions at ± 1.6 cM, corresponding to the critical level of $r^2 = 0.3$ (Maccaferri et al., 2015).

Bi-parental nested association mapping populations at ambient CO_2

We grew eight biparental NAM populations, each including 75 recombinant inbred lines (RILs), to validate the significant SNPs detected in the GWA. These populations were generated from crosses between the CIMMYT line Berkut (Irene/Babax//Pastor) as a female parent and eight lines (PBW343, Dharwar Dry, LR23 (PI 70613), LR3 (CItr 7635), RAC875, Kern, RSI5, and UC1419 (Patwin-515HP or PVP 201600390)) with diverse genetic backgrounds as male parents (Blake et al., 2019). These lines were genotyped with the Illumina wheat iSelect 90K SNP array (Blake et al., 2019; J. Zhang et al., 2018). We germinated and grew seedlings from July to September 2020 in the same greenhouse environments as the GWAS experiments described above. In each tub of the 25 genotypes being evaluated, we added reference genotypes: 3-5 plants of cv. Veery-10 and 2-3 plants of cv. Berkut and Hahn 1RS.

Linkage and joint linkage-association mapping

We calculated coefficients of dry mass for each genotype in a mixed linear model using the function lmer in R/lme4 (Bates et al., 2015) where we specified replicate and blocks within each replicate as random effects, genotype as a fixed effect, and only used the mean biomass of cv. Veery-10 in each tub as a covariate to correct for dry mass variation across blocks in different parts of the greenhouse. The mixed linear model for each nutrient treatment was mass (mg) ~ dry mass of Veery + genotype + (1|replication) + (1|replication:block). We then used the fitted biomass in each nutrient condition to calculate the dry mass ratio of interest.

As input for linkage mappings, we used an individual replication value, mean dry mass under NH_4^+ and NO_3^-, and dry mass ratio of plants under different concentrations and forms of nitrogen, of the 3 repeated experiments across summer months, each with 1 plant replicate. We performed QTL mapping for each trait in each individual population in R/qtl (Arends et al., 2010) and joint linkage-association mapping for all 8 populations in TASSEL, using Stepwise regression association analysis (Bradbury et al., 2007). For each trait, we identified SNPs with LOD > 2.0 in each population, or with p-value < 0.001 in joint linkage-association mapping.

Bi-parental nested association mapping populations at elevated CO_2

To evaluate how biomass changes under CO_2 enrichment, we germinated seedlings of six bi-parental nested association mapping populations with the same protocol above, but transplanted them into nutrient solution tubs placed in controlled environment facilities. The growth conditions in controlled environmental chambers (Conviron, Winnipeg, Canada), equipped with non-dispersive infrared analyzers, were as follows: 750 ppm CO_2 (concentration expected at the end of the 21st century (IPCC, 2021)), 50% relative humidity, 16 hr of 800 µmol m^{-2} s^{-1} PPFD at canopy height with 22/15°C light/dark temperature. We evaluated 25 genotypes in each tube, including 4 plants of the reference genotype cv. Veery-10.

We calculated coefficients of biomass traits with the same approach as described above: we specified replicate and blocks within each replicate as random effects, genotype as a fixed effect, and used the mean biomass of Veery-10 in each tub as a covariate to correct for dry mass variation across replications. The mixed linear model was as follows for each nutrient treatment: dry mass (mg) ~ dry mass of Veery + genotype + (1|replication) + (1|replication:block). We then used the fitted biomass in each nutrient condition to calculate dry mass ratio of interest. We performed QTL and joint linkage with the same approach as described above. We compared the significant QTL between ambient and elevated CO_2 levels.

Data processing and visualization

Unless otherwise noted, we performed all analyses in R (R Core Team, 2023) in an integrated development environment Rstudio (RStudio Team, 2020). We processed data with R/tidyverse version 2.0 (Wickham et al., 2019) and R/data.table version 1.14.8 (Dowle & Srinivasan, 2023), and visualized most of the numerical data with R/ggplot2 version 3.4.4 (Wickham, 2016). We employed packages R/see version 0.8.1 (Lüdecke et al., 2021), R/ggbeeswarm version 0.7.2 (Clarke et al., 2023), R/corrplot version 0.92 (Wei & Simko, 2021), and R/rMVP version 1.0.6 (Yin et al., 2020) for half violin plots, scattered violin plots, correlation plots, and Manhattan plots respectively.

Results

Wheat tolerated high levels of NH_4^+ and adapted to using either nitrogen forms

Whole seedling biomass of the worldwide spring wheat collection in an ambient CO_2 environment spanned 8-fold from 0.1 g to over 0.8 g (Figure 1). Monthly differences (experimental replicate) primarily accounted for the greatest portion of this biomass variation (Figure 1B). Biomass declined (Figure 1C) as light availability declined (Figure 1A), suggesting a response to changes in day length and temperature from the beginning of the summer (June) to late in the season (August) across both years of the experiment (Figure 1C).

The response to nitrogen form was consistent across seasonal changes (Figure 1D). We defined a moderate level 0.5 mM NH_4^+ as a reference treatment and compared the responses between nitrogen conditions by calculating the biomass ratio of the same genotype to the reference treatment. The biomass ratio under 0.5 mM NO_3^- to that under 0.5 mM NH_4^+ averaged about 1.0; the responses ranged from ~0.25 to ~1.5, suggesting potential adaptations to the specific nitrogen forms. Under 20 mM NH_4^+, the average response was about 80% of the biomass under 0.5 mM NH_4^+ and ranged from about 0.1 to 1.5, indicating larger variation in tolerance to high NH_4^+ levels within the germplasm. Seven subgroups (Maccaferri et al., 2015) within the germplasm that was used to correct for the population structure displayed slight different to nitrogen responses, but there is no clear association with the geographical origins of the accessions (Supplementary Figure 1).

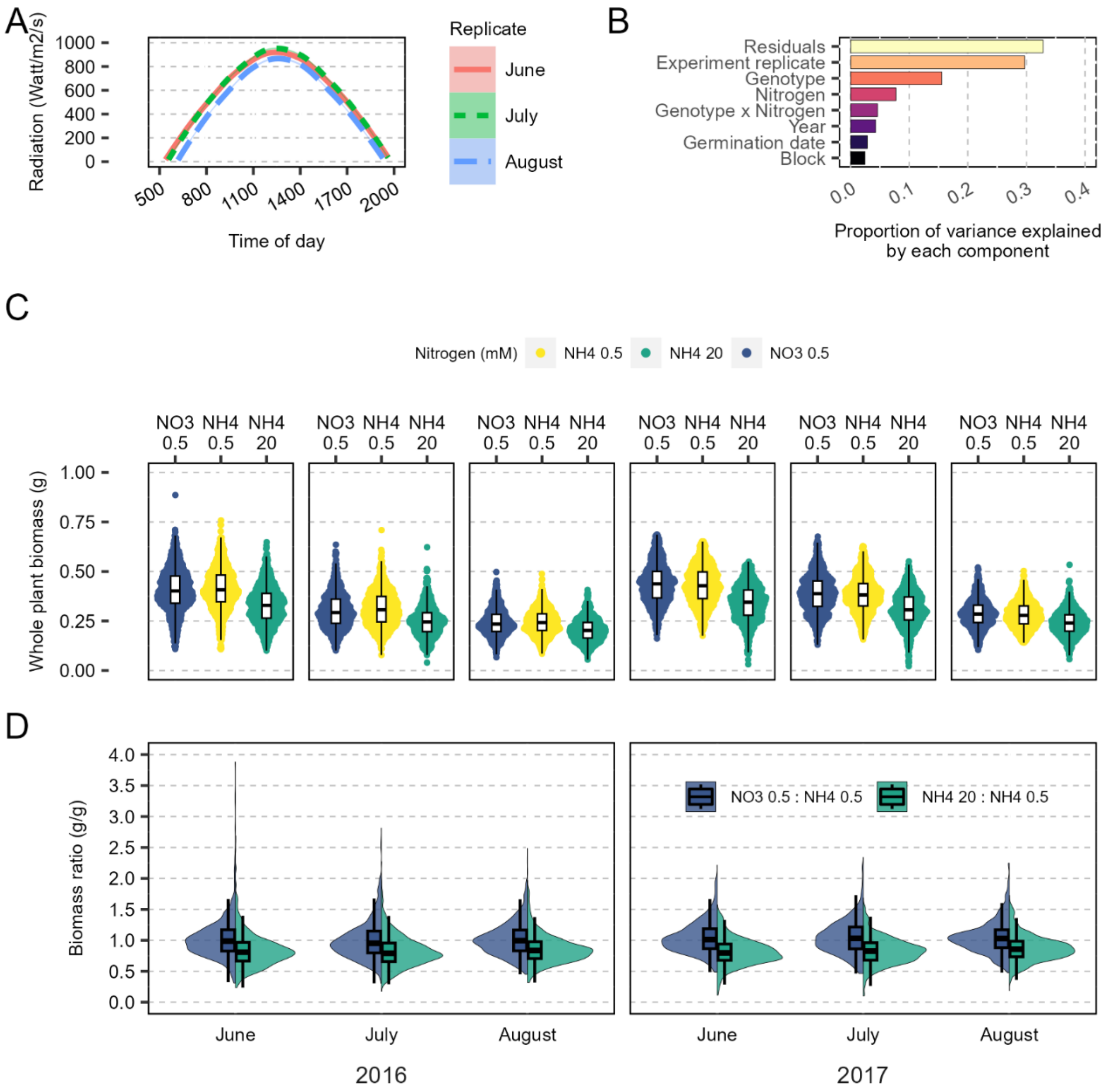


Figure 1 Wheat biomass varied with seasonal changes but nitrogen responsiveness to either form of nitrogen and high NH_4^+ level remained consistent.

(A) Seasonal variations in radiation, averaged across 2016 and 2017 (Watt m^{-2} s^{-1}), (B) Proportion of variance explained by each component in the experiment, (C) Distribution of whole plant biomass of 875 global wheat accessions receiving NO_3^- (0.5 mM) or NH_4^+ (0.5 or 20 mM) as a sole nitrogen source at 15 d after transplanting under ambient CO_2 concentrations) over 6 independent replicates in Summer months of 2016 and 2017. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima n=875 genotypes with 1 plant replicate in each month and year combination). Each dot represents a plant replicate. (D) Distribution of whole plant biomass ratio of 875 global wheat accessions under NO_3^- (0.5 mM) or NH_4^+ (20 mM) to the reference treatment NH_4^+ (0.5 mM) over 6 independent replicates in summer months of 2016 and 2017. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima n=875 genotypes with 1 plant replicate in each month and year combination). Figure C and D share the same x-axis.

Biomass is controlled by multiple loci across different nitrogen conditions

We performed genome-wide association analyses with biomass of each individual experiment replicate and across the whole experiment, accounting for biomass plasticity across the season. In total, 2419 SNPs were significantly associated with biomass in any given nitrogen condition and environment at the marker-wise threshold ($p<0.05$) (Supplementary Table 1). To narrow down candidate loci and limit false positive, we considered SNPs that were highly significant in at least one environment under a stringent experiment-wise p-value threshold (~p<0.0001). Experiment-wise, we identified 20 significant SNPs representing 14 loci that were either unique to or shared between the nitrogen conditions across multiple environments (Table 1). Different sets of SNPs were associated with biomass and biomass ratio. The majority of the significant SNPs were identified under 20 mM NH_4^+. Two loci associated with SNPs IWA2077 and IWA6647 were significant for biomass in all nitrogen treatments.

Table 1 Significant SNPs consistently associated with dry mass and dry mass ratio of wheat receiving 0.5 mM NH_4^+, 20 mM NH_4^+ or 0.5 mM NO_3^- at the marker-wise threshold ($p<0.05$; light red cells) and experimental-wise threshold ($p<0.0001$; red cells). Year and environment are indicated on the top rows. A and T indicates overall means within each year, and across the two years respectively.

SNP	0.5 mM NH_4^+									20 mM NH_4^+									0.5 mM NO_3^-									20:0.5 mM NH_4^+									0.5 NO_3^-:NH_4^+								
	2016				2017				T	2016				2017				T	2016				2017				T	2016				2017				T	2016				2017				T
	A	1	2	3	A	1	2	3	T	A	1	2	3	A	1	2	3	T	A	1	2	3	A	1	2	3	T	A	1	2	3	A	1	2	3	T	A	1	2	3	A	1	2	3	T
1B																																													
2077	▨	▨	▨	▨	▨	🟥	▨	▨	▨	▨	🟥	▨	▨	▨	🟥	▨	▨	▨	▨	▨	▨	▨	▨	🟥	▨	▨	▨																		
6647	▨	🟥	▨	▨	▨	🟥	▨	▨	▨	▨	🟥	▨	▨	▨	🟥	▨	▨	▨	▨	▨	▨	▨	▨	🟥	▨		▨																		
2B																																													
6767		▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	🟥	▨	▨	▨						▨									▨												
6768		▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	🟥	▨	▨	▨					▨	▨									▨												
8430		▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	🟥	▨	▨	▨					▨	▨									▨												
3B																																													
6930				▨						▨	▨	▨	🟥	▨		▨	▨	▨				▨						▨		▨		▨			▨	▨									
747		▨		▨	▨	▨	▨		▨	▨	▨	▨	▨	▨	▨	🟥	▨	▨				▨			▨									▨											
748		▨		▨	▨	▨	▨	▨	▨	▨	▨	▨	🟥	▨	▨	🟥	▨	▨				▨			▨									▨											
4A																																													
2383																						▨	▨	▨		▨	▨										▨		▨	▨	▨	▨	🟥	▨	▨
8432	▨		▨		▨		🟥		▨	▨	▨	▨		▨	▨	▨	▨	▨	▨		▨		▨	▨	▨		▨																		
5B																																													
1374				▨																								▨	▨	▨	🟥	▨	▨	▨	▨	▨	▨			▨	▨	▨		▨	▨
2453	▨			▨	▨	▨		▨	▨																			▨	▨	▨	🟥	▨	▨	▨	🟥	▨	▨	▨	▨	🟥	▨	▨	▨	▨	▨
2454	▨			▨	▨	▨		▨	▨																			▨	▨	▨	🟥	▨	▨	▨	🟥	▨	▨	▨	▨	🟥	▨	▨	▨	▨	▨
3359	▨		▨						▨	🟥	▨	▨	▨	▨	▨	▨		▨	▨								▨																		
4313	▨	▨		▨	▨		▨	▨	▨	▨	▨	▨	▨	▨		▨	▨	▨	▨	▨	▨		▨		🟥	▨	▨																		
6B																																													
5185		▨			▨	▨			▨																												▨	▨		▨	▨	🟥		▨	▨
8380																	▨		▨		▨	▨				▨	▨	▨	▨		▨	▨			▨	▨	▨	🟥	▨	▨	▨			▨	▨
7B																																													
1091				▨	▨		🟥	▨	▨		▨		▨	▨		▨	▨	▨				▨	▨		▨	▨	▨																		
1314	▨				▨		▨		▨	▨				▨	▨	🟥		▨					▨		▨																				
?																																													
3389																												▨	▨	▨	▨	▨	▨	🟥	▨	▨									

Biomass partitioning varied upon nitrogen sources, CO_2 levels and genetic backgrounds

The bi-parental populations, however, only presented a portion of the phenotypic variation previously observed in the global diversity panel (Figure 1). Genetic backgrounds and nitrogen conditions influenced the biomass of these bi-parental populations (Figure 2). Like the GWAS panel, season variation across multiple independent replicates significantly influenced the overall biomass (Figure 2A-B). Population Berkut x RAC875 produced the highest amount of biomass (Figure 2C). At the whole plant level, all populations exhibited comparable responses to nitrogen treatments (Figure 2C). Nitrogen responsiveness, however, varied between plant organs (Figure 2B). Interestingly, NO_3^--grown plants generally maintained comparable root growth to NH_4^{+-}-grown plants, but at a cost of the shoot growth (Figure 2E-F). As a result, seedlings under NO_3^- had lower whole plant biomass, but still partitioned more biomass into root than plants under NH_4^+ (Figure 2D,G). In contrast, biomass partitioning under different levels of NH_4^+ remained comparable for most populations (Figure 2G); the lower whole plant biomass under the high NH_4^+ was due to slower growth of both shoots and roots (Figure 2E-F).

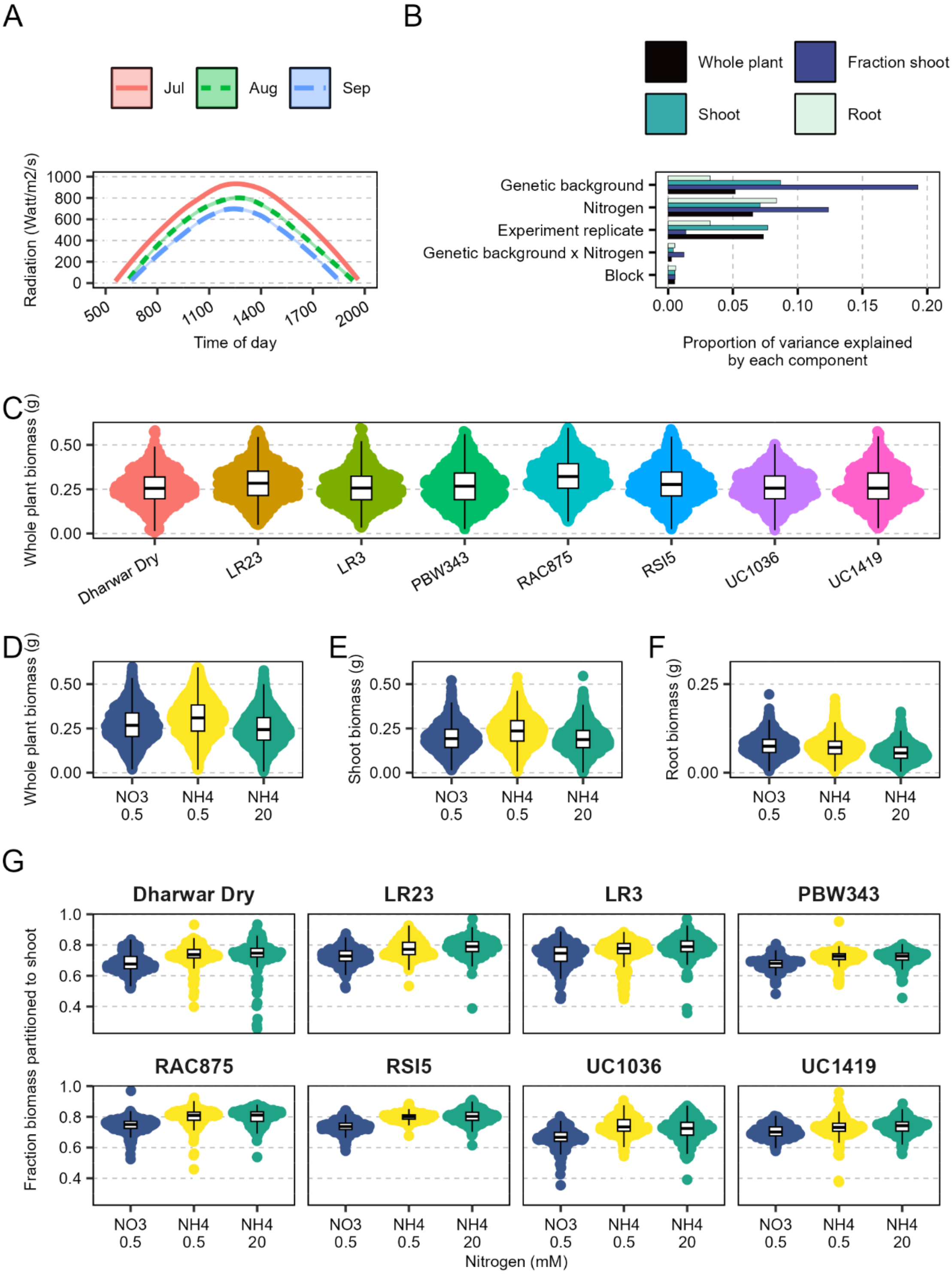


Figure 2 Genetic backgrounds determined biomass partitioning under different nitrogen source.

(A) Seasonal radiation variations in 2020 (Watt m^{-2} s^{-1}), (B) Proportion of variance explained by each component in the experiment. Each box represents different plant parts, including whole plant, shoot, root, and fraction of biomass partitioned to shoot. Variances explained by residuals are not shown. The sum of all components within the same plant part is 1. (C-G) Distribution of biomass by (C) population, (D-F) plant parts by nitrogen conditions (whole, D; shoot, E; root, F), and (G) fraction of biomass partitioned to shoot by nitrogen conditions of 8 bi-parental populations receiving NO_3^- (0.5 mM) or NH_4^+ (0.5 or 20 mM) as a sole nitrogen source at 15 d after transplanting under ambient CO_2 concentrations over 3 independent replicates in summer months of 2020. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima. Each dot represents an average of an individual genotype. (n=75 genotypes with up to 3 replicates). Although biomass significantly varied across environments as illustrated in (B), we showed here in C-G aggregated values across the environments. For QTL mapping, we used individual values for each environment to account for response plasticity across the season.

Comparing growth of the bi-parental populations under the ambient and elevated CO_2 levels, the growth environments explained the largest variations (64%) in biomass (Figure 3A and Supplementary Figure 2). In the controlled environment facilities, several growth factors differed from the ambient conditions in the greenhouse, including higher light intensity and longer day length. Nitrogen source and its interaction with CO_2 further determined the biomass responses to CO_2 enrichment (Figure 3A-B). Under ambient CO_2, the biparental populations were relatively more tolerant to high NH_4^+ and grew faster under NH_4^+ than under NO_3^-. In contrast, NO_3^- nutrition boosted higher whole plant biomass under CO_2 enrichment, showing a different nitrogen response than under the ambient CO_2 condition. The high NH_4^+ level also slowed down growth under CO_2 enrichment for all populations, except for population PBW343.

Correlations between biomass traits of NAM populations suggest genetic-dependent changes in carbon and nitrogen assimilation across the two CO_2 levels (Supplementary Figure 3 and Supplementary Figure 4). Biomass across different nitrogen conditions were positively correlated, with coefficients >0.78. NO_3^- preference, defined as the biomass ratio of growth at 0.5 mM NO_3^- over the growth at 0.5 mM NH_4^+, was also positively correlated with NH_4^+ tolerance, defined as the biomass ratio under high and moderate NH_4^+ concentrations (Supplementary Figure 3A). For individual populations, the correlations of biomass were also positive across nitrogen conditions (Supplementary Figure 3B-G). The correlations between biomass ratio were consistent in direction but varied in magnitude. NH_4^+ tolerance was generally not correlated with growth under NO_3^-, except for population UC1036 (Supplementary Figure 3F) and population PBW343 (Supplementary Figure 3C).

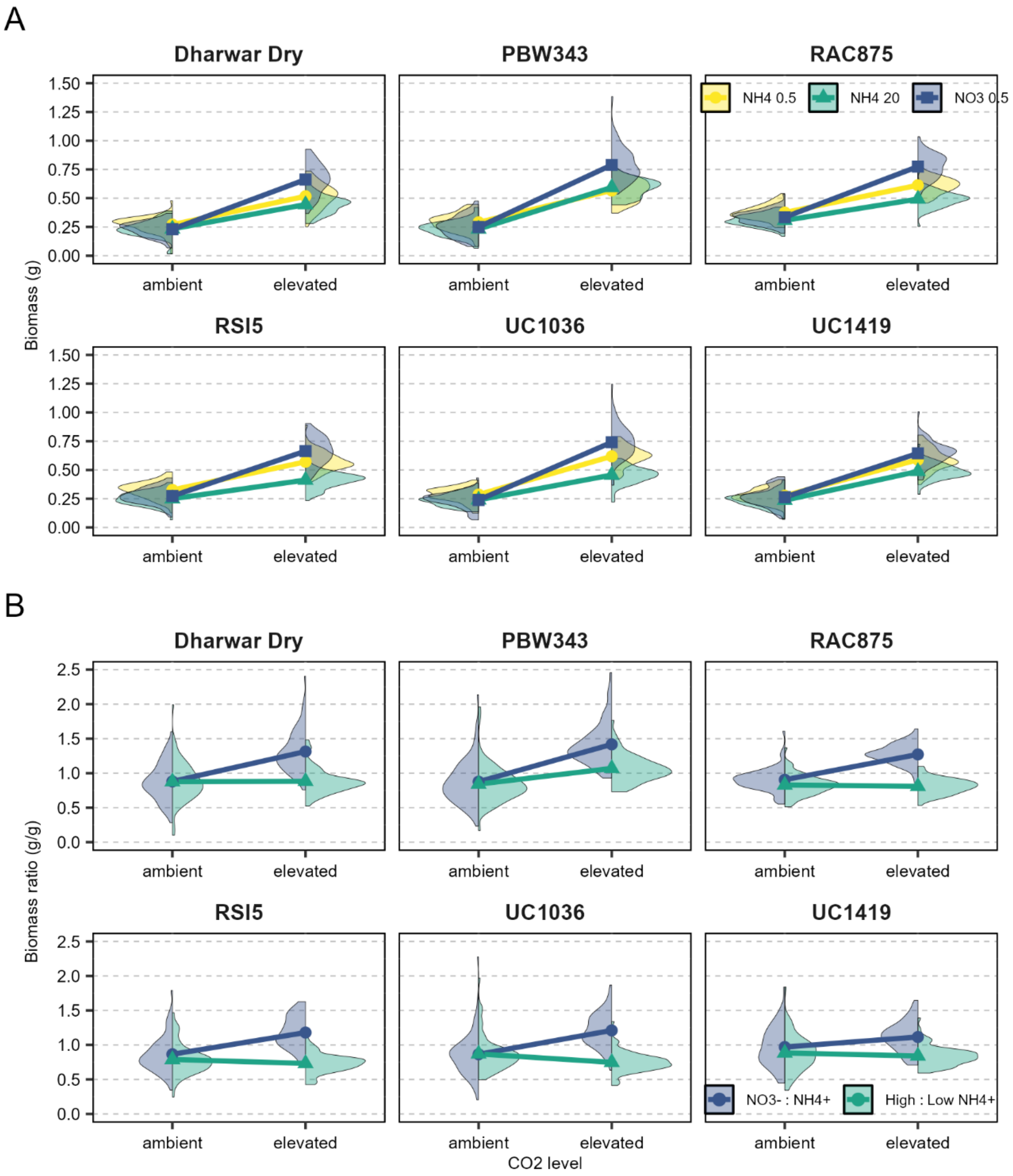


Figure 3 CO_2 enrichment changed wheat responses to inorganic nitrogen forms

(A) Distribution of whole plant biomass of 6 bi-parental populations receiving NO_3^- (0.5 mM) or NH_4^+ (0.5 or 20 mM) as a sole nitrogen source at 15 d after transplanting under ambient and 750 ppm CO_2 (n=75 genotypes with 4 plant replicate for each population). (B) Distribution of whole plant biomass ratio of 6 bi-parental populations under NO_3^- (0.5 mM) or NH_4^+ (20 mM) to the reference treatment NH_4^+ (0.5 mM) (n=75 genotypes with 4 plant replicate for each population). Symbols represent the average responses under different nitrogen conditions. Lines connect the averages between the two CO_2 levels.

Linkage mapping further identified new loci that are unique to genetic backgrounds.

Comparison between bi-parental populations further suggested that responses to different nitrogen forms may be primarily controlled by distinct sets of genes specific to genetic backgrounds. We identified 301 unique significant markers from the linkage mapping of individual populations, although the joint linkage mapping did not detect any common QTL that were shared across all bi-parental populations. We found only a few markers that were significant in multiple populations, although several markers were in close proximity to one another (Supplementary Figure 5). We detected 5 times more significant associations under the ambient CO_2 level because we included biomass data from both shoot and root and from all individual experiment replicates across the season, as opposed to only the average across four replicates with similar controlled environment settings (Supplementary Figure 6). For further studies, we selected a subset of 121 QTL that had at least one significant marker with LOD > 2.0 (Supplementary Table 2). Among these markers, the additive effect sizes ranged widely from -32 to 61. The proportions of variance explained by the markers (R^2) ranged from 0.12 to 0.21.

Discussion

Plants optimize assimilation and allocation of resources to maximize growth (Bloom et al., 1985). During the Green Revolution, the success of modern wheat cultivars came at an expense of superfluous nitrogen fertilization (Li et al., 2018). Despite significant increases in harvest index and grain yield, the introduction of dwarf phenotypes into modern varieties also made crops less sensitive to changes in nitrogen inputs (Gooding et al., 2012; Li et al., 2018). The increase in atmospheric CO_2 levels anticipated in the near future inhibits assimilation of NO_3^- into protein (Bloom et al., 2010), but promotes carbon assimilation (Walker et al., 2021), thereby further exacerbating the imbalance of crop carbon and nitrogen. Here, we demonstrated how changes in rhizosphere nitrogen form influence shoot carbon assimilation into biomass, and how an increase in carbon supply of the magnitude anticipated by the end of this century alters responses to nitrogen. Combining multiple mapping populations, we identified and cross-validated candidate loci underlying wheat carbon and nitrogen assimilation.

Understanding the natural variation in a crop nitrogen utilization is fundamental for breeding for enhanced nitrogen use efficiency. We particularly highlighted the importance of genetic regulation of carbon-nitrogen relations. Here, we established a wide range of biomass responses to NO_3^- versus NH_4^+ as nitrogen sources available within the global wheat germplasm. The available genetic resources offer great potential for improving nitrogen use efficiency to meet the challenge of the changing climate. Interestingly, the bi-parental populations only represented partial genetic diversity available in the global wheat germplasm and were more adapted to using ammonium under ambient CO_2 levels (Figure 2). Depending upon genetic background, the biomass cost of using a different nitrogen form may be as high from the effects of NH_4^+ toxicity, (Figure 2). Furthermore, genetic background also influenced responses to CO_2 enrichment. At 750 ppm CO_2, some genetic backgrounds grew even faster under an NH_4^+ concentration that was toxic to other genotypes (Figure 3). Because previous studies usually examined only a few genotypes, responses to nitrogen forms varied significantly (Cox & Reisenauer, 1973; Fuertes-Mendizábal et al., 2013; C. O'Sullivan et al., 2016; Sun et al., 2013). Employing a genetically diverse population, our study characterized both the general and the extreme responses to different nitrogen forms and NH_4^+ toxicity (Figure 1, Figure 2, and Figure 3). Genotypes with responses deviating from the averages may offer additional breeding values; for example, genotypes that tolerate the detrimental effects of elevated CO_2 levels (Cassan et al., 2023). Our results emphasize that understanding nitrogen responsiveness will require examination of genetically diverse materials (Katz et al. 2022).

Here, we demonstrated that the majority of the global bread wheat germplasm employ both nitrogen forms effectively and maintained robust growth despite experiencing NH_4^+ toxicity (Figure 1). But, how should breeders make selection based on nitrogen form and NH_4^+ toxicity as a breeding target? The positive correlations of biomass under different nitrogen conditions support that prioritizing selection of high biomass, perhaps under ample nitrogen supply, should ensure sufficient nitrogen responsiveness across different soil nitrogen availability (Cormier et al., 2013). Here, NO_3^- preference, defined as the ratio of biomass under both nitrogen forms at 0.5 mM, was also positively correlated with the biomass under 20 mM NH_4^+ for most populations (Supplementary Figure 3). Because these two values are independent, such relationship suggests connections between NO_3^- preference and NH_4^+ tolerance. Even plants that enjoy NO_3^- should generally perform decently under 20 mM NH_4^+. Such insights should allow breeders and farmers to maximize biomass production through manipulating soil nitrogen responses.

Breeding efforts guided by molecular genetic insights often attempt to alter expression of a few genes with large effects (Kasemsap & Bloom, 2022). Here, we demonstrated that small additive effects from a large number of regulatory elements may underlie biomass-nitrogen responsiveness. Heritability of wheat biomass is typically lower than other traits such as height or flowering time (Cormier et al., 2014). Still, the broad-sense heritability here is consistent with a previous field study determined at the same developmental stage (Molero et al., 2019). We identified large sets of candidate loci that were either unique to or stable across environments and mapping populations. Previous mappings in diversity panels of rice (Kasemsap et al., 2024) and Arabidopsis (Katz et al., 2022) underscore the complex genetics of nitrogen responsiveness. Relying on just a handful of genes may undermine the natural variations (Kasemsap & Bloom, 2022). A holistic understanding of the regulatory network that moderate carbon and nitrogen assimilation may better guide breeding for improved yield and grain quality simultaneously.

We identified both sets of candidate loci shared across and unique to nitrogen conditions across environments. Controlling false positive associations by enforcing stringent P-value thresholds may come at a cost of lower statistical power (Cormier et al., 2014). Here, we opted for a more lenient starting threshold with the Bonferroni correction, and prioritized associations that were consistent across multiple environments, which together were proven very stringent, yet effective in previous studies (Maccaferri et al., 2015; J. Zhang et al., 2018). Cross-validation between different mapping populations and studies are required to confirm the candidate loci identified in this study.

How adaptations to nitrogen form, NO_3^- versus NH_4^+, influence nitrogen responsiveness remains inconclusive. Superior growth under specific forms perhaps reflects the genetic adaptation to resources in natural habitats. Wheat is often assumed to be more adapted to NO_3^- perhaps because of the limited number of genotypes previously evaluated (He et al., 2022; S. Liu et al., 2019). In our study, the majority of the global germplasm perform well with either form of nitrogen (Figure 1), but the wide range of biomass responses in diverse genetic backgrounds also suggest potential adaptations (Figure 1 and Figure 2). For example, the natural abundance of rice *TCP19* functional allele follows gradients in soil nitrogen content and its gene expression follows changes in NO_3^- levels (Y. Liu et al., 2021). Here, however, we did not observe any clear evidence for potential local adaptation in this diversity panel, despite its diverse geographic origins (Maccaferri et al., 2015). A growing body of evidence suggest that plants may regulate soil nitrification rates, thereby balancing the two inorganic forms to meet changing demands (He et al., 2022; C. A. O’Sullivan et al., 2016). For example, weed species

(O’Sullivan et al., 2017) and wheat wild relatives (C. A. O’Sullivan et al., 2016) exude inhibitors to slow down the conversion of NO_3^- into NH_4^+. Aligning crop nitrogen preference with fertilizer inputs and environments should enhance nitrogen use efficiency (S. Liu et al., 2019).

A myriad of interacting physiological and environmental cues influence differential responses to nitrogen source (Britto & Kronzucker, 2013). Here, the growing environments accounted for the largest proportion of biomass variations (Figure 1 and Figure 2). We also observed little to no significant correlation of biomass traits between growth in the greenhouse under ambient CO_2 and in controlled environments under elevated CO_2 (Supplementary Figure 4). Biomass values were correlated across nitrogen conditions only within the same growth conditions. Such relationships confirmed that nitrogen responsiveness is highly dependent on other environmental factors. Inclusion of the climate conditions anticipated in the near future, like elevated CO_2 levels (Figure 3), is critical to assessing responsiveness to the inorganic nitrogen forms (Rubio-Asensio & Bloom, 2016). The influence of genetic adaptations to nitrogen sources on crop responses to climate change has significant implications on the future of food production and warrants further investigation.

Acknowledgements

We appreciate the assistance during data collection of Joshua J. Claxton, Dana R. Lawrence, Kungyao Wang, Jordan A. Stefani, Carly M. Miranda, Elias Potashov, Guilherme M. Silva, Jenna O’Kelley, Sydney Koebel, James Schmidt, Anna M. Gomes, Ariel Herrera, Steven Moreno, Temiloluwa Salako, Zion Congrave-Wilson, Lisa Tam, and Khine Z. Lin. We thank Ron Lane, Bill Werner, Jiangao Chen, Davith Hin, and Antonio Lopez for their continued support at the greenhouse facilities and, Kevin Roberts and P. Andre Nelson for their continued support at the controlled environmental facilities. We thank Jorge Dubcovsky for the feedback on the project and the manuscript, Matthew E. Gilbert for discussion on the experimental set-up, and Daniel E. Runcie for discussion on the experimental set-up and statistical analyses for GWAS.

Author contributions

Pornpipat Kasemsap and Arnold J. Bloom planned the research. Pornpipat Kasemsap collected data, analyzed data, and drafted the manuscript. Junli Zhang contributed to the genetic mapping. Daniel J. Kliebenstein contributed to statistical analyses and interpretation. All authors edited the manuscript.

Conflict of Interest

No conflict of interest declared.

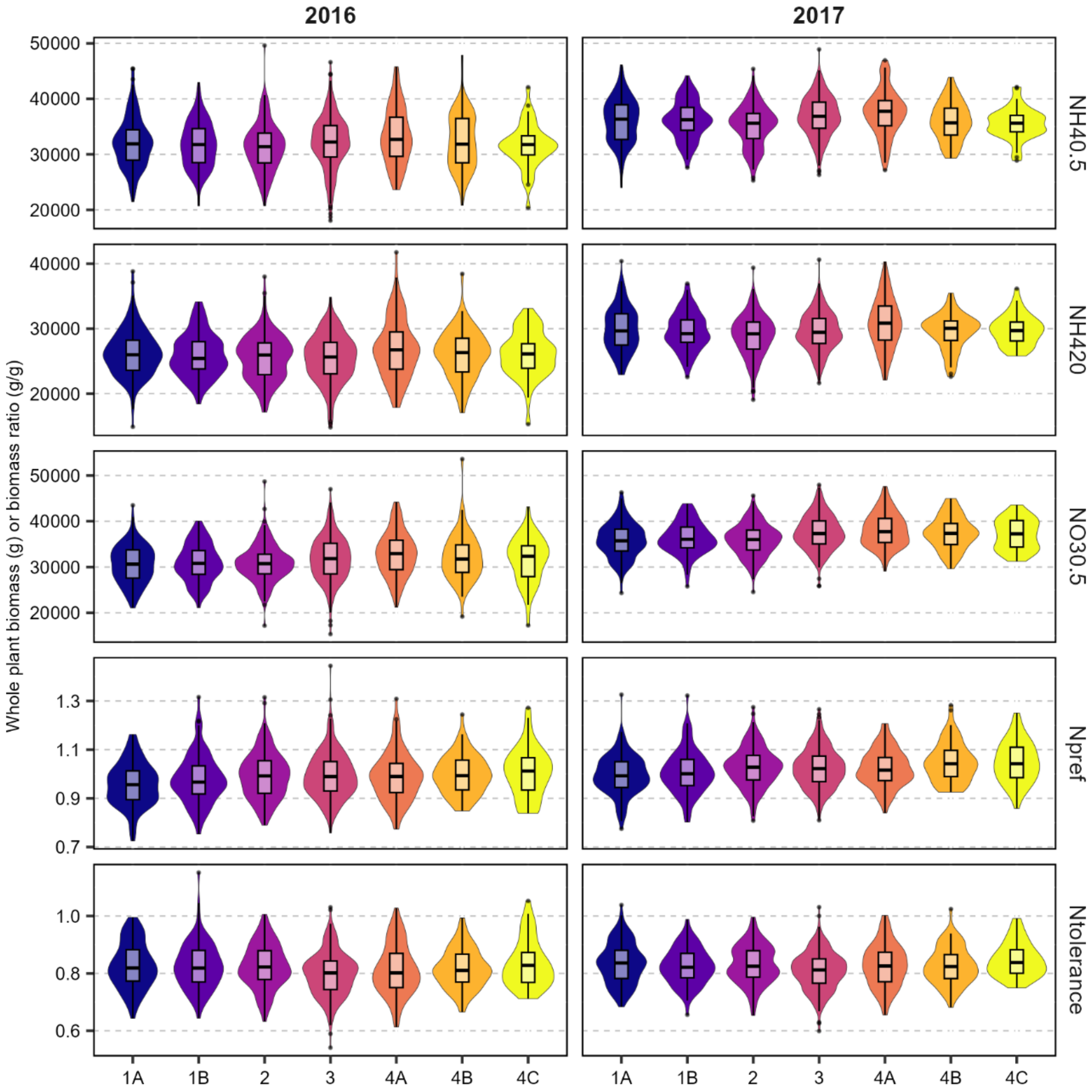
Funding

This work was supported in part by USDA-IWYP-16-06702, NSF IOS 13-58675, NSF IOS 16-55810, and the John B. Orr Endowment to Arnold J. Bloom. Pornpipat Kasemsap was supported by Thailand – United States Educational Foundation (TUSEF/Fulbright Thailand), UC Davis Department of Plant Sciences Graduate Student Researcher fellowship, and Henry A. Jastro research award, and Grad Innovator Fellowship from UC Davis Innovation Institute for Food and Health. Junli Zhang was supported by the United States Department of Agriculture, National Institute of Food and Agriculture (https://nifa.usda.gov/) competitive Grant 2022-68013-36439 (WheatCAP).

Data availability

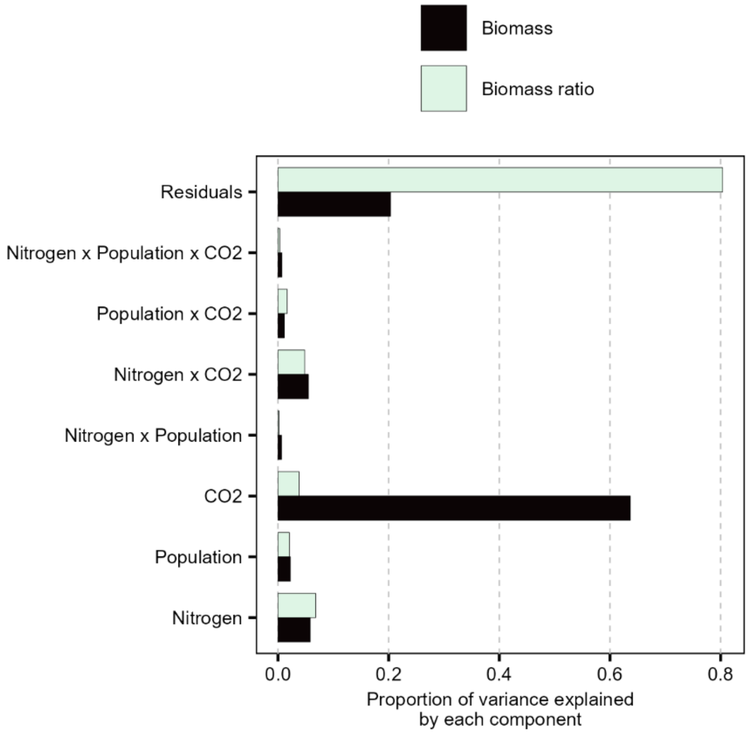
The raw data that support the findings of this study will be deposited and openly available on Dryad. Analysis R codes are available at https://www.github.com/paulkasemsap/Wheat_Nform_Mapping.

Supplementary Data



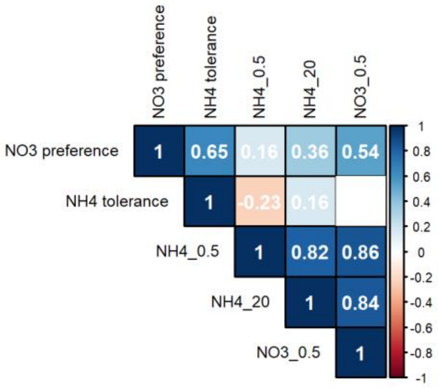
Supplementary Figure 1 Distribution of biomass or biomass ratio by seven subpopulations within the diversity panel

The seven populations were identified in the analysis of the population structure of 875 accessions from the National Small Grains Collection spring wheat core collection. The accessions distributed across 87 countries on six continents. Subpopulation 1A is prevalent in Europe and Africa. Subpopulation 1B and 3 are prevalent in Europe. Subpopulation 2 is prevalent in America. Subpopulation 4A, 4B and 4C are prevalent in Asia. See Figure 1 and 2 (Maccaferri et al., 2015) for more details.



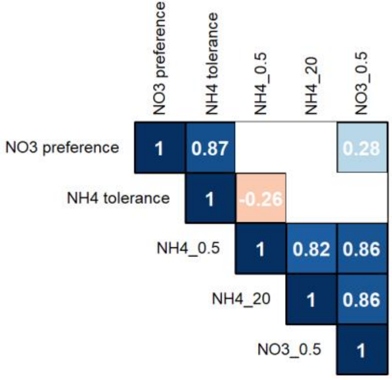
Supplementary Figure 2 Proportion of variance explained by each component in the Nested Association Mapping populations

A All 6 NAM populations



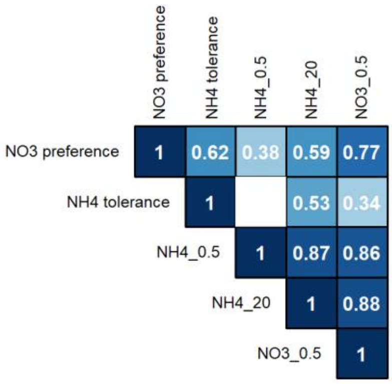
	NO3 preference	NH4 tolerance	NH4_0.5	NH4_20	NO3_0.5
NO3 preference	1	0.65	0.16	0.36	0.54
NH4 tolerance		1	-0.23	0.16	
NH4_0.5			1	0.82	0.86
NH4_20				1	0.84
NO3_0.5					1

B Dhwar Dry



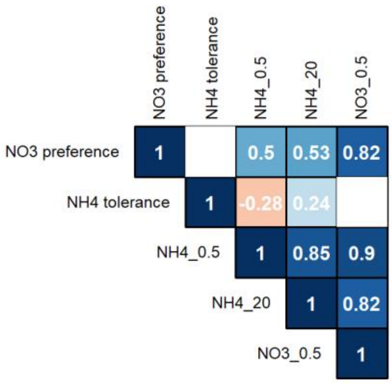
	NO3 preference	NH4 tolerance	NH4_0.5	NH4_20	NO3_0.5
NO3 preference	1	0.87			0.28
NH4 tolerance		1	-0.26		
NH4_0.5			1	0.82	0.86
NH4_20				1	0.86
NO3_0.5					1

C PBW343



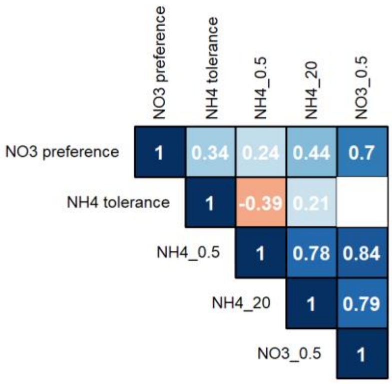
	NO3 preference	NH4 tolerance	NH4_0.5	NH4_20	NO3_0.5
NO3 preference	1	0.62	0.38	0.59	0.77
NH4 tolerance		1		0.53	0.34
NH4_0.5			1	0.87	0.86
NH4_20				1	0.88
NO3_0.5					1

D RAC875



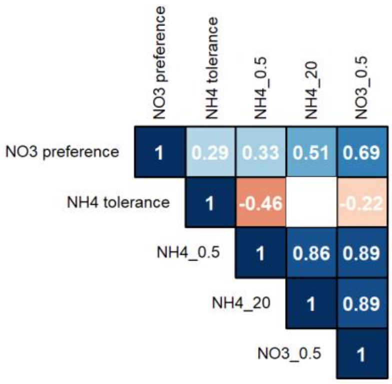
	NO3 preference	NH4 tolerance	NH4_0.5	NH4_20	NO3_0.5
NO3 preference	1		0.5	0.53	0.82
NH4 tolerance		1	-0.28	0.24	
NH4_0.5			1	0.85	0.9
NH4_20				1	0.82
NO3_0.5					1

E RSI5



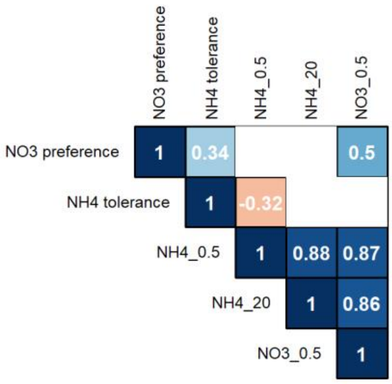
	NO3 preference	NH4 tolerance	NH4_0.5	NH4_20	NO3_0.5
NO3 preference	1	0.34	0.24	0.44	0.7
NH4 tolerance		1	-0.39	0.21	
NH4_0.5			1	0.78	0.84
NH4_20				1	0.79
NO3_0.5					1

F UC1036



	NO3 preference	NH4 tolerance	NH4_0.5	NH4_20	NO3_0.5
NO3 preference	1	0.29	0.33	0.51	0.69
NH4 tolerance		1	-0.46		-0.22
NH4_0.5			1	0.86	0.89
NH4_20				1	0.89
NO3_0.5					1

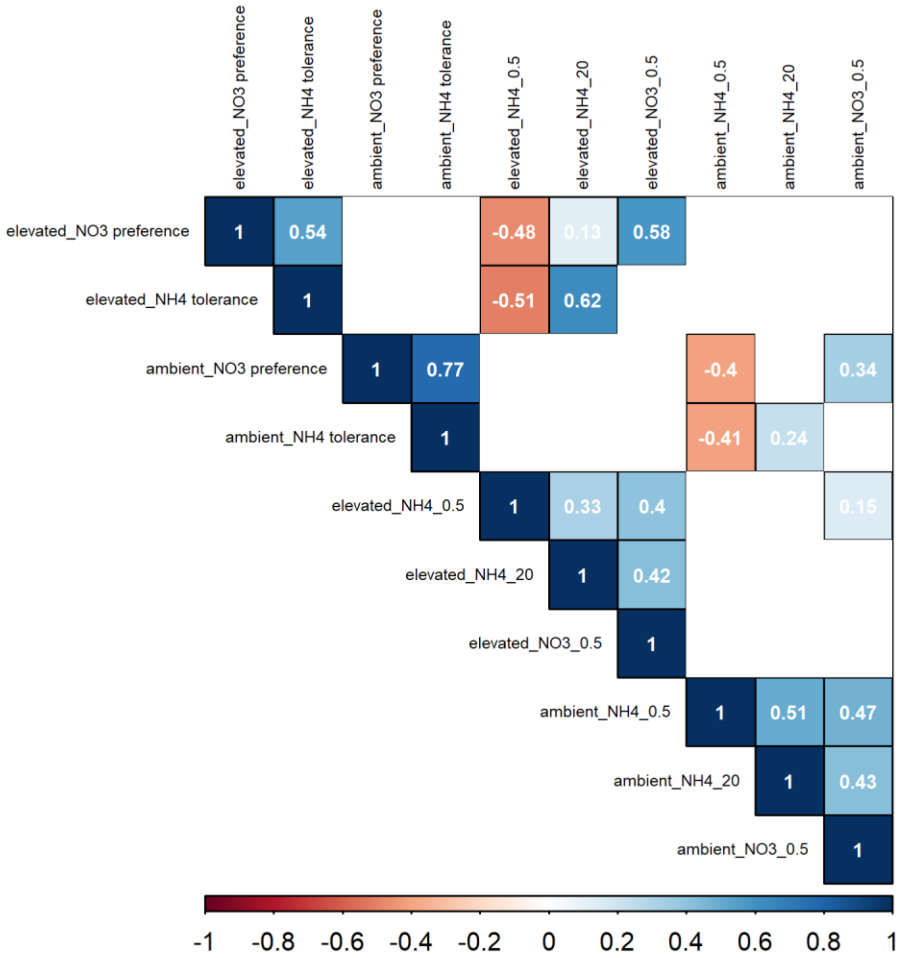
G UC1419



	NO3 preference	NH4 tolerance	NH4_0.5	NH4_20	NO3_0.5
NO3 preference	1	0.34			0.5
NH4 tolerance		1	-0.32		
NH4_0.5			1	0.88	0.87
NH4_20				1	0.86
NO3_0.5					1

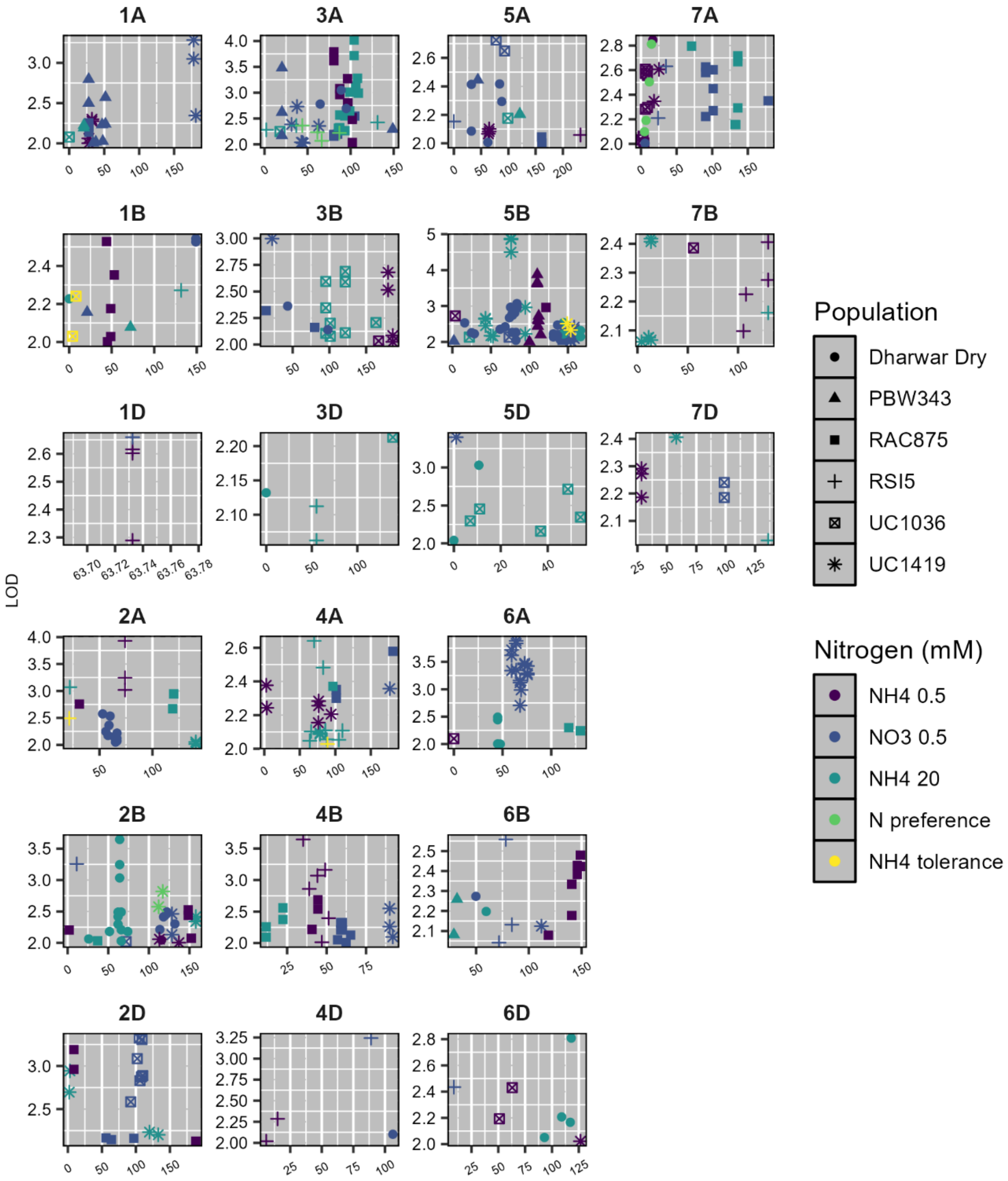
Supplementary Figure 3 Correlation between biomass traits of 6 Nested Association Mapping populations

Pearson's correlation coefficients at p-value < 0.01 between biomass traits across the CO_2 concentrations (A) across 6 NAM populations and (B) – (G) by each NAM population. Insignificant correlations are not displayed. The horizontal bar displays reference color for negative correlations (red) to positive correlations (blue).



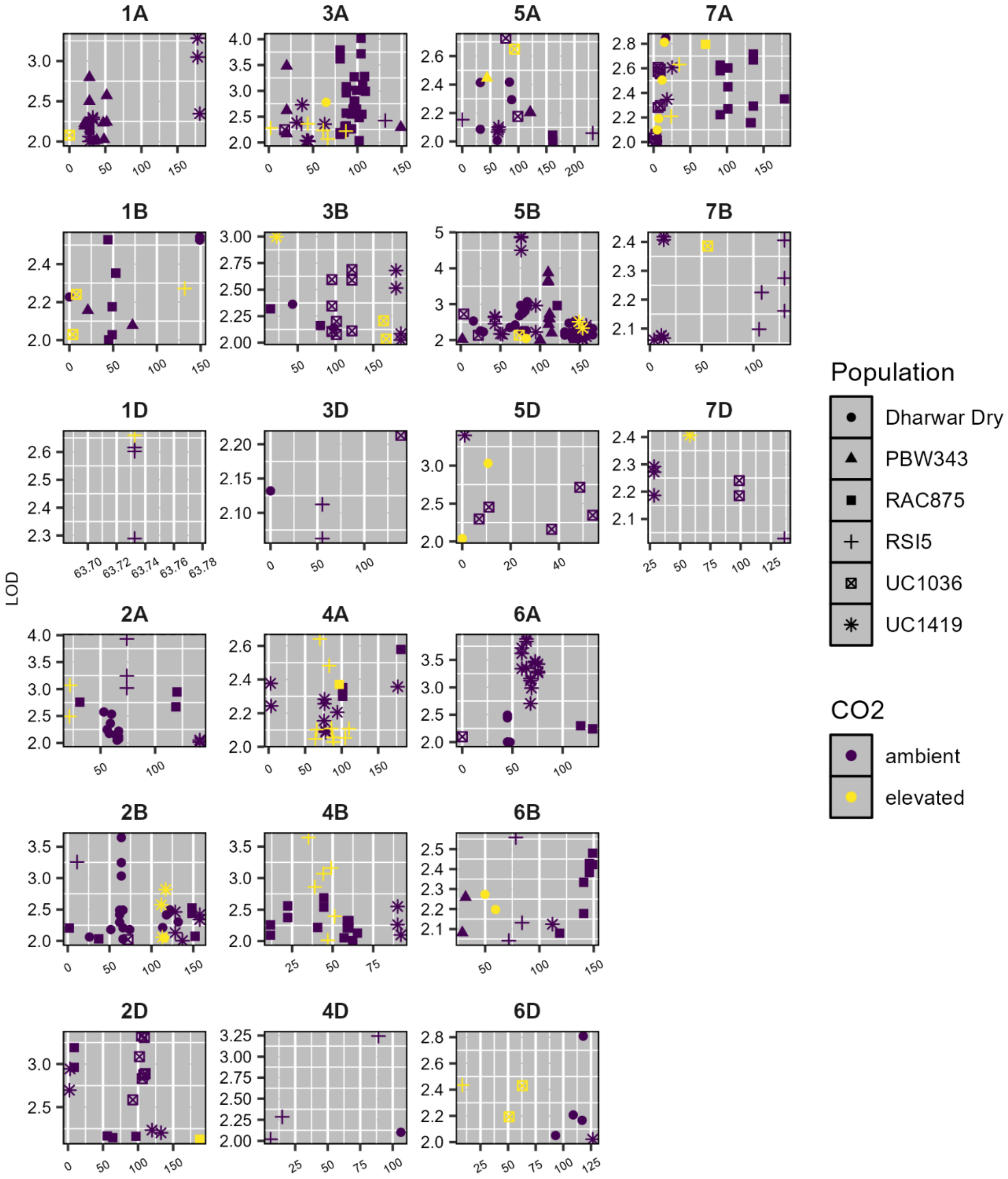
Supplementary Figure 4 Correlation between biomass traits of 6 Nested Association Mapping populations by CO_2 concentration

Pearson’s correlation coefficients at p-value < 0.01 between biomass traits by CO_2 concentration. Insignificant correlations are not displayed. The horizontal bar displays reference color for negative correlations (red) to positive correlations (blue).



Supplementary Figure 5 QTL mapping identified significant markers specific to populations and nitrogen forms across the genome

Distribution of significant markers (LOD > 2.0) across the wheat genome. Each panel represents a chromosome. Symbols represent individual bi-parental populations. Symbol colors represent nitrogen treatments or traits.



Supplementary Figure 6 QTL mapping identified relatively more significant markers under ambient than elevated CO₂ conditions.

Distribution of significant markers (LOD = 2.0) across the wheat genome. Each panel represents a chromosome. Symbols represent individual bi-parental populations. Symbol colors represent CO₂ levels.

Supplementary Table 1 Significant SNPs with p-value < 0.05

This table is available as a supplementary file.

Supplementary Table 2 SNPs with LOD > 2.0 identified in QTL mapping of 6 biparental mapping populations at ambient and elevated CO_2

CO_2	Pop	Chr	Pos	LOD	Organ	Replicate	Nitrogen	Marker	Effect	R^2	Closest marker	Closest marker LOD
ambient	PBW343	1A	47.00	2.23	shoot	September	NO3_0.5	c1A.loc47	-0.037	0.13	IWB8167_1A_522.5	2.18
ambient	UC1419	1A	178.00	3.05	whole	August	NO3_0.5	c1A.loc178	-0.068	0.17	IWB36704_1A_564.4	2.46
ambient	UC1419	1A	178.00	3.28	shoot	August	NO3_0.5	c1A.loc178	-0.050	0.18	IWB36704_1A_564.4	2.37
ambient	UC1419	1A	181.00	2.35	root	August	NO3_0.5	c1A.loc181	-0.017	0.14	IWB36704_1A_564.4	2.31
elevated	UC1036	1B	4.00	2.03	NA	NA	NH4_tolerance	c1B.loc4	0.070	0.13	IWB7923_1B_38.9	2.01
elevated	UC1036	1B	8.00	2.24	NA	NA	NH4_tolerance	c1B.loc8	0.080	0.14	IWB36013_1B_94.8	2.11
ambient	RAC875	1B	44.00	2.53	root	September	NH4_0.5	c1B.loc44	-0.013	0.16	IWB22869_1B_189.9	2.33
ambient	RAC875	1B	53.00	2.35	root	September	NH4_0.5	c1B.loc53	-0.013	0.15	IWB7186_1B_163.1	2.35
elevated	RSI5	2A	22.00	3.07	NA	NA	NH4_20	c2A.loc22	30.710	0.18	IWB12622_2A_21.3	3.05
ambient	DD	2A	53.00	2.58	root	September	NO3_0.5	c2A.loc53	-0.009	0.15	IWA5793_2A_78.3	2.49
ambient	DD	2A	56.00	2.25	whole	September	NO3_0.5	c2A.loc56	-0.026	0.13	IWB10000_2A_161.4	2.22
ambient	DD	2A	58.00	2.18	shoot	September	NO3_0.5	c2A.loc58	-0.018	0.13	IWB54979_2A_611.9	2.12
ambient	DD	2A	59.00	2.36	whole	September	NO3_0.5	c2A.loc59	-0.027	0.14	IWB54979_2A_611.9	2.22
ambient	DD	2A	60.00	2.53	root	September	NO3_0.5	c2A.loc60	-0.009	0.15	IWA2831_2A_205.2	2.45
ambient	DD	2A	64.00	2.15	root	September	NO3_0.5	c2A.loc64	-0.008	0.13	IWB3592_2A_598.6	2.09
ambient	RAC875	2A	119.00	2.67	whole	September	NH4_20	c2A.loc119	-0.035	0.16	IWA8385_2A_728.8	2.14
ambient	RAC875	2A	120.00	2.95	shoot	September	NH4_20	c2A.loc120	-0.028	0.18	IWA8385_2A_728.8	2.52
ambient	RAC875	2B	2.00	2.21	root	August	NH4_0.5	c2B.loc2	0.008	0.14	IWB32316_2B_3.4	2.14
ambient	DD	2B	66.00	2.21	whole	September	NH4_20	c2B.loc66	0.033	0.13	IWB2702_2B_57.7	2.03
ambient	DD	2B	66.00	2.49	root	September	NH4_20	c2B.loc66	0.009	0.14	IWB2702_2B_57.7	2.22
elevated	UC1419	2B	112.00	2.57	NA	NA	N_preference	c2B.loc112	-0.100	0.15	IWB7223_2B_730.2	2.19
ambient	DD	2B	118.00	2.41	root	July	NO3_0.5	c2B.loc118	0.012	0.14	IWA6966_2B_449.8	2.41
ambient	DD	2B	132.00	2.30	root	July	NO3_0.5	c2B.loc132	0.012	0.13	IWB34793_2B_524.2	2.27
ambient	UC1419	2D	2.00	2.70	whole	August	NH4_20	c2D.loc2	-0.036	0.15	IWB15863_2D_9.3	2.60
ambient	UC1419	2D	3.00	2.94	shoot	August	NH4_20	c2D.loc3	-0.030	0.17	IWB15863_2D_9.3	2.79
ambient	RAC875	2D	56.00	2.17	root	August	NO3_0.5	c2D.loc56	-0.007	0.13	IWB24243_2D_71.8	2.13
ambient	RAC875	2D	64.00	2.15	root	August	NO3_0.5	c2D.loc64	-0.008	0.13	IWB24243_2D_71.8	2.13
ambient	UC1036	2D	102.00	3.09	root	July	NO3_0.5	c2D.loc102	0.017	0.19	IWB60045_2D_622.9	2.64

ambient	UC1036	2D	105.00	3.32	whole	July	NO3_0.5	c2D.loc105	0.047	0.20	IWB60045_2D_622.9	3.17
ambient	UC1036	2D	106.00	2.83	shoot	July	NO3_0.5	c2D.loc106	0.029	0.17	IWB60045_2D_622.9	2.80
ambient	UC1036	2D	108.00	2.87	shoot	July	NO3_0.5	c2D.loc108	0.029	0.18	IWB60045_2D_622.9	2.80
ambient	UC1036	2D	109.00	3.31	whole	July	NO3_0.5	c2D.loc109	0.043	0.20	IWB60045_2D_622.9	3.17
ambient	UC1036	2D	110.00	2.89	root	July	NO3_0.5	c2D.loc110	0.016	0.18	IWB48503_2D_598.6	2.74
elevated	RAC875	2D	188.00	2.13	NA	NA	NH4_0.5	c2D.loc188	28.790	0.13	IWB64108_2D_648.3	2.07
elevated	RSI5	3A	2.00	2.28	NA	NA	NH4_20	c3A.loc2	27.280	0.14	IWB8501_3A_12.3	2.28
ambient	UC1419	3A	44.00	2.04	fshoot	July	NO3_0.5	c3A.loc44	0.016	0.12	IWB6013_3A_56.4	2.02
ambient	UC1419	3A	45.00	2.02	fshoot	July	NO3_0.5	c3A.loc45	0.016	0.12	IWB6013_3A_56.4	2.02
ambient	UC1419	3A	63.00	2.35	fshoot	July	NO3_0.5	c3A.loc63	0.018	0.14	IWA7319_3A_513.6	2.04
ambient	RAC875	3A	85.00	2.20	root	September	NO3_0.5	c3A.loc85	0.009	0.14	IWB6861_3A_471.4	2.16
elevated	RSI5	3A	87.00	2.22	NA	NA	N_preference	c3A.loc87	-0.090	0.14	IWB65332_3A_627.3	2.21
ambient	DD	3A	89.00	3.05	fshoot	July	NO3_0.5	c3A.loc89	0.026	0.17	IWB73345_3A_624.5	2.64
ambient	DD	3A	95.00	2.69	fshoot	July	NO3_0.5	c3A.loc95	0.024	0.15	IWB8499_3A_638.4	2.62
ambient	RAC875	3A	97.00	2.80	root	September	NH4_0.5	c3A.loc97	0.013	0.17	IWB30296_3A_586.9	2.78
ambient	RAC875	3A	99.00	2.65	whole	September	NH4_20	c3A.loc99	0.031	0.16	IWB39719_3A_605.8	2.61
ambient	RAC875	3A	99.00	3.01	shoot	September	NH4_20	c3A.loc99	0.026	0.18	IWB39719_3A_605.8	2.95
ambient	RAC875	3A	102.00	2.48	root	September	NH4_0.5	c3A.loc102	0.013	0.15	IWA3078_3A_611.7	2.21
ambient	RAC875	3A	102.00	2.52	whole	September	NH4_0.5	c3A.loc102	0.041	0.15	IWA3078_3A_611.7	2.31
ambient	RAC875	3A	105.00	2.55	root	September	NO3_0.5	c3A.loc105	0.010	0.16	IWB26018_3A_624.9	2.44
ambient	RAC875	3A	106.00	3.12	shoot	September	NH4_20	c3A.loc106	0.027	0.19	IWB5751_3A_625.5	3.12
ambient	RAC875	3A	108.00	3.28	shoot	September	NH4_20	c3A.loc108	0.027	0.20	IWB23609_3A_636.8	3.08
ambient	RAC875	3A	109.00	2.99	whole	September	NH4_20	c3A.loc109	0.033	0.18	IWB23609_3A_636.8	2.71
ambient	RAC875	3B	14.00	2.32	fshoot	July	NO3_0.5	c3B.loc14	0.009	0.14	IWB39254_3B_15	2.28
elevated	UC1419	3B	22.00	3.00	NA	NA	NO3_0.5	c3B.loc22	44.950	0.17	IWB56030_3B_825.8	2.92
elevated	UC1036	3B	167.00	2.03	NA	NA	NH4_0.5	c3B.loc167	37.520	0.13	IWB3151_3B_785.6	2.02
elevated	RSI5	4A	64.00	2.05	NA	NA	NH4_20	c4A.loc64	25.820	0.13	IWB58408_4A_597.9	2.04
elevated	RSI5	4A	66.00	2.10	NA	NA	NH4_20	c4A.loc66	26.990	0.13	IWB61099_4A_600.9	2.04
ambient	UC1419	4A	76.00	2.15	shoot	July	NH4_0.5	c4A.loc76	-0.031	0.13	IWB71770_4A_611.6	2.15
elevated	RSI5	4A	86.00	2.11	NA	NA	NH4_20	c4A.loc86	27.380	0.13	IWB47072_4A_585.7	2.02
elevated	RSI5	4A	88.00	2.06	NA	NA	NH4_20	c4A.loc88	27.090	0.13	IWB47072_4A_585.7	2.02

ambient	UC1419	4A	94.00	2.21	fshoot	July	NH4_0.5	c4A.loc94	-0.013	0.13	IWB42362_4A_622.2	2.00
ambient	RAC875	4A	181.00	2.58	root	July	NO3_0.5	c4A.loc181	0.008	0.16	IWA7765_4A_710.7	2.58
elevated	RSI5	4B	39.00	2.86	NA	NA	NH4_0.5	c4B.loc39	36.040	0.17	IWB73001_4B_26.5	2.81
ambient	RAC875	4B	57.00	2.05	shoot	July	NO3_0.5	c4B.loc57	0.029	0.13	IWB61846_4B_531.3	2.05
ambient	RAC875	4B	59.00	2.21	whole	July	NO3_0.5	c4B.loc59	0.036	0.14	IWB55457_4B_535.1	2.20
ambient	RAC875	4B	59.00	2.32	shoot	July	NO3_0.5	c4B.loc59	0.030	0.14	IWB55457_4B_535.1	2.29
ambient	RAC875	4B	60.00	2.21	whole	July	NO3_0.5	c4B.loc60	0.037	0.14	IWB55457_4B_535.1	2.20
ambient	RAC875	4B	60.00	2.33	shoot	July	NO3_0.5	c4B.loc60	0.031	0.14	IWB55457_4B_535.1	2.29
ambient	UC1419	4B	90.00	2.55	shoot	July	NO3_0.5	c4B.loc90	0.033	0.15	IWB38092_4B_646.7	2.17
ambient	RSI5	4D	6.00	2.02	shoot	July	NH4_0.5	c4D.loc6	0.039	0.12	IWB49180_4D_6	2.02
ambient	RSI5	4D	89.00	3.24	fshoot	August	NO3_0.5	c4D.loc89	-0.016	0.19	IWB28897_4D_484.7	3.24
elevated	PBW343	5A	44.00	2.45	NA	NA	NO3_0.5	c5A.loc44	61.140	0.15	IWA3190_5A_41.4	2.02
ambient	UC1036	5A	99.00	2.18	fshoot	September	NH4_20	c5A.loc99	0.019	0.14	IWA3370_5A_538.3	2.17
ambient	PBW343	5A	121.00	2.20	whole	July	NH4_20	c5A.loc121	0.046	0.13	IWA2836_5A_598.7	2.08
ambient	PBW343	5A	121.00	2.21	shoot	July	NH4_20	c5A.loc121	0.035	0.13	IWA2836_5A_598.7	2.07
ambient	RAC875	5A	161.00	2.04	shoot	July	NO3_0.5	c5A.loc161	0.029	0.13	IWB51122_5A_584.1	2.04
ambient	DD	5B	16.00	2.53	root	August	NO3_0.5	c5B.loc16	-0.010	0.15	IWB30458_5B_12.6	2.36
ambient	DD	5B	25.00	2.26	root	August	NO3_0.5	c5B.loc25	-0.009	0.13	IWB73825_5B_13.7	2.23
ambient	UC1419	5B	50.00	2.17	root	July	NH4_20	c5B.loc50	-0.009	0.13	IWB39949_5B_275	2.17
ambient	DD	5B	62.00	2.35	fshoot	September	NO3_0.5	c5B.loc62	0.017	0.14	IWB29709_5B_440.5	2.30
ambient	DD	5B	74.00	2.67	fshoot	September	NO3_0.5	c5B.loc74	0.019	0.15	IWB13598_5B_487.5	2.55
ambient	DD	5B	78.00	2.74	shoot	July	NO3_0.5	c5B.loc78	0.045	0.16	IWB11214_5B_526.6	2.73
ambient	DD	5B	84.00	3.00	fshoot	September	NO3_0.5	c5B.loc84	0.019	0.17	IWA7024_5B_546.8	2.99
ambient	PBW343	5B	111.00	2.61	root	August	NH4_0.5	c5B.loc111	0.009	0.16	IWB39017_5B_696	2.61
ambient	PBW343	5B	112.00	2.59	shoot	August	NH4_0.5	c5B.loc112	0.030	0.15	IWA4329_5B_694.5	2.48
ambient	PBW343	5B	112.00	2.72	whole	August	NH4_0.5	c5B.loc112	0.039	0.16	IWA4329_5B_694.5	2.53
ambient	UC1419	5B	158.00	2.41	root	September	NO3_0.5	c5B.loc158	-0.016	0.14	IWB46235_5B_583.2	2.38
ambient	UC1419	5D	1.00	3.40	fshoot	July	NO3_0.5	c5D.loc1	-0.019	0.19	IWA2821_5D_540.3	3.38
ambient	UC1036	5D	7.00	2.30	shoot	August	NH4_20	c5D.loc7	0.021	0.14	IWB5734_5D_3.6	2.23
ambient	UC1036	5D	11.00	2.45	whole	August	NH4_20	c5D.loc11	0.028	0.15	IWB5734_5D_3.6	2.25
ambient	UC1036	5D	37.00	2.16	fshoot	August	NH4_20	c5D.loc37	-0.016	0.14	IWB31037_5D_107.6	2.00

ambient	UC1036	5D	54.00	2.35	fshoot	August	NH4_20	c5D.loc54	-0.019	0.15	IWB45442_5D_312.6	2.30
ambient	DD	6A	45.00	2.44	whole	September	NH4_20	c6A.loc45	0.034	0.14	IWB40242_6A_31.4	2.26
ambient	DD	6A	45.00	2.49	shoot	September	NH4_20	c6A.loc45	0.026	0.14	IWB40242_6A_31.4	2.31
ambient	UC1419	6A	64.00	3.84	shoot	July	NO3_0.5	c6A.loc64	-0.041	0.21	IWB40867_6A_67.5	3.83
ambient	UC1419	6A	64.00	3.88	whole	July	NO3_0.5	c6A.loc64	-0.055	0.21	IWB40867_6A_67.5	3.88
ambient	UC1419	6A	68.00	2.70	root	July	NO3_0.5	c6A.loc68	-0.012	0.15	IWB12868_6A_73.7	2.60
ambient	UC1419	6A	68.00	3.12	shoot	July	NO3_0.5	c6A.loc68	-0.037	0.18	IWB12868_6A_73.7	2.99
ambient	UC1419	6A	68.00	3.16	whole	July	NO3_0.5	c6A.loc68	-0.050	0.18	IWB12868_6A_73.7	3.01
ambient	UC1419	6A	69.00	2.99	shoot	July	NO3_0.5	c6A.loc69	-0.037	0.17	IWB62987_6A_61	2.99
ambient	RAC875	6A	118.00	2.30	fshoot	August	NH4_20	c6A.loc118	-0.007	0.14	IWB3096_6A_592.5	2.25
ambient	PBW343	6B	32.00	2.26	fshoot	September	NH4_20	c6B.loc32	0.023	0.14	IWB49791_6B_154	2.19
ambient	RSI5	6B	84.00	2.13	fshoot	July	NO3_0.5	c6B.loc84	-0.012	0.13	IWA4484_6B_519.1	2.12
ambient	RAC875	6B	146.00	2.38	whole	September	NH4_0.5	c6B.loc146	0.042	0.15	IWB27572_6B_720.5	2.21
ambient	RAC875	6B	146.00	2.43	shoot	September	NH4_0.5	c6B.loc146	0.032	0.15	IWB27572_6B_720.5	2.28
ambient	RAC875	6B	149.00	2.42	whole	September	NH4_0.5	c6B.loc149	0.039	0.15	IWB23603_6B_718.3	2.21
ambient	RAC875	6B	149.00	2.48	shoot	September	NH4_0.5	c6B.loc149	0.030	0.15	IWB23603_6B_718.3	2.28
elevated	UC1036	6D	63.00	2.43	NA	NA	NH4_0.5	c6D.loc63	-32.340	0.15	IWA4042_6D_425	2.07
ambient	DD	6D	117.00	2.17	whole	September	NH4_20	c6D.loc117	-0.036	0.13	IWB35491_6D_389.6	2.12
ambient	DD	6D	118.00	2.81	root	September	NH4_20	c6D.loc118	-0.010	0.16	IWB35491_6D_389.6	2.76
elevated	DD	7A	7.00	2.19	NA	NA	N_preference	c7A.loc7	0.110	0.13	IWB28886_7A_15	2.08
ambient	UC1036	7A	9.00	2.56	shoot	September	NH4_0.5	c7A.loc9	-0.032	0.16	IWB46914_7A_12.4	2.55
elevated	RSI5	7A	24.00	2.21	NA	NA	NO3_0.5	c7A.loc24	46.590	0.14	IWA8032_7A_26.4	2.18
elevated	RSI5	7A	35.00	2.63	NA	NA	NO3_0.5	c7A.loc35	50.740	0.16	IWB12296_7A_34.7	2.55
elevated	RAC875	7A	71.00	2.80	NA	NA	NH4_20	c7A.loc71	30.460	0.17	IWB7484_7A_76.1	2.76
ambient	RAC875	7A	136.00	2.29	root	July	NH4_20	c7A.loc136	0.009	0.14	IWB9574_7A_689.3	2.03
ambient	RAC875	7A	136.00	2.67	shoot	July	NH4_20	c7A.loc136	0.043	0.16	IWB9574_7A_689.3	2.20
ambient	RAC875	7A	136.00	2.72	whole	July	NH4_20	c7A.loc136	0.052	0.17	IWB9574_7A_689.3	2.25
ambient	RSI5	7B	130.00	2.16	fshoot	September	NH4_20	c7B.loc130	-0.010	0.13	IWB2238_7B_721.2	2.15
ambient	RSI5	7B	130.00	2.27	whole	September	NH4_0.5	c7B.loc130	-0.049	0.14	IWB2238_7B_721.2	2.27
ambient	RSI5	7B	130.00	2.41	shoot	September	NH4_0.5	c7B.loc130	-0.041	0.15	IWB2238_7B_721.2	2.40

elevated	UC1419	7D	58.00	2.41	NA	NA	NH4_20	c7D.loc58	26.950	0.14	IWB63887_7D_565.2	2.28

References

Arends, D., Prins, P., Jansen, R. C., & Broman, K. W. (2010). R/qtl: High-throughput multiple QTL mapping: Fig. 1. *Bioinformatics*, *26*(23), 2990–2992. https://doi.org/10.1093/bioinformatics/btq565

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using **lme4**. *Journal of Statistical Software*, *67*(1). https://doi.org/10.18637/jss.v067.i01

Blake, N. K., Pumphrey, M., Glover, K., Chao, S., Jordan, K., Jannick, J.-L., Akhunov, E. A., Dubcovsky, J., Bockelman, H., & Talbert, L. E. (2019). Registration of the Triticeae-CAP Spring Wheat Nested Association Mapping Population. *Journal of Plant Registrations*, *13*(2), 294–297. https://doi.org/10.3198/jpr2018.07.0052crmp

Bloom, A. J. (2010). Energetics of Nitrogen Acquisition. In *Annual Plant Reviews Volume 42* (pp. 63–81). Wiley-Blackwell.

Bloom, A. J., Burger, M., Asensio, J. S. R., & Cousins, A. B. (2010). Carbon Dioxide Enrichment Inhibits Nitrate Assimilation in Wheat and Arabidopsis. *Science*, *328*(5980), 899–903. https://doi.org/10.1126/science.1186440

Bloom, A. J., Chapin, F. S., & Mooney, H. A. (1985). Resource Limitation in Plants-An Economic Analogy. *Annual Review of Ecology and Systematics*, *16*(1), 363–392. https://doi.org/10.1146/annurev.es.16.110185.002051

Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., & Buckler, E. S. (2007). TASSEL: Software for association mapping of complex traits in diverse samples. *Bioinformatics*, *23*(19), 2633–2635. https://doi.org/10.1093/bioinformatics/btm308

Britto, D. T., & Kronzucker, H. J. (2002). NH4+ toxicity in higher plants: A critical review. *Journal of Plant Physiology*, *159*(6), 567–584. http://dx.doi.org/10.1078/0176-1617-0774

Britto, D. T., & Kronzucker, H. J. (2013). Ecological significance and complexity of N-source preference in plants. *Annals of Botany*, *112*(6), 957–963. https://doi.org/10.1093/aob/mct157

Cassan, O., Pimparé, L.-L., Mozzanino, T., Fizames, C., Devidal, S., Roux, F., Milcu, A., Lèbre, S., Gojon, A., & Martin, A. (2023). *Natural genetic variation underlying the negative effect of* *elevated CO_2 on ionome composition in* Arabidopsis thaliana [Preprint]. Plant Biology. https://doi.org/10.1101/2023.06.05.543678

Clarke, E., Sherrill-Mix, S., & Dawson, C. (2023). *ggbeeswarm: Categorical Scatter (Violin Point) Plots* (Version R package version 0.7.2) [Computer software]. https://CRAN.R-project.org/package=ggbeeswarm

Cormier, F., Faure, S., Dubreuil, P., Heumez, E., Beauchêne, K., Lafarge, S., Praud, S., & Le Gouis, J. (2013). A multi-environmental study of recent breeding progress on nitrogen use efficiency in wheat (Triticum aestivum L.). *Theoretical and Applied Genetics*, *126*(12), 3035–3048. https://doi.org/10.1007/s00122-013-2191-9

Cormier, F., Foulkes, J., Hirel, B., Gouache, D., Moënne-Loccoz, Y., & Le Gouis, J. (2016). Breeding for increased nitrogen-use efficiency: A review for wheat (*T. aestivum* L.). *Plant Breeding*, *135*(3), 255–278. https://doi.org/10.1111/pbr.12371

Cormier, F., Le Gouis, J., Dubreuil, P., Lafarge, S., & Praud, S. (2014). A genome-wide identification of chromosomal regions determining nitrogen use efficiency components in wheat (Triticum aestivum L.). *Theoretical and Applied Genetics*, *127*(12), 2679–2693.

Cox, W. J., & Reisenauer, H. M. (1973). Growth and ion uptake by wheat supplied nitrogen as nitrate, or ammonium, or both. *Plant and Soil*, *38*(2), 363–380. https://doi.org/10.1007/BF00779019

Dowle, M., & Srinivasan, A. (2023). *data.table: Extension of `data.frame`* [Computer software]. https://r-datatable.com, https://Rdatatable.gitlab.io/data.table, https://github.com/Rdatatable/data.table

Epstein, E., & Bloom, A. J. (2005). *Mineral Nutrition of Plants: Principles and Perspectives*. Sinauer Associates, Incorporated.

Fox, J., & Weisberg, S. (2019). *An R companion to applied regression* (Third edition). SAGE.

Fuertes-Mendizábal, T., González-Torralba, J., Arregui, L. M., González-Murua, C., González-Moro, M. B., & Estavillo, J. M. (2013). Ammonium as sole N source improves grain quality in wheat. *Journal of the Science of Food and Agriculture*, *93*(9), 2162–2171. https://doi.org/10.1002/jsfa.6022

Gao, Y., & Cabrera Serrenho, A. (2023). Greenhouse gas emissions from nitrogen fertilizers could be reduced by up to one-fifth of current levels by 2050 with combined interventions. *Nature Food*, *4*(2), 170–178. https://doi.org/10.1038/s43016-023-00698-w

Gooding, M. J., Addisu, M., Uppal, R. K., Snape, J. W., & Jones, H. E. (2012). Effect of wheat dwarfing genes on nitrogen-use efficiency. *The Journal of Agricultural Science*, *150*(1), 3–22. https://doi.org/10.1017/S0021859611000414

Hawkesford, M. J. (2014). Reducing the reliance on nitrogen fertilizer for wheat production. *Journal of Cereal Science*, *59*(3), 276–283. https://doi.org/10.1016/j.jcs.2013.12.001

He, X., Chi, Q., Meng, L., Zhao, C., He, M., Dan, X., Huang, X., Zhao, J., Cai, Z., Zhang, J., & Müller, C. (2022). Plants with nitrate preference can regulate nitrification to meet their nitrate demand. *Soil Biology and Biochemistry*, *165*, 108516. https://doi.org/10.1016/j.soilbio.2021.108516

Huang, M., Liu, X., Zhou, Y., Summers, R. M., & Zhang, Z. (2019). BLINK: A package for the next level of genome-wide association studies with both individuals and markers in the millions. *GigaScience*, *8*(2). https://doi.org/10.1093/gigascience/giy154

IPCC. (2021). *Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* (V. Masson-Delmotte, P. Zhai, A. Pirani, S. L. Connors, C. Péan, S. Berger, N. Caud, Y. Chen, L. Goldfarb, M. I. Gomis, M. Huang, K. Leitzell, E. Lonnoy, J. B. R. Matthews, T. K. Maycock, T. Waterfield, Ö. Yelekçi, R. Yu, & B. Zhou, Eds.). Cambridge University Press. https://doi.org/10.1017/9781009157896

Kasemsap, P., & Bloom, A. J. (2022). Breeding for Higher Yields of Wheat and Rice through Modifying Nitrogen Metabolism. *Plants*, *12*(1), 85. https://doi.org/10.3390/plants12010085

Kasemsap, P., Cohen, I., & Bloom, A. J. (2024). *Genome-wide Association Study of Rice Vegetative Biomass under Different Inorganic Nitrogen Forms—Ammonium or Nitrate*. https://doi.org/10.1101/2024.08.12.607622

Katz, E., Knapp, A., Lensink, M., Keller, C. K., Stefani, J., Li, J.-J., Shane, E., Tuermer-Lee, K., Bloom, A. J., & Kliebenstein, D. J. (2022). Genetic variation underlying differential ammonium and nitrate responses in *Arabidopsis thaliana*. *The Plant Cell*, *34*(12), 4696–4713. https://doi.org/10.1093/plcell/koac279

Kong, L., Xie, Y., Hu, L., Feng, B., & Li, S. (2016). Remobilization of vegetative nitrogen to developing grain in wheat (Triticum aestivum L.). *Field Crops Research*, *196*, 134–144. https://doi.org/10.1016/j.fcr.2016.06.015

Korte, A., & Farlow, A. (2013). The advantages and limitations of trait analysis with GWAS: A review. *Plant Methods*, *9*(1), 29. https://doi.org/10.1186/1746-4811-9-29

Lammerts Van Bueren, E. T., & Struik, P. C. (2017). Diverse concepts of breeding for nitrogen use efficiency. A review. *Agronomy for Sustainable Development*, *37*(5), 50. https://doi.org/10.1007/s13593-017-0457-3

Langsrud, Ø. (2003). ANOVA for unbalanced data: Use Type II instead of Type III sums of squares. *Statistics and Computing*, *13*(2), 163–167. https://doi.org/10.1023/A:1023260610025

Li, S., Tian, Y., Wu, K., Ye, Y., Yu, J., Zhang, J., Liu, Q., Hu, M., Li, H., Tong, Y., Harberd, N. P., & Fu, X. (2018). Modulating plant growth–metabolism coordination for sustainable agriculture. *Nature*, *560*(7720), 595–600. https://doi.org/10.1038/s41586-018-0415-5

Lipka, A. E., Tian, F., Wang, Q., Peiffer, J., Li, M., Bradbury, P. J., Gore, M. A., Buckler, E. S., & Zhang, Z. (2012). GAPIT: Genome association and prediction integrated tool. *Bioinformatics*, *28*(18), 2397–2399. https://doi.org/10.1093/bioinformatics/bts444

Liu, S., Chi, Q., Cheng, Y., Zhu, B., Li, W., Zhang, X., Huang, Y., Müller, C., Cai, Z., & Zhang, J. (2019). Importance of matching soil N transformations, crop N form preference, and climate to enhance crop yield and reducing N loss. *Science of The Total Environment*, *657*, 1265–1273. https://doi.org/10.1016/j.scitotenv.2018.12.100

Liu, Y., Wang, H., Jiang, Z., Wang, W., Xu, R., Wang, Q., Zhang, Z., Li, A., Liang, Y., Ou, S., Liu, X., Cao, S., Tong, H., Wang, Y., Zhou, F., Liao, H., Hu, B., & Chu, C. (2021). Genomic basis of geographical adaptation to soil nitrogen in rice. *Nature*. https://doi.org/10.1038/s41586-020-03091-w

Lüdecke, D., Patil, I., Ben-Shachar, M., Wiernik, B., Waggoner, P., & Makowski, D. (2021). see: An R Package for Visualizing Statistical Models. *Journal of Open Source Software*, *6*(64), 3393. https://doi.org/10.21105/joss.03393

Maccaferri, M., Zhang, J., Bulli, P., Abate, Z., Chao, S., Cantu, D., Bossolini, E., Chen, X., Pumphrey, M., & Dubcovsky, J. (2015). A Genome-Wide Association Study of Resistance to Stripe Rust (Puccinia striiformis f. Sp. Tritici) in a Worldwide Collection of Hexaploid Spring Wheat (Triticum aestivum L.). *G3: Genes|Genomes|Genetics*, *5*(3), 449–465. PMC. https://doi.org/10.1534/g3.114.014563

Malik, P. L., Janss, L., Nielsen, L. K., Borum, F., Jørgensen, H., Eriksen, B., Schjoerring, J. K., & Rasmussen, S. K. (2019). Breeding for dual-purpose wheat varieties using marker–trait associations for biomass yield and quality traits. *Theoretical and Applied Genetics*, *132*(12), 3375–3398. https://doi.org/10.1007/s00122-019-03431-z

McCarthy, M. I., & Hirschhorn, J. N. (2008). Genome-wide association studies: Potential next steps on a genetic journey. *Human Molecular Genetics*, *17*(R2), R156–R165. https://doi.org/10.1093/hmg/ddn289

McMullen, M. D., Kresovich, S., Villeda, H. S., Bradbury, P., Li, H., Sun, Q., Flint-Garcia, S., Thornsberry, J., Acharya, C., Bottoms, C., Brown, P., Browne, C., Eller, M., Guill, K., Harjes, C., Kroon, D., Lepak, N., Mitchell, S. E., Peterson, B., … Ware, D. (2009). Genetic properties of the maize nested association mapping population. *Science*, *325*. https://doi.org/10.1126/science.1174320

Molero, G., Joynson, R., Pinera-Chavez, F. J., Gardiner, L., Rivera-Amado, C., Hall, A., & Reynolds, M. P. (2019). Elucidating the genetic basis of biomass accumulation and radiation use efficiency in spring wheat and its role in yield potential. *Plant Biotechnology Journal*, *17*(7), 1276–1288. https://doi.org/10.1111/pbi.13052

Nishina, K., Ito, A., Hanasaki, N., & Hayashi, S. (2017). Reconstruction of spatially detailed global map of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>−</sup> application in synthetic nitrogen fertilizer. *Earth System Science Data*, *9*(1), 149–162. https://doi.org/10.5194/essd-9-149-2017

Norton, J. M. (2015). Nitrification in Agricultural Soils. In J. S. Schepers & W. R. Raun (Eds.), *Agronomy Monographs* (pp. 173–199). American Society of Agronomy, Crop Science Society of America, Soil Science Society of America. https://doi.org/10.2134/agronmonogr49.c6

Norton, J., & Ouyang, Y. (2019). Controls and Adaptive Management of Nitrification in Agricultural Soils. *Frontiers in Microbiology*, *10*. https://www.frontiersin.org/article/10.3389/fmicb.2019.01931

O'Sullivan, C. A., Fillery, I. R. P., Roper, M. M., & Richards, R. A. (2016). Identification of several wheat landraces with biological nitrification inhibition capacity. *Plant and Soil*, *404*(1–2), 61–74. https://doi.org/10.1007/s11104-016-2822-4

O'Sullivan, C. A., Whisson, K., Treble, K., Roper, M. M., Micin, S. F., & Ward, P. R. (2017). Biological nitrification inhibition by weeds: Wild radish, brome grass, wild oats and annual ryegrass decrease nitrification rates in their rhizospheres. *Crop and Pasture Science*, *68*(8), 798. https://doi.org/10.1071/CP17243

O'Sullivan, C., Palta, J., Farrell, M., & Treble, K. (2016). Genotypic variability in wheat for preference of NH4 + , NO3—Or NH4 + NO3. *Proceedings of the 2016 International Nitrogen Initiative Conference*. 7th International Nitrogen Initiative Conference, Melbourne, 4th to 8th Dec 2016. http://www.ini2016.com/pdf-papers/INI2016_Osullivan_Cathryn.pdf

Qaseem, M. F., Qureshi, R., Muqaddasi, Q. H., Shaheen, H., Kousar, R., & Röder, M. S. (2018). Genome-wide association mapping in bread wheat subjected to independent and combined high temperature and drought stress. *PLOS ONE*, *13*(6), e0199121. https://doi.org/10.1371/journal.pone.0199121

R Core Team,. (2023). *R: A Language and Environment for Statistical Computing* [Computer software]. R Foundation for Statistical Computing. https://www.R-project.org/

Ren, C., Zhang, X., Reis, S., Wang, S., Jin, J., Xu, J., & Gu, B. (2023). Climate change unequally affects nitrogen use and losses in global croplands. *Nature Food*. https://doi.org/10.1038/s43016-023-00730-z

RStudio Team. (2020). *RStudio: Integrated Development for R* [Computer software]. http://www.rstudio.com/.

Rubio-Asensio, J. S., & Bloom, A. J. (2016). Inorganic nitrogen form: A major player in wheat and Arabidopsis responses to elevated CO2. *Journal of Experimental Botany*, erw465.

Saini, D. K., Chopra, Y., Singh, J., Sandhu, K. S., Kumar, A., Bazzer, S., & Srivastava, P. (2022). Comprehensive evaluation of mapping complex traits in wheat using genome-wide association studies. *Molecular Breeding*, *42*(1), 1. https://doi.org/10.1007/s11032-021-01272-7

Shi, H., Chen, M., Gao, L., Wang, Y., Bai, Y., Yan, H., Xu, C., Zhou, Y., Xu, Z., Chen, J., Tang, W., Wang, S., Shi, Y., Wu, Y., Sun, D., Jia, J., & Ma, Y. (2022). Genome-wide association study of agronomic traits related to nitrogen use efficiency in wheat. *Theoretical and Applied Genetics*. https://doi.org/10.1007/s00122-022-04218-5

Subbarao, G. V., Kishii, M., Bozal-Leorri, A., Ortiz-Monasterio, I., Gao, X., Ibba, M. I., Karwat, H., Gonzalez-Moro, M. B., Gonzalez-Murua, C., Yoshihashi, T., Tobita, S., Kommerell, V., Braun, H.-J., & Iwanaga, M. (2021). Enlisting wild grass genes to combat nitrification in wheat farming: A nature-based solution. *Proceedings of the National Academy of Sciences*, *118*(35), e2106595118. https://doi.org/10.1073/pnas.2106595118

Sukumaran, S., Dreisigacker, S., Lopes, M., Chavez, P., & Reynolds, M. P. (2015). Genome-wide association study for grain yield and related traits in an elite spring wheat population grown in temperate irrigated environments. *Theoretical and Applied Genetics*, *128*(2), 353–363. https://doi.org/10.1007/s00122-014-2435-3

Sun, J., Guo, Y., Zhang, G., Gao, M., Zhang, G., Kong, F., Zhao, Y., & Li, S. (2013). QTL mapping for seedling traits under different nitrogen forms in wheat. *Euphytica*, *191*(3), 317–331. https://doi.org/10.1007/s10681-012-0834-6

Walker, A. P., De Kauwe, M. G., Bastos, A., Belmecheri, S., Georgiou, K., Keeling, R. F., McMahon, S. M., Medlyn, B. E., Moore, D. J. P., Norby, R. J., Zaehle, S., Anderson-Teixeira, K. J., Battipaglia, G., Brienen, R. J. W., Cabugao, K. G., Cailleret, M., Campbell, E., Canadell, J. G., Ciais, P., … Zuidema, P. A. (2021). Integrating the evidence for a terrestrial carbon sink caused by increasing atmospheric CO2. *New Phytologist*, *229*(5), 2413–2445. https://doi.org/10.1111/nph.16866

Wang, X., Mao, W., Wang, Y., Lou, H., Guan, P., Chen, Y., Peng, H., & Wang, J. (2023). Breeding design in wheat by combining the QTL information in a GWAS panel with a general genetic map and computer simulation. *The Crop Journal*, S2214514123001368. https://doi.org/10.1016/j.cj.2023.10.001

Wei, T., & Simko, V. (2021). *R package "corrplot": Visualization of a Correlation* (Version Version 0.92) [Computer software]. https://github.com/taiyun/corrplot

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis* (2nd ed. 2016). Springer International Publishing : Imprint: Springer. https://doi.org/10.1007/978-3-319-24277-4

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T., Miller, E., Bache, S., Müller, K., Ooms, J., Robinson, D., Seidel, D., Spinu, V., … Yutani, H. (2019). Welcome to the Tidyverse. *Journal of Open Source Software, 4*(43), 1686. https://doi.org/10.21105/joss.01686

Xu, G., Fan, X., & Miller, A. J. (2012). Plant Nitrogen Assimilation and Use Efficiency. *Annual Review of Plant Biology*, *63*(1), 153–182. https://doi.org/10.1146/annurev-arplant-042811-105532

Yin, L., Zhang, H., Tang, Z., Xu, J., Yin, D., Zhang, Z., Yuan, X., Zhu, M., Zhao, S., Li, X., & Liu, X. (2020). *rMVP: A Memory-efficient, Visualization-enhanced, and Parallel-accelerated tool for Genome-Wide Association Study* [Preprint]. Genetics. https://doi.org/10.1101/2020.08.20.258491

Yu, J., Holland, J. B., McMullen, M. D., & Buckler, E. S. (2008). Genetic Design and Statistical Power of Nested Association Mapping in Maize. *Genetics*, *178*(1), 539–551. https://doi.org/10.1534/genetics.107.074245

Zhang, J., Gizaw, S. A., Bossolini, E., Hegarty, J., Howell, T., Carter, A. H., Akhunov, E., & Dubcovsky, J. (2018). Identification and validation of QTL for grain yield and plant water status under contrasting water treatments in fall-sown spring wheats. *Theoretical and Applied Genetics*, *131*(8), 1741–1759. https://doi.org/10.1007/s00122-018-3111-9

Zhang, X., Zou, T., Lassaletta, L., Mueller, N. D., Tubiello, F. N., Lisk, M. D., Lu, C., Conant, R. T., Dorich, C. D., Gerber, J., Tian, H., Bruulsema, T., Maaz, T. M., Nishina, K., Bodirsky, B. L., Popp, A., Bouwman, L., Beusen, A., Chang, J., … Davidson, E. A. (2021). Quantification of global and national nitrogen budgets for crop production. *Nature Food*, *2*(7), 529–540. https://doi.org/10.1038/s43016-021-00318-5

Chapter 4

Genetic adaptation to ammonium sustains wheat grain quality and alleviates acclimation to CO_2 enrichment

Submitted for publication and deposited on *biorxiv* (https://doi.org/10.1101/2023.11.21.568194)

Genetic adaptation to ammonium sustains wheat grain quality and alleviates acclimation to CO_2 enrichment

Pornpipat Kasemsap[a]*, Arnold J. Bloom[a]

[a]University of California at Davis, Department of Plant Sciences, Davis, CA 95616 USA

*Corresponding author, e-mail pkasemsap@ucdavis.edu

Running title: Adaptation to ammonium alleviates CO_2 acclimation

Abstract

Plants synthesize protein through assimilating inorganic nitrogen. Yet, the extent to which soil nitrogen sources alter crop responses to atmospheric CO_2 remains uncertain. We assessed wheat (*Triticum aestivum* L.) biomass under CO_2 enrichment in genotypes that demonstrated a preference for ammonium (NH_4^+) or nitrate (NO_3^-), and contrasting degrees of NH_4^+ tolerance. Nitrogen-form preference, but not NH_4^+ tolerance, correlated with CO_2 responses. Notably, NH_4^+-preferring genotypes maintained higher biomass and sustained grain nitrogen concentrations, thus avoiding CO_2 acclimation, the decline in biomass stimulation after prolonged exposure to CO_2 enrichment. Furthermore, NH_4^+ nutrition accelerated flowering and increased spike biomass. Breeding for NH_4^+-adapted genotypes may not only improve climate resilience, but also potentially accelerate development and increase yield without any penalty on grain quality. Because wheat provides 20% of the protein and carbohydrate in the human diet, our study provided strategies to sustain food security under the atmospheric conditions anticipated in the future.

Keywords

biomass, climate change, fertilizer, flowering, genetic, nitrate, nitrogen, nutrition, protein, yield

Introduction

Anthropogenic climate change poses significant challenges to food security. Crop nutritional values decline under CO_2 enrichment (Al-Hadeethi et al., 2019; Loladze, 2014). For example, wheat (*Triticum aestivum* L.), the staple crop that supplies 20% of protein and calories in the human diet (FAO, 2022), suffers from sharp declines in grain protein, Fe, and Zn under elevated CO_2 concentrations (Myers et al., 2014). Maintaining crop nutritional qualities as climate changes is vital to global food security.

Declining grain protein under CO_2 enrichment does not simply derive from dilution of organic nitrogen by additional biomass: in non-leguminous C_3 plants, CO_2 enrichment decreased nitrogen concentration about twice as much as the concentrations of other elements and almost three times more than the increase in carbon (Gifford et al., 2000; Loladze, 2014; Myers et al., 2014; Seibert et al., 2021; Taub et al., 2008; Uddling et al., 2018). Plants in which CO_2 enrichment did not stimulate growth still had lower nitrogen concentrations (Broberg et al., 2017; Feng et al., 2015; Wujeska-Klause et al., 2019). Even when total nitrogen remained high under CO_2 enrichment because of unassimilated NO_3^-, grain protein declined (Bloom et al., 2002). Wheat grown in California during the past 35 years compromised biomass to avoid changes in protein concentration as atmospheric CO_2 levels increased (Bloom & Plant, 2021). Farmers who already substantially fertilize their wheat (Raun & Johnson, 1999) may need unsustainable quantities of fertilizers to maintain high yields (Pleijel et al., 2019).

Wheat responses to CO_2 depend on nitrogen source (Bloom, 2015; Rubio-Asensio & Bloom, 2016). Crops acquire soil nitrogen primarily in the inorganic forms nitrate (NO_3^-) and ammonium (NH_4^+) (Xu et al., 2012). Elevated CO_2 concentrations during the day (Bloom et al., 2002, 2010, 2012, 2014) and night (Asensio et al., 2015) inhibit the assimilation of NO_3^- into protein in shoots, but NH_4^+ assimilation remains unaffected (Bloom et al., 2002). Reliance on NO_3^- as the primary nitrogen source diminishes grain protein under CO_2 enrichment in controlled environments (Bloom et al., 2010) and in the field (Bloom et al., 2014). By contrast, reliance on NH_4^+ sustains grain protein at elevated CO_2 levels (Fernando et al., 2017). Breeding cultivars for a greater reliance on NH_4^+ might ensure future productivity (Bloom et al., 2010, 2014).

Exposure to high NH_4^+ concentrations, however, can be toxic (Britto & Kronzucker, 2002). Plants expend up to 40% of root respiration to prevent excessive NH_4^+ accumulation (Britto et al., 2001).

NH_4^+-sensitive plants experience physiological and morphological disorders that limit growth and development (Esteban et al., 2016). To avoid toxicity, plants assimilate most of the absorbed soil NH_4^+ into organic compounds in roots and transport them to other organs (Britto & Kronzucker, 2002). Sensitivity to nitrogen forms, especially exposure to high NH_4^+ concentrations, varies significantly among species (Bloom et al., 2012; Britto & Kronzucker, 2013; Miller & Cramer, 2005). Nevertheless, information on the genetic basis of such responses remains sparse.

Rarely do studies account for different inorganic nitrogen forms (Kasemsap & Bloom, 2022). Only a handful of studies have contrasted the physiological responses of wheat to NH_4^+ versus NO_3^- and CO_2 levels (Bloom et al., 2002; Carlisle et al., 2012; Rubio-Asensio & Bloom, 2016), and none of the studies selected the plant materials based primarily on the degree of preference for a nitrogen form or NH_4^+ tolerance. The limited genetic diversity in any given study (Kasemsap & Bloom, 2022) and the difficulties in precisely controlling the nitrogen forms in the root zone (Bloom et al., 2012) may underlie the lack of understanding about this topic.

Here, we characterized biomass under CO_2 enrichment of 12 genotypes from diverse mapping populations that demonstrated a preference for NH_4^+ or NO_3^- and high tolerance or susceptibility to NH_4^+ toxicity. Given the strong interactions between carbon and nitrogen metabolisms, we hypothesized that plants would be more responsive to their preferred nitrogen form under CO_2 enrichment. These traits can guide breeders in selecting genotypes that ensure grain yield and quality under the edaphic and atmospheric conditions anticipated in the near future.

Materials and methods

Plant materials

In a preliminary study, we assessed the biomass of 15-day (d) old hexaploid wheat plants under either NH_4^+ or NO_3^- nutrition at ambient CO_2 concentrations and calculated the ratio of plant biomass under 1) 20 mM vs. 0.5 mM NH_4^+ (NH_4^+ tolerance) and 2) 0.5 mM NO_3^- vs. 0.5 mM NH_4^+ (nitrogen-form preference). Here, based on the nitrogen responses, we selected 3 genotypes from a panel of 875 spring hexaploid wheat (*Triticum aestivum* L.) accessions from the USDA-ARS National Small Grain Core Collection (NSGCC) (Maccaferri et al., 2015) and 8 genotypes from 2 bi-parental Nested-Association

Mapping (NAM) populations (Blake et al., 2019; Zhang et al., 2018). We included two additional genotypes: cv. Veery 10, which we have used in previous studies, as a reference genotype (Asensio et al., 2015; Bloom et al., 2002, 2012, 2020; Carlisle et al., 2012; Rubio-Asensio & Bloom, 2016), and cv. Berkut, the common parent of the bi-parental populations.

We defined inorganic nitrogen-form preference for each genotype as "NH_4^+-preference", "NO_3^--preference", or potentially adapted to "both forms" when the biomass ratio under 0.5 mM NH_4^+ vs. NO_3^- was higher than 1.1, lower than 0.9, or between 0.9 – 1.1, respectively. We defined NH_4^+ tolerance for each genotype as either "tolerant" or "susceptible" when the biomass ratio of plants under 20 mM vs. 0.5 mM NH_4^+ was higher or lower than 0.9, respectively. Based on these criteria, our experimental panel consisted of one reference genotype, one common parent of the bi-parental mapping populations and 11 additional genotypes for which we had sufficient seeds and that showed diverse responses to the nitrogen source (Table 1).

Table 1 Selected genotypes based on the ratio of 15 d plant biomass at ambient CO_2 levels under 1) 0.5 mM NO_3^- vs. 0.5 mM NH_4^+ (nitrogen-form preference) and 2) 20 mM vs. 0.5 mM NH_4^+ (NH_4^+ tolerance) from the USDA-National Small Grain Core Collection (NSGCC; 875 genotypes) and bi-parental Nested Association Mapping populations for which cv. Berkut was the common female parent (NAM; 75 genotypes each). Accession or line ID are from the original studies where the populations were developed.

#	Population (Accession/line ID) and name used in this study		Biomass ratio NO_3^-: NH_4^+	Form preference	Biomass ratio High: Low NH_4^+	NH_4^+ tolerance
1	NSGCC (PI83733)	74	0.96	Both	0.98	Tolerant
2	NSGCC (PI182077)	166	1.07	Both	1.02	Tolerant
3	NSGCC (PI285944)	459	1.05	Both	0.68	Susceptible
4	NAM Parent	Berkut	1.16	NO_3^-	0.71	Susceptible
5	Berkut×PBW343 (PBW343_15)	P10	0.71	NH_4^+	0.45	Susceptible
6	Berkut×PBW343 (PBW343_40)	P22	1.19	NO_3^-	1.12	Tolerant
7	Berkut×PBW343 (PBW343_52)	P33	0.78	NH_4^+	1.13	Tolerant
8	Berkut×PBW343 (PBW343_58)	P39	1.24	NO_3^-	0.48	Susceptible
9	Berkut×RAC875 (RAC875_45)	R33	0.58	NH_4^+	1.02	Tolerant
10	Berkut×RAC875 (RAC875_57)	R39	0.82	NH_4^+	0.58	Susceptible
11	Berkut×RAC875 (RAC875_70)	R49	1.33	NO_3^-	0.94	Tolerant
12	Berkut×RAC875 (RAC875_99)	R69	0.78	NH_4^+	0.50	Susceptible
13	Reference	Veery 10	1.03	Both	0.75	Susceptible

Growth conditions

For each genotype, we used seeds from the same mother plant grown in a controlled environmental chamber at Davis, California, USA. We disinfected seeds with 10% v/v $NaHClO_3$ for 10 minutes, rinsed them multiple times with deionized water, and placed them on a germination paper soaked with deionized water. We kept the seeds on the germination paper at 4°C for at least 48 hr and later moved them to room temperature for 4 d until transplanting. We transplanted uniform seedlings into 20 dm^3 opaque tubs containing an aerated nutrient solution composed of 1 mM K_2HPO_4, 1 mM KH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 1 ppm Fe-NaDTPA (Sequestrene 330, Becker Underwood), and 50% of other micronutrients (B, Mn, Zn, Cu, Mo) in a Hoagland solution (Epstein & Bloom, 2005) with custom nitrogen concentrations. We adjusted the solution pH in the tubs initially to 6.0 and replenished the tubs with freshly made solution twice in the first week and every 1 to 2 days in the following weeks to maintain pH and sufficient nutrient levels. The tubs were kept in controlled environmental chambers under 16 h of 900 $\mu mol\ m^{-2}\ s^{-1}$ of light at plant height and 22/15°C and 60/83 % relative humidity during the light and dark period respectively (Conviron, Winnipeg, Canada). We passed industrial CO_2 gas from a compressed gas tank through a column filled with $KMnO_4$-covered clay pebbles to remove any C_2H_2 contaminants and injected it into the growth chambers to regulate the CO_2 concentration of two controlled environment chambers at either ~420 ppm ("ambient") or 750 ppm as monitored by non-dispersive infrared analyzers.

We used a completely randomized design (Supplementary Figure S1) to evaluate the influence of CO_2 levels and inorganic nitrogen forms on the growth of the 13 genotypes. We placed a group of 12 tubs into each of the two growth chambers. Six tubs in each chamber received either 0.5 mM NH_4^+ from $(NH_4)_2SO_4$ or 0.5 mM NO_3^- from KNO_3 as a sole nitrogen source. Each tub represented an experimental unit and contained 42 plants: 3 seedlings of each of the 12 genotypes and 6 seedlings of the control genotype (cv Veery 10) placed in the middle of the tub. Each group of seedlings of the same genotype was randomly transplanted into the tub. We rotated the tubs regularly within each chamber to minimize positional effects. The randomization of genotypes within each tub should have alleviated shading among heterogeneous canopy architectures.

Biomass assessment

To determine biomass production, we harvested one plant of each genotype from each tub at 21 d during the tillering stage and at 60 d after transplanting when most genotypes had completed the heading stage. We took 6 biological replicates per genotype per nitrogen treatment, one plant from each tub, in each destructive harvest. We harvested 2 and 4 plants of Veery 10 at each harvest, respectively. As we harvested plants to measure growth, the planting density changed from 217 plants m^{-2} (42 plants per a 20” × 15” tub surface) at the beginning of the experiment to 145 plants m^{-2} (28 plants per tub) after the first harvest. Planting density in this experiment remained within the range of other controlled environmental studies as well as the field settings of 80-400 plant m^{-2} (Fischer et al., 2019). We harvested the roots and shoots separately during the first harvest. Because the roots became entangled after 3 weeks of growth, we assessed only shoot biomass at the second harvest. We rinsed plant samples with deionize water and dried them at 60°C for at least 48 h before weighing them.

To quantify changes in yield components, we determined tiller number, tiller producing spike number, spike number, spike biomass, spikelet number and grain nitrogen content as proxies for potential grain yield. Spike biomass refers to dry mass of all harvested spikes, including both chaffs and grains. We did not measure mass of individual spike components, because the kernels were not fully developed across all genotype and nitrogen treatments. We selected the longest (most developed) and the shortest (least developed) spikes of each plant to determine the number of rachis and spikelets. We define spikelets as rachis whose florets developed into at least one seed. We calculated spike fertility as a ratio of spikelet number to total rachis number. Except for genotype “74”, “166”, “459”, we sampled developing grains randomly from the longest spike of each plant and measured nitrogen concentration with a Leco TrusSpec N (Leco Corporation, St. Joseph, USA) that uses a combustion method (Association of Official Analytical Chemists, 2000) coupled with an infrared detection system. The kernels of genotype “P10”, “P33” and “P39” developed slower under NO_3^- than NH_4^+ nutrition but were large enough for the analysis. We were unable to collect data on the mature grains because our growth chamber failed and subjected the plants to temperatures above 70°C. Nonetheless, the relative differences in nitrogen concentrations of developing grains allowed us to compare nitrogen and carbon translocations to the grains among the treatments.

Statistical modeling

We quantified the influence of nitrogen form, CO_2 concentration, and genotype with a linear model: biomass ~ nitrogen form × CO_2 concentration × genotype. Because of differences in sample sizes, we analyzed biomass of the control genotype, Veery 10, separately. We conducted an analysis of variance

(ANOVA) and calculated type II sums of squares (Langsrud, 2003) using R/car version 3.1 (Fox & Weisberg, 2019). We checked the ANOVA assumptions for homogeneity of variance and normal distribution of model residuals with Levene's test (Levene, 1960) and Shapiro-Wilk Normality test (Royston, 1982), respectively. Based on ANOVA outputs, we determined mean separations with Tukey's Honest Significant Difference (Tukey's HSD) method in R/emmeans version 1.8.6 (Lenth, 2023) and R/multcomp version 1.4-23 (Hothorn et al., 2008).

To determine whether the classification of inorganic nitrogen-form preference and NH_4^+ tolerance of individual genotypes were associated with biomass production, we updated the linear regression model with the categorical variables based on the classification of nitrogen-form adaptation and tolerance, as followed: (*a*) biomass ~ nitrogen form × CO_2 concentration × inorganic nitrogen-form preference or (*b*) biomass ~ nitrogen form × CO_2 concentration × NH_4^+ tolerance.

Validation of the reference genotype, Veery 10

Before we examined genotypic variations of biomass and partitioning, we verified growth and development of the reference genotype, Veery 10, a semi-dwarf cultivar commonly used for experiments (Bishop & Bugbee, 1998; Waines & Ehdaie, 2007). Studies in controlled environments have often used cv Veery 10 due to its compact structure and fast growth rate (Supplementary Table S1). We analyzed growth of Veery 10 separately from the other genotypes because this reference genotype experienced a slightly different growth environment due to the experimental design and had 2 to 4 times as many replicates. Since an early study from our group that used Veery 10 (Smart et al., 1998), atmospheric CO_2 levels have risen from about 366 ppm to over 420 ppm (IPCC, 2021). As such, controlled experiments have also increased the "ambient" concentrations about ~15% to keep up with natural atmospheric changes (Supplementary Table S1). Despite the increased ambient CO_2 levels and slight differences in growth conditions across studies, we still observed consistent trends in responses to CO_2 enrichment (Supplementary Figure S2, Supplementary Figure S3, and Supplementary Table S1). Veery 10 produced similar biomass under 0.5 mM NO_3^- and 0.5 mM NH_4^+ at ambient CO_2 (Supplementary Figure S2 and Supplementary Figure S3). In our previous experiments, we also observed that Veery 10 at ambient CO_2 produced comparable biomass under different nitrogen forms (Table 1).

The influence of nitrogen form and CO_2 enrichment, however, differed as plants transitioned into the reproductive stages. In Veery 10, during the vegetative stage at 21 d, CO_2 enrichment increased shoot biomass 61% and 37% under NH_4^+ and NO_3^-, respectively (Supplementary Figure S2B). Veery 10 partitioned significantly more biomass to shoot growth and had a higher shoot-to-root ratio under NH_4^+ than NO_3^- under CO_2 enrichment, although differences in absolute shoot and whole plant mass were not statistically significant between the two nitrogen forms (Supplementary Figure S2A-E). Our results still supported that elevated CO_2 concentrations enhanced biomass of shoots under NH_4^+ nutrition and roots under NO_3^- nutrition, respectively (Rubio-Asensio & Bloom, 2016). Veery 10 was most responsive to changes in CO_2 level when receiving NH_4^+ as a sole nitrogen source (Carlisle et al., 2012). Yet we observed a slight increase in biomass under NH_4^+ (Bloom et al., 2012; Rubio-Asensio & Bloom, 2016), rather than the decrease previously reported (Carlisle et al., 2012).

At the reproductive stage, we can only compare shoot growth because we could not separate roots from individual plants (Supplementary Figure S3). For Veery 10 under elevated CO_2 exposure, the average shoot growth stimulation by CO_2 across nitrogen forms decreased from +50% at 21 d to +18% at 60 d (Supplementary Figure S3A). Changes in CO_2 and nitrogen form did not affect tiller and spike number but had profound consequences on relative development of Veery 10 (Supplementary Figure S3C-G). On one hand, under CO_2 enrichment ~25% tillers bore no spikes under NH_4^+ (Supplementary Figure S3G), although total spike mass remained comparable to plants under NO_3^- (Supplementary Figure S3C). On the other hand, CO_2 enrichment enhanced shoot biomass partitioning to spikes more under NO_3^- than NH_4^+, primarily because the fraction of spike biomass at ambient CO_2 levels under $NO_3^-$was significantly lower than under NH_4^+ (Supplementary Figure S3D). In the main result, we focus on the phenotypic diversity in the panel of 12 genotypes that had responses distinct from those we observed in the reference genotype Veery 10.

Results

The genetic influence was highly significant across all traits, but its interactions varied (Supplementary Table S1). Responses of the reference genotype Veery 10 were consistent with previous studies (see Methods) but failed to represent the significant, much larger biomass variation exhibited among other genotypes (Figure 1). Both nitrogen forms had a similar influence on most genotypes at ambient CO_2 levels, but biomass under CO_2 enrichment varied among genotypes and between NO_3^- and NH_4^+ nutrition (Figure 1A).

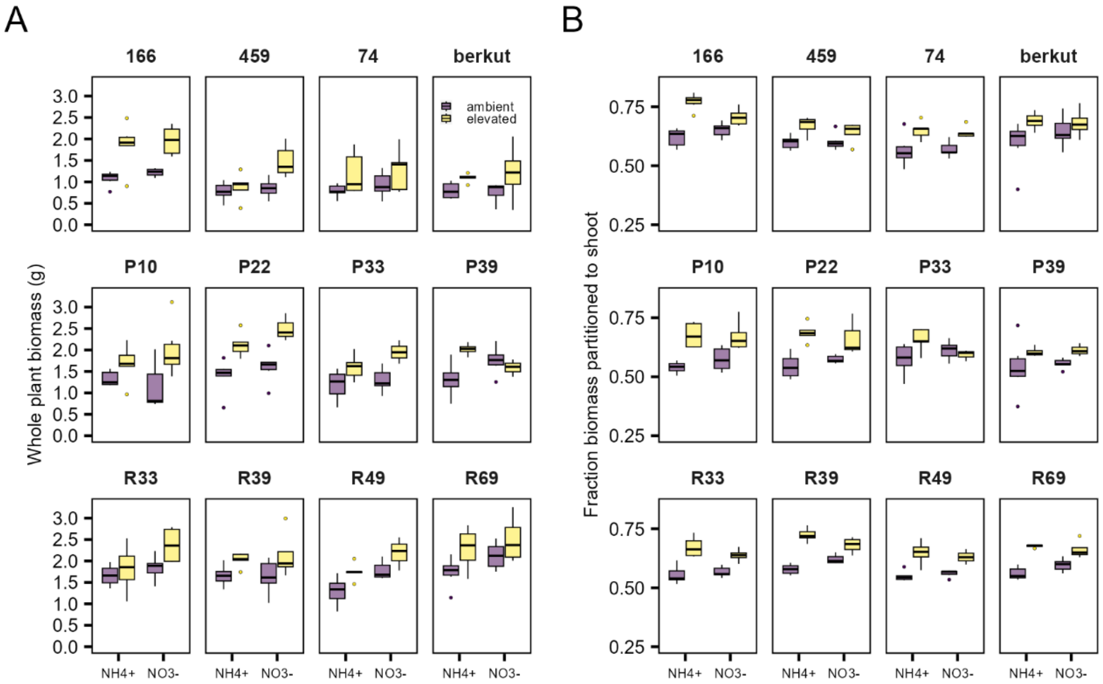


Figure 1 CO_2 enrichment enhanced vegetative biomass production and favored partitioning to shoot under NH_4^+ nutrition.

Distribution of whole plant biomass (A) or fraction biomass partitioned to root (B) of 12 wheat genotypes under NH_4^+ or NO_3^- as a sole nitrogen source at 21 d after transplanting in ambient (purple box) or elevated (yellow box) CO_2 concentrations. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima (n=6). Each dot represents outliers.

Biomass partitioning under CO_2 enrichment depends on nitrogen source

CO_2 enrichment favored biomass partitioning to shoots under NH_4^+ nutrition for 4 out of 12 genotypes, whereas the remaining genotypes partitioned similar proportions to roots and shoots under both forms (Figure 1B and Supplementary Figure S4). Genetics interacted with either nitrogen and CO_2 in determining growth of the organ involved in their assimilation: for root biomass in response to nitrogen forms (Figure 2A) and for shoot biomass in response to CO_2 levels (Figure 3A & B). Linear relationships between shoot and root biomass across all genotypes demonstrated that plants under CO_2 enrichment grew more root with NO_3^- nutrition and more shoot with NH_4^+ nutrition, and the differences increased as the plant size increased (Figure 2B).

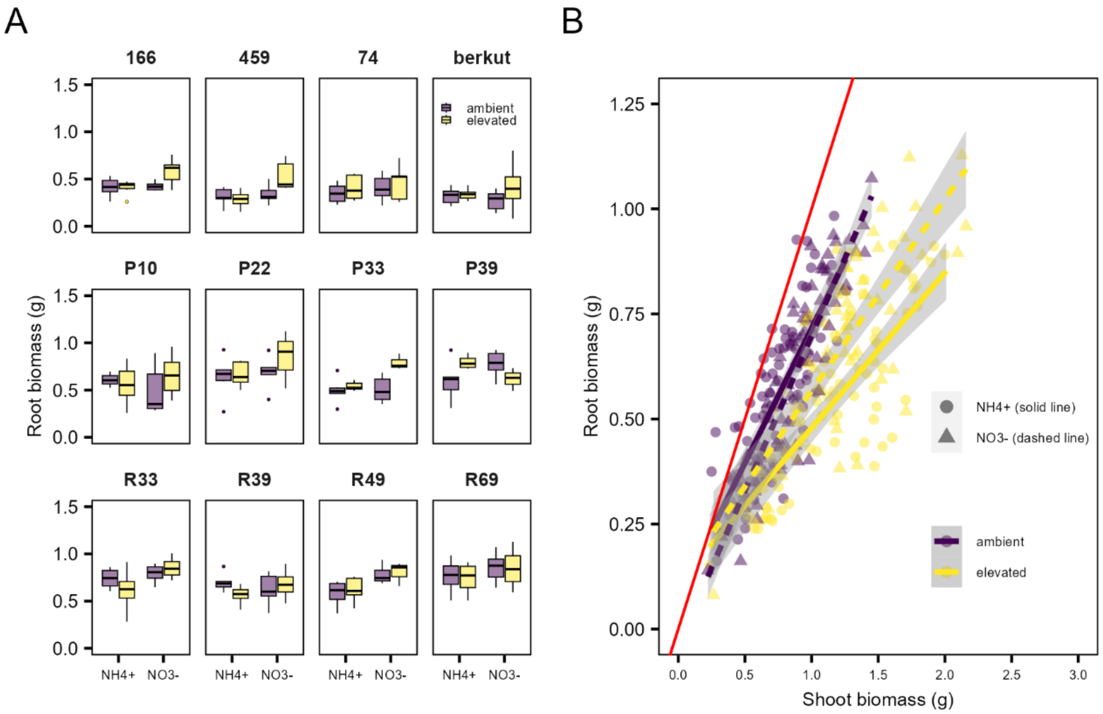
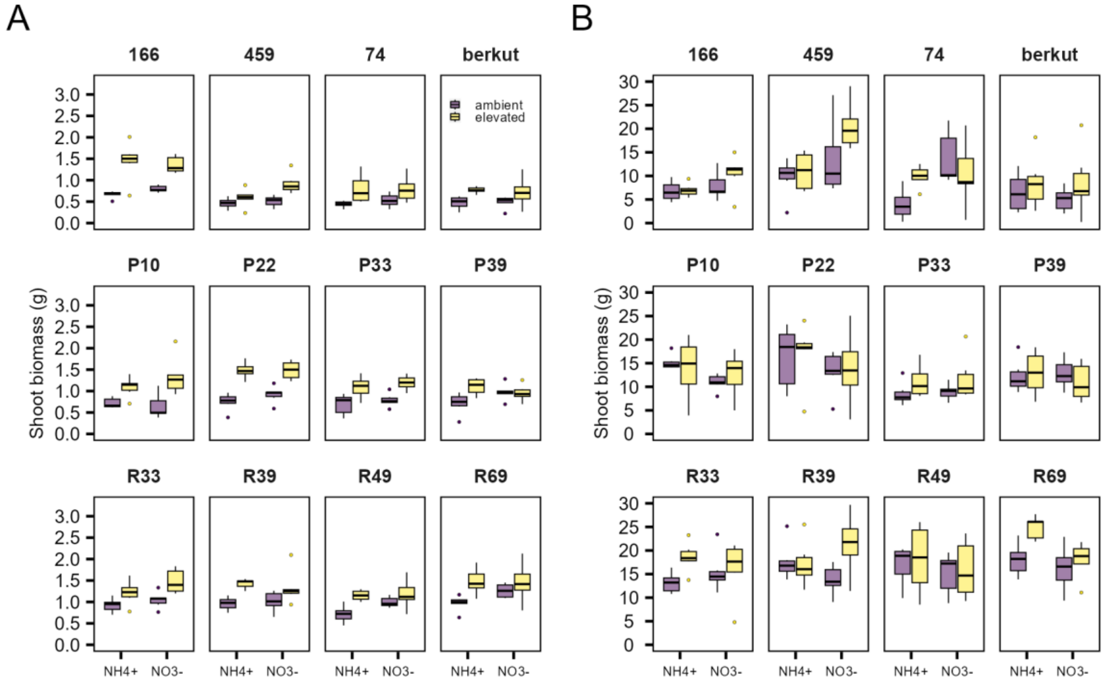


Figure 2 Elevated CO_2 enhancement of root growth under NO_3^- and shoot growth under NH_4^+ nutrition amplified with plant size.

Distribution of root biomass (A) under NH_4^+ or NO_3^- as a sole nitrogen source at 21 d after transplanting in ambient (purple box) or elevated (yellow box) CO_2 concentrations. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima (n=6). Each dot represents outliers. Scatter plots and linear relationships between shoot and root biomass across 12 wheat genotypes (B) under NH_4^+ (purple line) or NO_3^- (yellow line) at ambient (circle, solid line) or 750 ppm CO_2 (triangle, dashed line). The red line represents a 1:1 ratio. Gray areas represent 95% confidence intervals.



C
3 d after transplanting

D
18 d after transplanting

E
37 d after transplanting

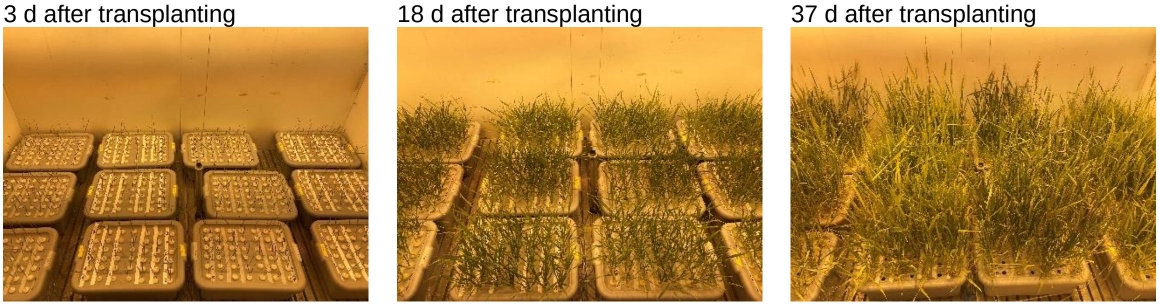


Figure 3 As plants transitioned into the reproductive stage, effects of CO_2 interactions with nitrogen forms varied among genotypes.

Distribution of shoot biomass at 21 d (A) and 60 d (B) after transplanting of 12 wheat genotypes under NH_4^+ or NO_3^- as a sole nitrogen source at 21 d after transplanting in ambient (purple box) or elevated (yellow box) CO_2 concentrations. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima (n=6). Each dot represents outliers. Time course canopy change in the ambient CO_2 chamber at 3, 18 and 37 d after transplanting respectively (C-E). Leaves covered most of the tub surface by 3 weeks (D), and the canopy fully closed by 5 weeks after transplanting (E).

Genotypic variations determined CO_2 acclimation

We focused on the influence of CO_2 enrichment on shoot growth as the plants transitioned into reproduction (Figure 3). Genotypic variations in acclimation to CO_2 was apparent after exposure to elevated CO_2 for 60 d (Figure 3A-B and Supplementary Table S1). The biomass stimulation of CO_2 diminished or even became negative (Figure 3B). For example, genotype “166”, the biomass increase under CO_2 enrichment with NH_4^+ nutrition went from 117% at 21 d to negligible at 60 d, whereas with

NO_3^- nutrition the effects halved (71%→33%) (Figure 3A-B). On average, NH_4^+-grown plants were more responsive to changes in CO_2 concentrations (Figure 3A-B): the biomass stimulation from 21 d to 60 d changed from 61% to 19% under NH_4^+ and 42% to 18% under NO_3^-. Notably, not all genotypes suffered from CO_2 acclimation (Figure 3B); a few genotypes maintained biomass stimulation. For example, biomass stimulation increased from 31% to 41% with NH_4^+ nutrition for "R33" and from 32% to 55% with NO_3^- nutrition for "R39" (Figure 3B).

NH_4^+ accelerated reproductive development

The reference genotype, Veery 10 reached anthesis about 7 d earlier under NH_4^+ than under NO_3^-. Similarly, NH_4^+ nutrition accelerated the flowering of most other genotypes (Figure 4). We cannot distinguish whether final grain yield differed because we did not have complete data on the precise differences in flowering time or biomass at physiological maturity, but the growth data at 60 d provided a "snapshot" into crop development that can serve as a proxy for potential yield.

We examined how resource allocation shifted between the vegetative and the reproductive stages. Vegetative tiller development was controlled by nitrogen form and the interactions between CO_2 level and genotype (Supplementary Table S1). Tiller numbers were slightly higher under NO_3^- than under NH_4^+ (Supplementary Figure S5A). Because tiller buds almost fully developed by the first harvest, we observed just a few more tillers at 60 d (Supplementary Figure S5B). Similar to biomass, tiller growth enhancement by CO_2 was slower at 60 d than at 21 d (Supplementary Figure S5A-B). Interactions of the nitrogen and genotype became more important than CO_2 in controlling the reproductive tiller number (Supplementary Table S1). With NO_3^- nutrition, 4 out of 13 genotypes had slightly lower tiller number under CO_2 enrichment than under the ambient treatment (Supplementary Figure S5B). The number of spikes did not differ between nitrogen forms nor CO_2 levels (Supplementary Figure S5D), although the proportion of tillers that produced spikes varied across treatments (Supplementary Figure S5C).

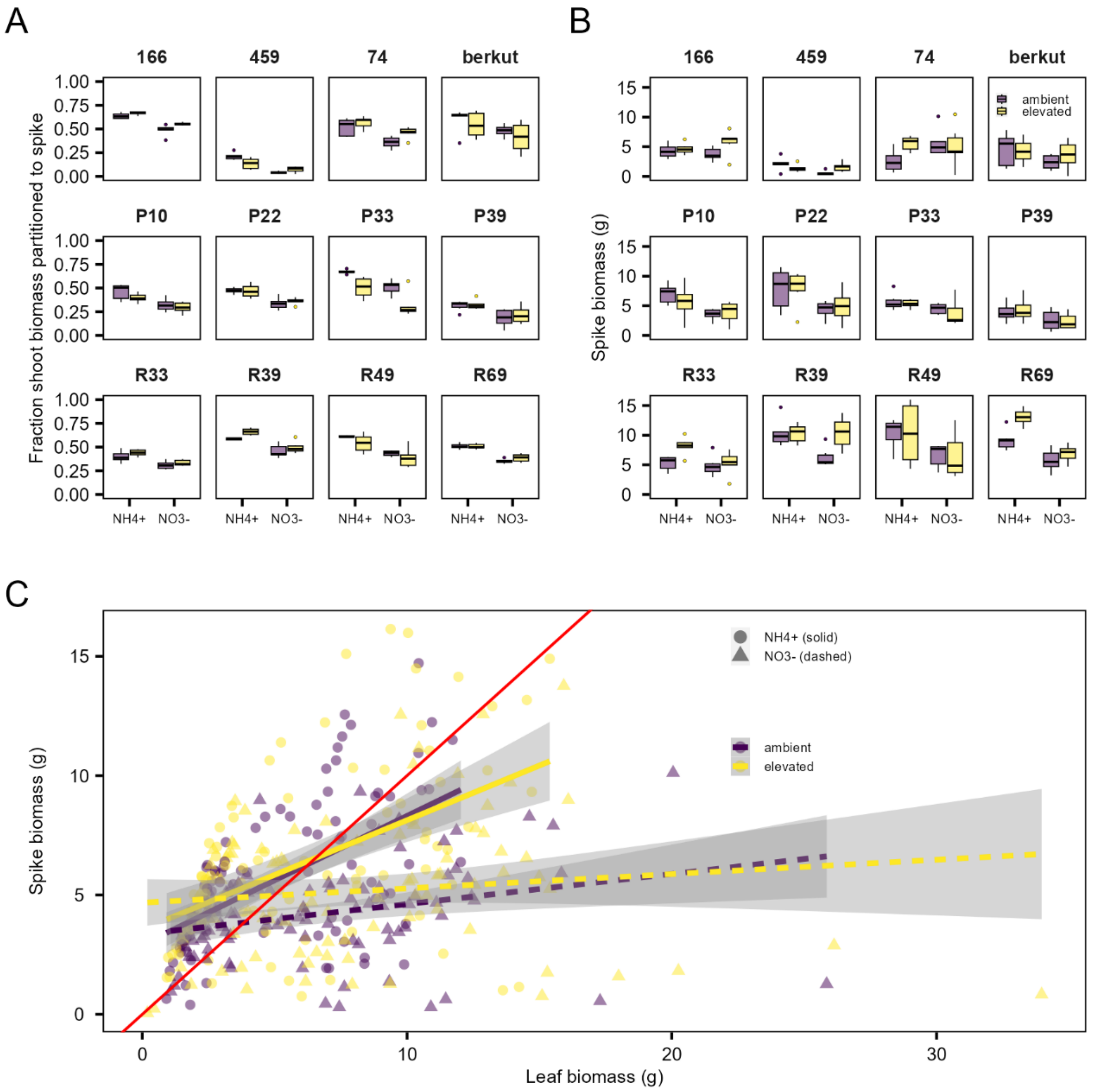


Figure 4 NH_4^+ nutrition influenced flowering and accelerated spike development.

Distribution of fraction shoot biomass partitioned to spike (A), spike biomass at 60 d (B) after transplanting of 12 wheat genotypes under NH_4^+ or NO_3^- as a sole nitrogen source at 21 d after transplanting in ambient (purple box) or elevated (yellow box) CO_2 concentrations. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima (n=6). Each dot represents outliers. Scatter plots and linear relationships between leaf and spike biomass at 60 d across 12 wheat genotypes (C) under NH_4^+ (purple line) or NO_3^- (yellow line) at ambient (circle, solid line) versus 750 ppm CO_2 (triangle, dashed line). The red line represents a 1:1 ratio. Gray areas represent 95% confidence intervals.

The influence of nitrogen was most pronounced in the allocation of shoot biomass to spikes (Figure 4A). At 60 d, plants allocated more biomass to spikes with NH_4^+ than with NO_3^- nutrition, suggesting faster development under NH_4^+ nutrition (Figure 4B). CO_2 enrichment increased absolute spike mass ~23% across both nitrogen forms. (Figure 4B), but the influence on spike mass allocation varied with genotype. Most genotypes increased or showed no change in spike partitioning, except for “Berkut”, "P33" and "R49" (Figure 4A). The influence of nitrogen form on spike mass also varied with genotype. Most genotypes produced higher spike mass with NH_4^+ nutrition, with a few exceptions (Figure 4B); for example, "74" had 53% more spike mass with NO_3^- than with NH_4^+ across the CO_2 levels, or more than double the size if we consider only the ambient CO_2 treatment.

We determined rachis and spikelet number in the most and least developed spike of each plant (Supplementary Figure S6). Nitrogen form influenced the rachis number and the proportion of rachis that developed into spikelets, an indicator of spike fertility (Supplementary Figure S6). Although the rachis numbers were generally higher in NO_3^--grown plants (Supplementary Figure S6A-B), spikelet numbers were relatively similar under both nitrogen forms when spikes reached maturity (Supplementary Figure S6C). We observed that basal spikelets were more likely to abort. As a result, spike fertility of the most developed spike was relatively higher under NH_4^+ nutrition for most genotypes (Supplementary Figure S7).

Spike biomass was positively correlated with leaf biomass (Figure 4C) although there was no clear pattern on how nitrogen form and CO_2 level influenced leaf biomass (Supplementary Figure S8). CO_2 enrichment generally increased leaf biomass, but the extent of increase depended on nitrogen form and genotype (Supplementary Table S1). Interestingly, as leaf biomass increased, plants receiving NH_4^+ produced significantly higher spike mass than plants receiving NO_3^- (Figure 4C). Such relationships affirm the positive influence of NH_4^+ on spike growth and development.

We measured nitrogen concentration of kernels in the most developed spikes (Figure 5). To limit developmental differences, we focused on the genotypes from the two bi-parental populations and their common parent Berkut because their developmental rates were somewhat similar, although spikes of genotype "P10", "P33", and "P39" in the NO_3^- treatment still developed much slower than those in the NH_4^+ treatment (Figure 4B). Grain nitrogen concentration was lower in NO_3^- (2.32%) than NH_4^+ (3.07%) and their responses to CO_2 enrichment depended on genotype (Figure 5). Under CO_2 enrichment, nitrogen concentrations either decreased or did not change (Figure 5). Grains of genotype "P10" and "P33" had higher nitrogen concentration under NO_3^- at the elevated CO_2 level, but such an anomalous trend likely derived from nitrogen form-driven differences in developmental stages because these kernels were much smaller than other genotypes and the same genotype under NH_4^+ (Figure 5).

When we exclude extremely small kernels with higher concentrations from these genotypes, the data pattern of the subset genotypes remain consistent with the whole dataset (Supplementary Figure S9).

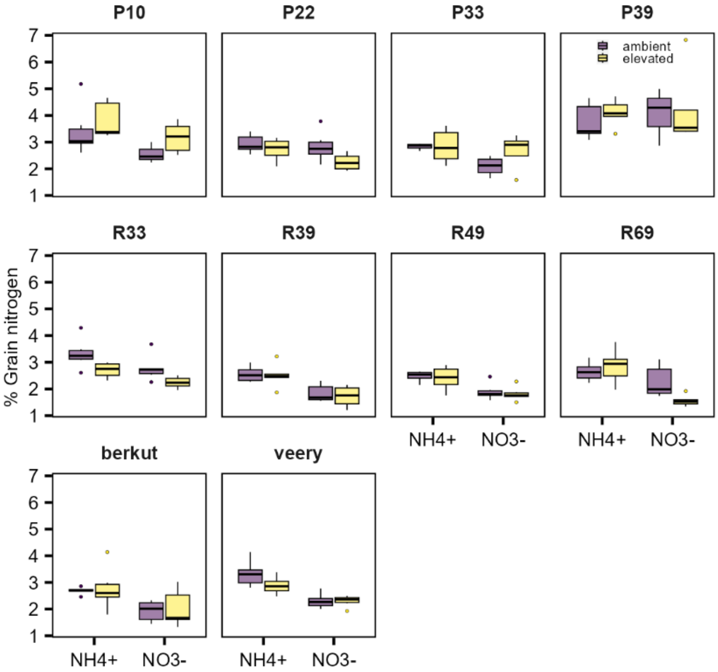


Figure 5 Nitrogen concentrations in developed grains were lower under NO_3^- than NH_4^+ and dependent upon genetics and CO_2 concentrations interactions.

Distribution of nitrogen concentrations in developing grains in the most developed spike of 10 wheat genotypes under NH_4^+ or NO_3^- as a sole nitrogen source at 60 d after transplanting in ambient (purple box) or elevated (yellow box) CO_2 concentrations. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima (n=6). Each dot represents outliers. Genotype “74”, “166” and “459” were excluded from nitrogen analysis due to significant differences in developmental stages.

Nitrogen adaptation explained responses to CO_2 enrichment

We examined whether nitrogen-form preference or NH_4^+ tolerance can explain biomass change under CO_2 enrichment (Supplementary Table S2). Because the three genotypes from the NSGCC panel responded similarly to both nitrogen forms (Table 1), we retained only genotypes from the two bi-parental populations and their common parent that had a preference for either nitrogen form in the analysis. Nitrogen preference accounted for ~4% of the variation in biomass (Supplementary Table S2). Genotypes that preferred NH_4^+ produced more whole plant biomass at 21 d (Figure 6A), shoot biomass at 60 d (Figure 6B), and spike biomass (Figure 6C), independent of nitrogen form and CO_2 level. But, nitrogen-form preference did not alter grain nitrogen (Figure 6D). Moreover, tolerance to high NH_4^+ levels was not associated with biomass under the non-toxic concentrations in the current experiment except for grain nitrogen that differed slightly between tolerant and susceptible genotypes (P=0.046) (Supplementary Figure S10).

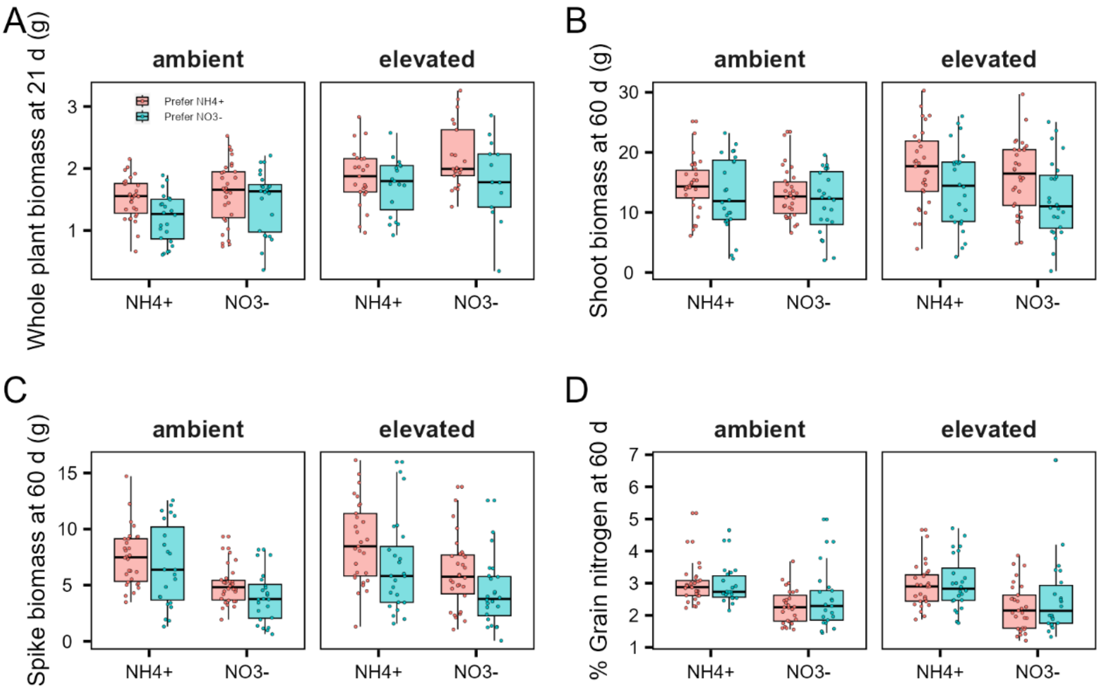


Figure 6 Adaptation to nitrogen forms explained biomass production under both nitrogen forms and CO_2 levels.

The box colors denote classification of nitrogen-form preference at ambient CO_2 with NH_4^+ (pink box) or NO_3^- (blue box)-preferred. Distribution of whole plant biomass at 21 d (a), shoot biomass at 60 d (b), spike biomass at 60 d (c) and developing grain nitrogen concentration at 60 d (d) after transplanting of 9 wheat genotypes under NH_4^+ or NO_3^- as a sole nitrogen source in ambient or elevated CO_2 concentrations. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima. Each dot represents each plant replicate across genotypes with the same nitrogen preference classifications.

Relationships among growth traits indicated that several changes across yield components were unique to an individual nitrogen form (Supplementary Figure S11-15). For example, the relationship between tiller fertility and shoot biomass was positive under NH_4^+ nutrition, but negative under NO_3^- nutrition (Supplementary Figure S12-13). Furthermore, changes in tiller and rachis number correlated with spike biomass only under NH_4^+ nutrition (Supplementary Figure S12). Combined changes in yield components, particularly spike number, proportion of fertile tiller, spikelet number, and spike fertility contributed to the observed variations in spike biomass. Lastly, although biomass traits of genotypes with different NH_4^+ tolerance levels did not differ (Supplementary Figure S10), we observed weak but significant correlations, between tolerance levels and some yield components (Supplementary Figure S11).

Discussion

Shoot carbon assimilation converges with root nitrogen assimilation during biomass production. Changes in atmospheric CO_2 concentrations likely favor C_3 species in NH_4^+-dominated environments but C_4 species in NO_3^--dominated environments (Bloom et al., 2012). CO_2 enrichment lowers plant capacity to use NO_3^- by 16.2% but has a negligible influence on NH_4^+ usage (Cheng et al., 2012). Adaptations to such changes includes shifts in species distribution (Bloom et al., 2012) and trade-offs between carbohydrates and proteins to maintain grain quality (Bloom & Plant, 2021). This study highlights the importance of incorporating diverse genetic materials and provides further evidence that increased crop reliance on NH_4^+ may mitigate the detrimental effects of rising CO_2 levels.

Here, genotype accounted for most of the biomass variation (Supplementary Table S1). Because most studies are limited to a few species or genotypes within one species, adaptations to nitrogen forms or tolerance to toxic concentrations have rarely been considered. Genotype-specific responses are likely responsible for contradictory results among studies that used a single genotype to represent a species. For example, the wheat variety Yercora Rojo slowed nitrate assimilation under a free-air CO_2 enrichment in a potentially high NO_3^- soil (Bloom et al., 2014). In contrast, the variety Batis showed higher nitrogen acquisition in NO_3^--fertilized soil than NH_4^+-fertilized soil under elevated daytime CO_2 levels (Dier et al., 2018). Furthermore, researchers often assume that wheat prefers NO_3^- over NH_4^+ or may supply the plants with potentially toxic concentrations of NH_4^+. For example, the variety Chuanmai 58 showed significantly slower growth with NH_4^+ than NO_3^- at 5 mM (Wang et al., 2020), a concentration 10 times higher than the present study. Our experiment on different genotypes of the same species and near-isogenic lines from the same background allowed us to compare and contrast the genotypic effects. By accounting for potential genetic adaptation to nitrogen forms, we showed that the nitrogen-form preference and NH_4^+ tolerance level can explain responses to CO_2 enrichment (Figure 6).

Preference for a nitrogen form varies with genetic backgrounds and environmental conditions (Britto & Kronzucker, 2013; Miller & Cramer, 2005). Species develop at different rates under NH_4^+ or NO_3^- (Bloom et al., 2012). Plants should be adapted to the prevalent nitrogen form in their natural habitats (Gigon & Rorison, 1972; Maathuis, 2009). Soil inorganic nitrogen distribution and relative availability may vary 2 to 3 orders of magnitude (R. B. Jackson & Caldwell, 1993). Microbial activities are responsible for the transient nature of soil nitrogen (L. E. Jackson et al., 1989) that interferes with determining crop nitrogen-form preference based solely on relative availability in the soil. Given the high uptake rates by both plants and microbes, the relative availability may not be a major selective pressure (L. E. Jackson et al., 1989). Perhaps, plants benefit most from being generalists that can employ both forms (Houlton et al., 2007). Despite being statistically significant, nitrogen-form preference may only account for a small proportion of biomass variation (Supplementary Table S2). Plant responses to different nitrogen forms indicate the desirability of including genetically and phenotypically diverse materials when testing for physiological responses that may underlie crop local adaptations to soil nutrients.

Nutrient deficiencies or exposures to high nutrient concentrations usually constrain species distribution range and drive local adaptation (Baxter et al., 2010; Terés et al., 2019). Such adaptations to extreme conditions may diminish overall fitness when these stressors are absent (Mauro & Ghalambor, 2020). Here, after plants were exposed to moderate NH_4^+ levels, we observed weak correlations between NH_4^+ tolerance and several yield components that suggest potential fitness trade-offs (Supplementary Figure S11). The high light intensity we employed may have mitigated both NH_4^+ toxicity (Esteban et al., 2016) and CO_2 inhibition of NO_3^- photoassimilation (Rubio-Asensio & Bloom, 2016). To evaluate the influence of NH_4^+ tolerance on fitness, subsequent investigations should carefully select genetic materials exposed to both high and optimal nitrogen concentrations.

CO_2 acclimation lowered the biomass stimulation effects of CO_2 enrichment over time (Bloom et al., 2012). Interestingly, genotypes classified as NH_4^+-preferring maintained the biomass stimulation effects after prolonged CO_2 exposure (Figure 3). Moreover, NH_4^+-adapted genotypes also showed higher biomass than NO_3^--adapted genotypes when receiving either form as a sole nitrogen source and at both ambient and elevated CO_2 levels (Figure 6). These results suggest that plants adapted to NH_4^+

may be more resilient to changes in CO_2 levels (Bloom et al., 2010; Fernando et al., 2017; Subbarao et al., 2021; Subbarao & Searchinger, 2021). Experiments on additional materials should verify whether this finding is universal.

The influence of nitrogen forms on crop development, particularly flowering time, remains unresolved (Kasemsap & Bloom, 2022). Typically, CO_2 enrichment accelerates development and senescence through rubisco and photosynthesis acclimation (Sicher & Bunce, 1997). Here, nitrogen form had a stronger influence than CO_2 enrichment as plants transitioned into reproduction, such that biomass responses were similar under both CO_2 conditions but diverged under different nitrogen forms (Supplementary Table S1 and Figure 4). Regardless of CO_2 levels, NH_4^+ nutrition stimulated flower development and spike biomass accumulation (Figure 4). Several yield components contributed to the increased spike biomass under NH_4^+ nutrition (Supplementary Figures 11 & 12). The positive correlations of grain number and spikelet number (Rawson, 1970), and spike biomass (Rivera-Amado et al., 2019; Sierra-Gonzalez et al., 2021; Slafer et al., 2015), allowed us to estimate grain number and potential yield , even though we did not directly measure the grain number and grain yield. Moreover, enhanced flower development under NH_4^+ nutrition may have reduced the number of floret abortion in basal spikelets, resulting in relatively higher spike fertility (Supplementary Figure S7). Such action may counteract delayed development, an underlying cause of spikelet abortion (Backhaus et al., 2023). Different nitrogen forms likely triggered a distinct suite of developmental changes (Supplementary Figure S12-13), as previously reported in the model species (Katz et al., 2022).

Improving nitrogen assimilation in elevated CO_2 atmospheres is critical to human nutrition (Myers et al., 2014) and climate change mitigation (Gao & Cabrera Serrenho, 2023). Wheat may have already sacrificed carbon gain to maintain stable nitrogen concentration as atmospheric CO_2 levels increased (Bloom & Plant, 2021) . Here, adaptations to NH_4^+ increased spike mass but did not change grain nitrogen concentrations (Figure 6). Grain nitrogen concentrations in this study are within a range observed in other field settings (Gaju et al., 2014). As such, the ability to maintain protein with no penalty on carbon yield may be advantageous under CO_2 enrichment. Still, we were unable to assess the nutritional quality of mature grains because of a malfunctioning growth chamber. During advanced grain-filling stages, nitrogen concentrations may continue to change as plants approach senescence

(Salgó & Gergely, 2012). Early flowering would most effectively improve yield when coupled with delayed but short senescence to allow longer grain filling time (Xie et al., 2016). Given urea and NH_4^+-based fertilizers are already dominant forms in agricultural production (Nishina et al., 2017), information on translocation of assimilated resources to sink organs at maturity would have major implications for food production under climate conditions anticipated in the near future. Breeding for cultivars with improved NH_4^+ adaptation and assimilation capacity may not only increase crop climate resilience, but also potentially accelerate development and improve yield without sacrificing nutritional values.

Acknowledgments

We thanked Joshua Hegarty for discussion on grain measurements. We thanked Teng Vang and the California Wheat Commission for their help on grain protein measurements. We appreciate the assistance of Joshua J. Claxton, Francesco G. Riga, Abenezer D. Shankute, Zennin G. Casl, Steven A. Kunze, Dana R. Lawrence, and James K. Schmidt during data collection.

Author contributions

Pornpipat Kasemsap and Arnold J. Bloom planned the research and edited the manuscript. Pornpipat Kasemsap collected, analyzed data and wrote the first draft of the manuscript.

Conflict of Interest

Not applicable.

Funding

This work was supported in part by USDA-IWYP-16-06702, NSF IOS 13-58675, NSF IOS 16-55810, and the John B. Orr Endowment to Arnold J. Bloom. Pornpipat Kasemsap was supported by Thailand – United States Educational Foundation (TUSEF/Fulbright Thailand), UC Davis Department of Plant Sciences Graduate Student Researcher fellowship, and Henry A. Jastro research award, and Grad Innovator Fellowship from UC Davis Innovation Institute for Food and Health.

Data availability statement

The data that support the findings of this study are openly available on Dryad at DOI: 10.5061/dryad.00000008p and in supplementary materials. Analysis R codes are available at https://www.github.com/paulkasemsap/Wheat_NxCO2_13gen.

Supplementary Data

Supplementary Table S1 P-values of ANOVA for biomass traits

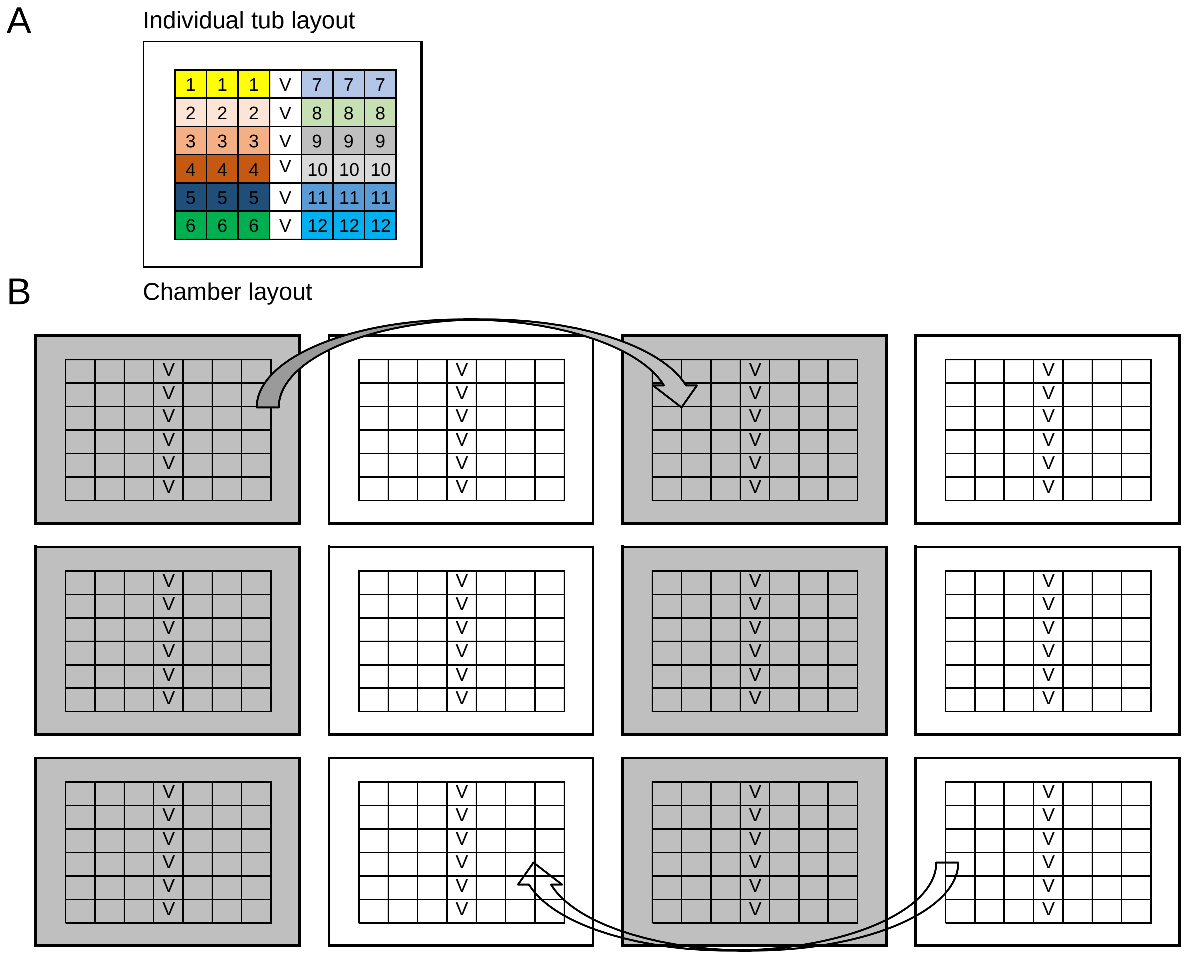
	Phenotype											
Source of Variation	d21 TotalDW	d21 ShootDW	d21 RootDW	d21 f_root	d21 tiller	d21 Shoot:Root	d60 ShootDW	d60 LeafDW	d60 SeedDW	d60 f_seed	d60 tiller	d60 spike
Veery 10												
Nitrogen form	0.4337	0.0986	0.4305	0.0004	0.2412	0.0001	0.1978	0.0011	0.6803	0.0000	0.2329	0.2702
CO_2	0.0003	0.0000	0.0220	0.0001	0.3203	0.0001	0.0159	0.6765	0.0016	0.0000	0.2967	0.4323
Nitrogen form × CO_2	0.6442	0.2202	0.3765	0.0023	0.7032	0.0009	0.2299	0.6176	0.1439	0.0013	0.5506	0.2382
Shapiro-Wilk test	0.0345	0.0194	0.0716	0.0309	0.3440	0.0013	0.3517	0.2638	0.2807	0.4657	0.0004	0.0018
Levene’s test	0.1193	0.1069	0.4276	0.6436	0.5283	0.9496	0.5003	0.8005	0.2866	0.2559	0.0144	0.3882
12 Genotypes												
Nitrogen form	0.000	0.002	0.000	0.461	0.000	0.514	0.625	0.000	0.000	0.000	0.014	0.417
CO_2	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.001	0.003	0.286	0.019	0.318
Genotype	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Nitrogen form × CO_2	0.309	0.175	0.010	0.000	0.060	0.000	0.839	0.805	0.924	0.211	0.031	0.266
Nitrogen form × Genotype	0.646	0.844	0.575	0.968	0.464	0.711	0.000	0.000	0.000	0.799	0.002	0.221
CO_2 × Genotype	0.400	0.004	0.729	0.092	0.000	0.000	0.449	0.034	0.133	0.000	0.309	0.275
Nitrogen form × CO_2 × Genotype	0.227	0.155	0.157	0.867	0.105	0.231	0.016	0.018	0.166	0.874	0.579	0.074
Shapiro-Wilk test	0.175	0.000	0.639	0.000	0.000	0.000	0.084	0.000	0.027	0.000	0.000	0.057
Levene’s test	0.693	0.319	0.546	0.227	0.298	0.185	0.136	0.005	0.000	0.000	0.117	0.650

Supplementary Table S1 P-values of ANOVA for biomass traits (cont.)

	Phenotype							
Source of Variation	d60 Tiller Fertility	d60 Rachis Old	d60 Rachis Young	d60 Spikelet Old	d60 Spikelet Young	d60 Spike Fertility	d60 Spike Fertility Young	d60 % Grain nitrogen
Veery 10								
Nitrogen form	0.0002	0.0000	0.0000	0.0013	0.9623	0.0000	0.0030	0.0000
CO_2	0.0025	0.0000	0.0191	0.0004	0.0951	0.3474	0.2844	0.0012
Nitrogen form × CO_2	0.0058	0.0001	0.8044	0.0000	0.4238	0.0046	0.3695	0.0006
Shapiro-Wilk test	0.0000	0.0000	0.0019	0.0000	0.0000	0.0000	0.0000	0.0008
Levene’s test	0.0000	0.1521	0.4834	0.0704	0.0001	0.0000	0.4906	0.0091
12 Genotypes								
Nitrogen form	0.0005	0.0000	0.0000	0.0005	0.0002	0.0000	0.0411	0.0000
CO_2	0.0000	0.2971	0.4689	0.3923	0.0404	0.7981	0.0003	0.4974
Genotype	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Nitrogen form × CO_2	0.0178	0.1006	0.2059	0.9283	0.1289	0.0931	0.5442	0.5875
Nitrogen form × Genotype	0.0000	0.0034	0.0155	0.4715	0.8477	0.1676	0.7906	0.0659
CO_2 × Genotype	0.1574	0.0050	0.2777	0.0119	0.0268	0.0281	0.1280	0.0250
Nitrogen form × CO_2 × Genotype	0.3491	0.7517	0.2577	0.1432	0.0052	0.1086	0.0443	0.5801
Shapiro-Wilk test	0.0000	0.0018	0.0105	0.0026	0.0000	0.0003	0.0000	0.0000
Levene’s test	0.0775	0.3695	0.9283	0.0903	0.6022	0.0279	0.0000	0.5881

Supplementary Table S2 P-values of ANOVA for nitrogen preference and NH_4^+ tolerance

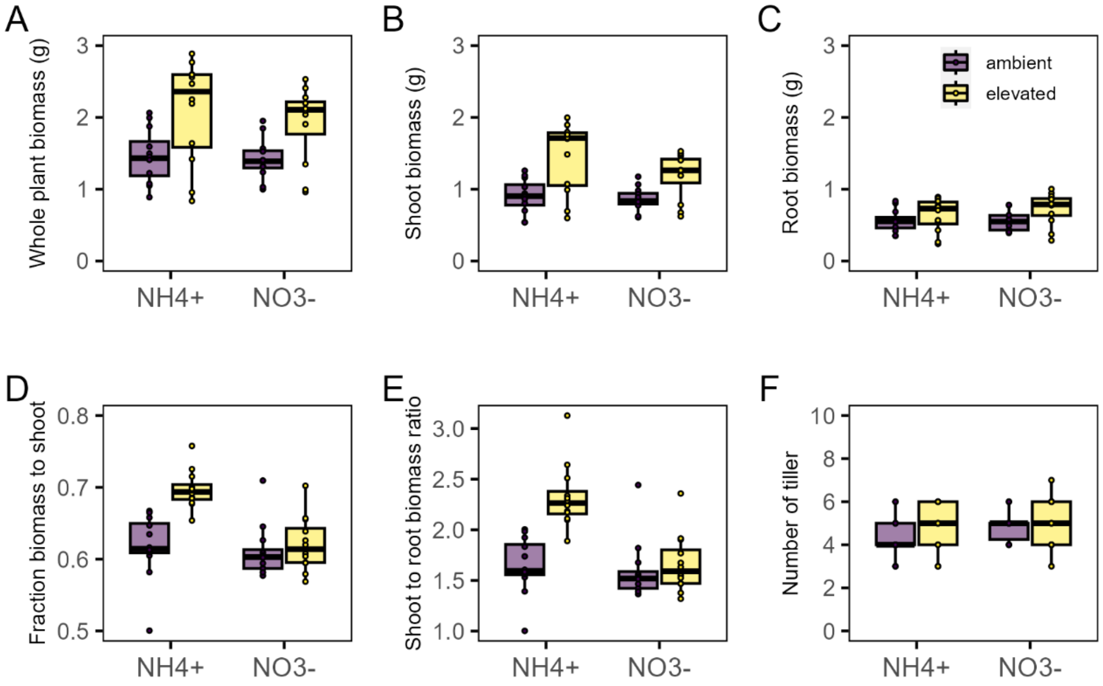
	Phenotype							
Source of Variation	d21 TotalDW Preference	d60 ShootDW Preference	d60 SeedDW Preference	d60 % Grain N Preference	d21 TotalDW Tolerance	d60 ShootDW Tolerance	d60 SeedDW Tolerance	d60 % Grain N Preference
Nitrogen form	0.0064	0.0513	0.0000	0.0000	0.0064	0.0513	0.0000	0.0000
CO2	0.0000	0.0093	0.0836	0.9415	0.0000	0.0093	0.0836	0.9727
Preference or tolerance	0.0015	0.0021	0.0012	0.2105	0.0015	0.0021	0.0012	0.0461
Nitrogen form x CO2	0.7217	0.9027	0.9365	0.9720	0.7217	0.9027	0.9365	0.9663
Nitrogen form x Preference or tolerance	0.8958	0.8819	0.9370	0.3437	0.8958	0.8819	0.9370	0.3581
CO2 x Preference	0.9344	0.2260	0.2167	0.6772	0.9344	0.2260	0.2167	0.0749
Nitrogen form x CO2 x Preference or tolerance	0.1518	0.8798	0.6952	0.9723	0.1518	0.8798	0.6952	0.8827



1	1	1	V	7	7	7
2	2	2	V	8	8	8
3	3	3	V	9	9	9
4	4	4	V	10	10	10
5	5	5	V	11	11	11
6	6	6	V	12	12	12

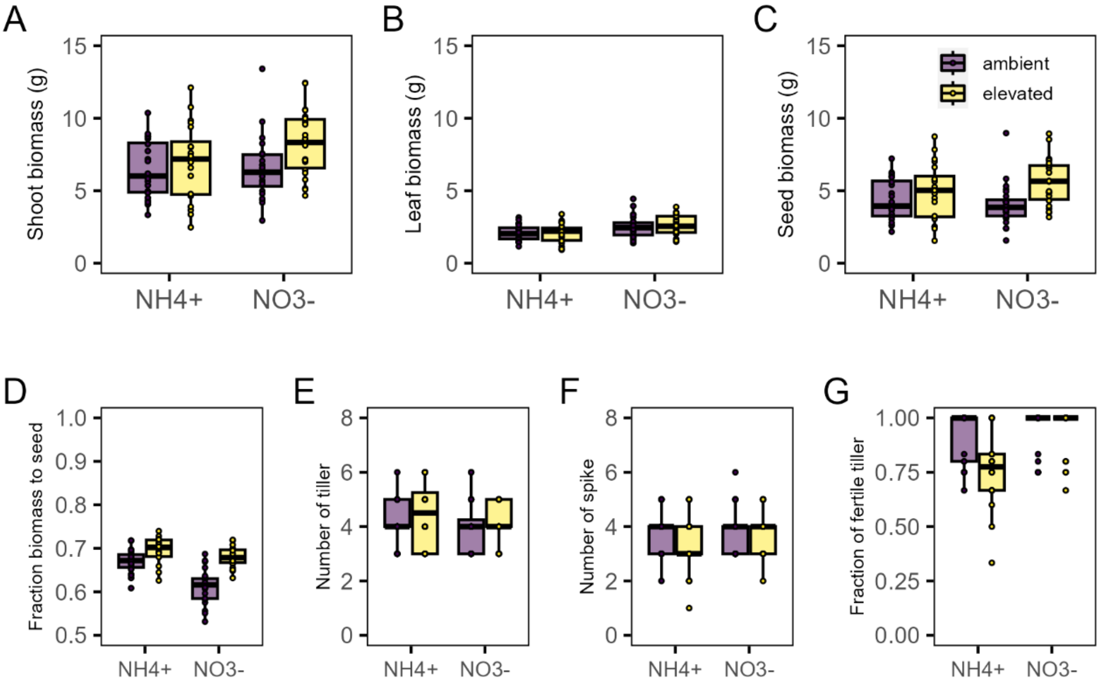
Supplementary Figure S1 Experimental design.

Completely randomized design with 3 factors: 2 nitrogen forms, 2 CO$_2$ concentrations and 12 genotypes (+ 1 control genotype; “V” for Veery 10). We transplanted randomly a group of 3 uniform seedlings per genotype into each of twelve tubs (A) placed in a controlled environmental chamber (B). Six tubs in each group received either 0.5 mM NH$_4^+$ from (NH$_4$)$_2$SO$_4$ or 0.5 mM NO$_3^-$ from KNO$_3$ as a sole nitrogen source. We rotated tubs of the same nitrogen source regularly within each chamber to ensure comparable light interception and avoid positional effects.



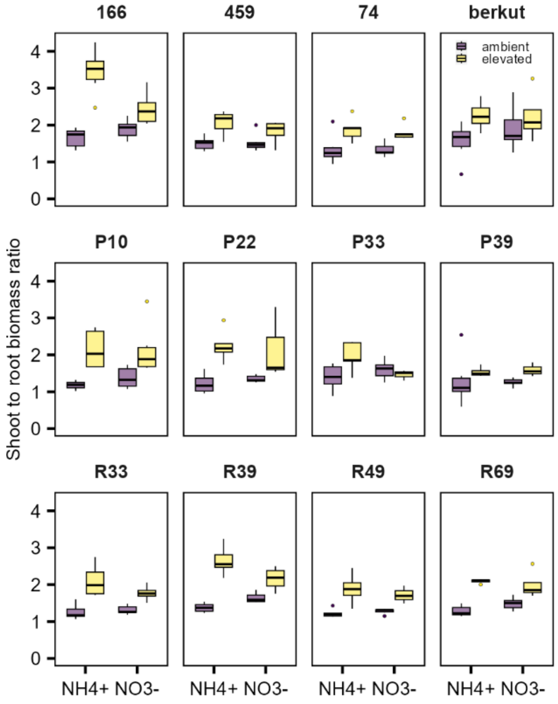
Supplementary Figure S2 As CO_2 level increases during the vegetative stage, Veery 10 under NH_4^+ nutrition partitioned more biomass to shoot growth.

Distribution of biomass (A, C, D), fraction shoot biomass (B), shoot to root biomass ratio (E), and number of tiller (F)-at 21 d after transplanting of cv Veery 10 under NH_4^+ or NO_3^- as a sole nitrogen source in ambient (purple box) or elevated CO_2 level (yellow box). The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima. Each dot represents each of the12 biological plant replicates.



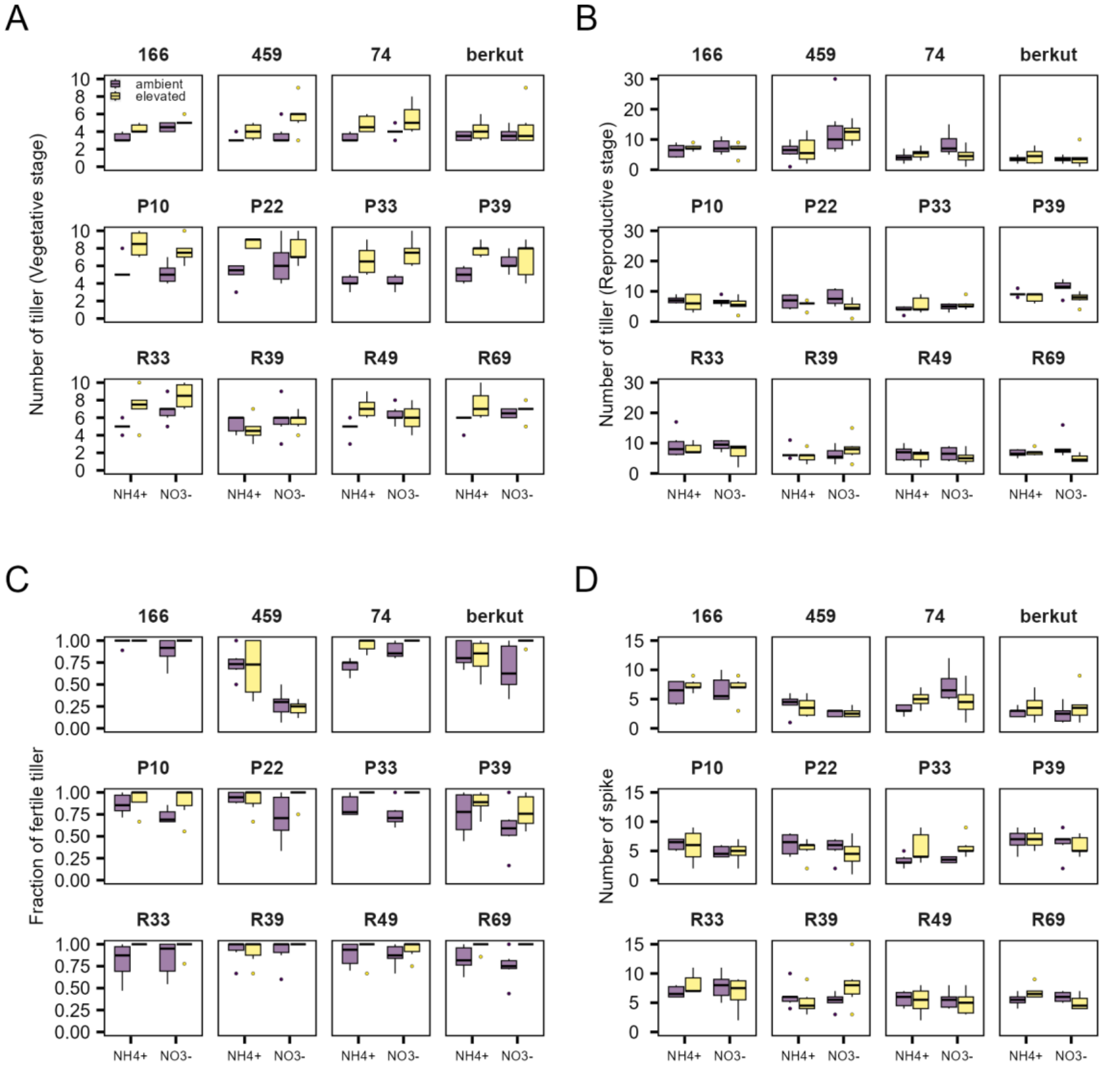
Supplementary Figure S3 At 60 d of elevated CO_2 exposure, biomass stimulation slowed down, but CO_2 still influenced tiller development and seed biomass partitioning under different nitrogen forms.

Distribution of biomass (A, C, D), fraction seed biomass (B), number of tiller (E), number of spike (F), and proportion of fertile tiller (G) at 60 d after transplanting of cv Veery 10 under NH_4^+ or NO_3^- as a sole nitrogen source in ambient (purple box) or elevated CO_2 level (yellow box). The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima. Each dot represents each of the 24 biological plant replicates.



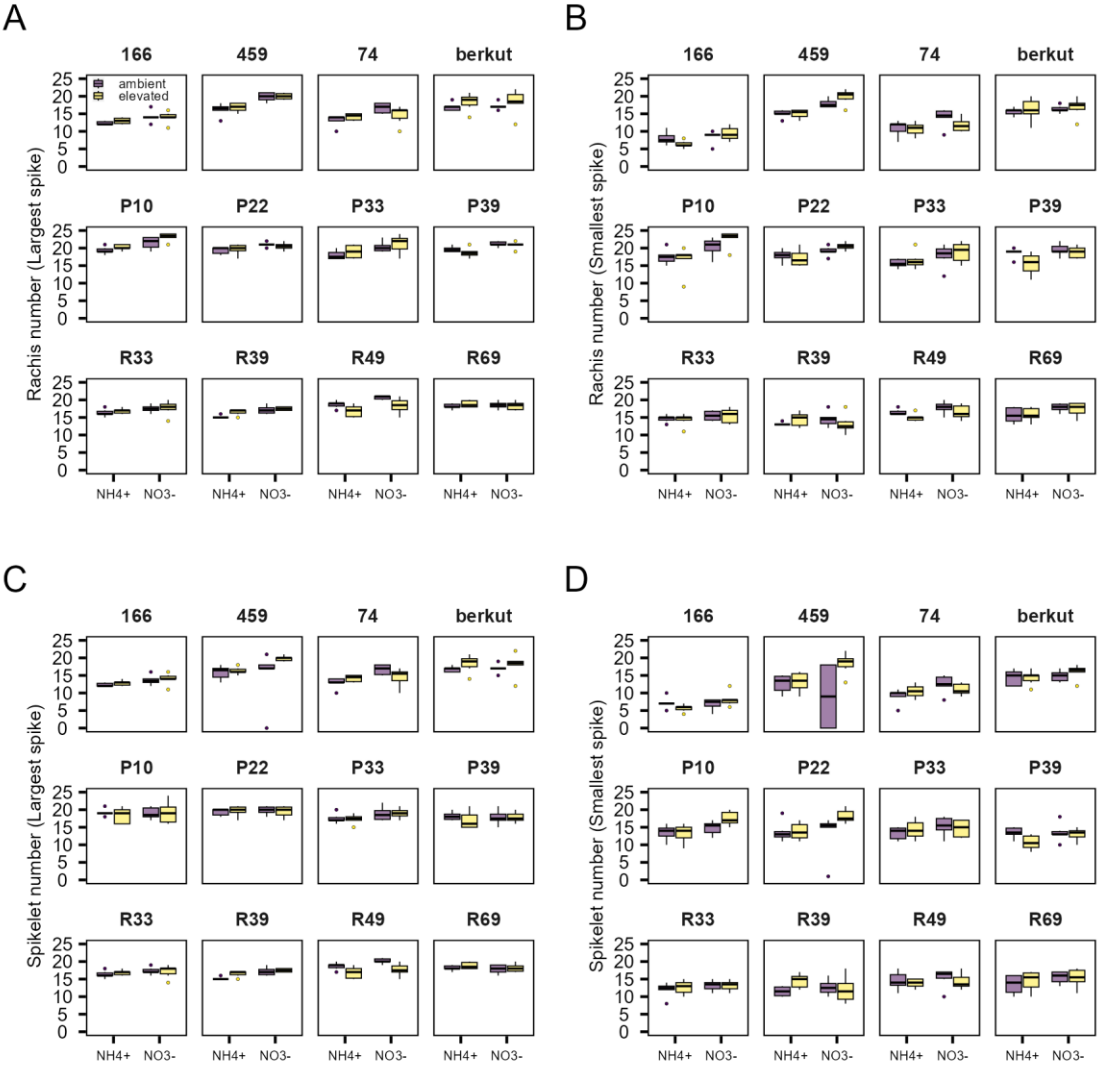
Supplementary Figure S4 Shoot to root ratio consistent with biomass partitioned to shoot

Distribution of shoot to root biomass ratio of 12 wheat genotypes under NH_4^+ or NO_3^- as a sole nitrogen source at 21 d after transplanting in ambient (purple box) or elevated (yellow box) CO_2 concentrations. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima (n=6). Each dot represents outliers.



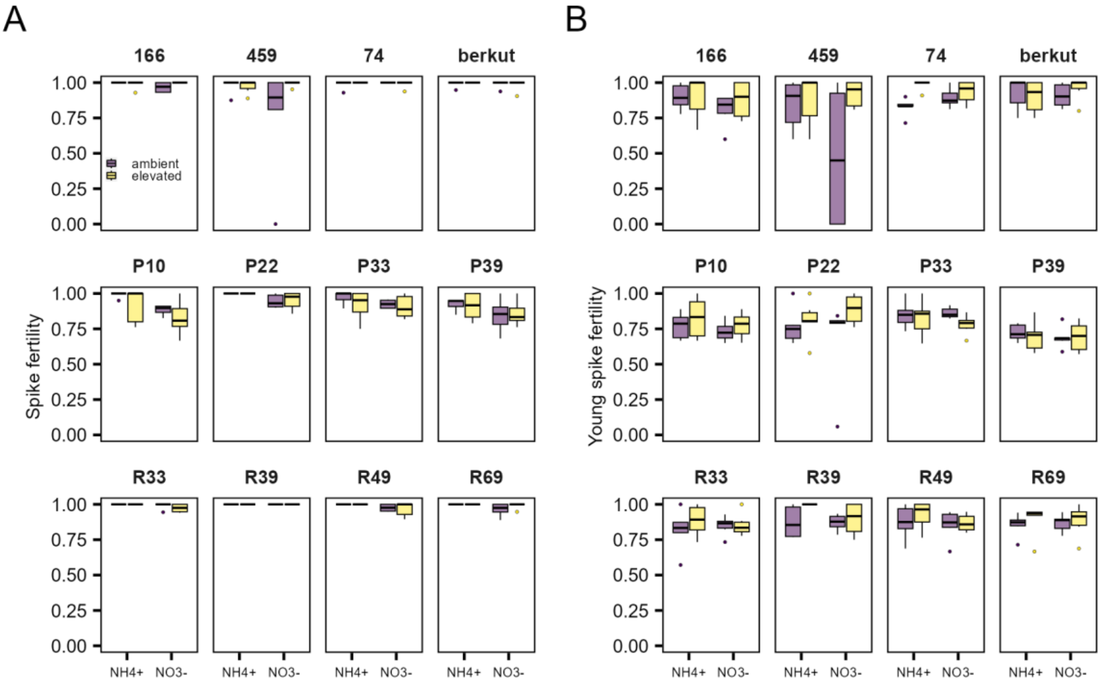
Supplementary Figure S5 Tiller and spike development

Distribution of tiller number at 21 d (A) and 60 d (B) after transplanting, proportion of seed producing tiller (C), and spike number (D) in ambient (purple box) or elevated (yellow box) CO_2 concentrations. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima (n=6). Each dot represents outliers.



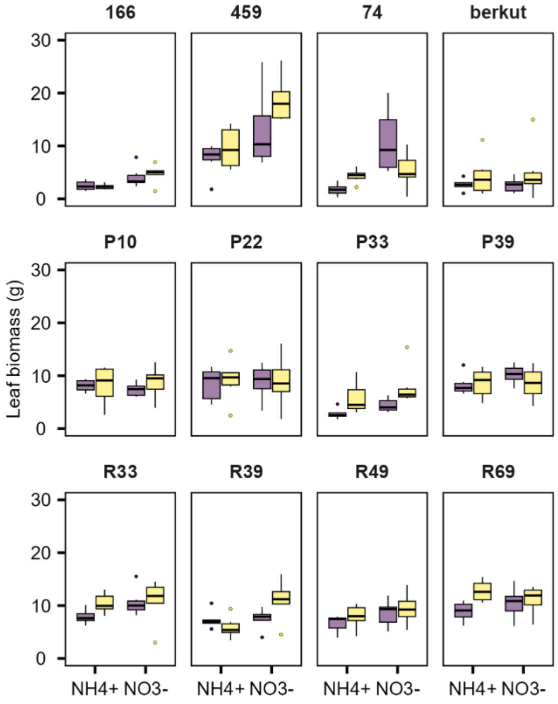
Supplementary Figure S6 Rachis and spikelet development

Distribution of rachis number (A,B) and spikelet number (C,D) of the most (A,C) and least (B,D) developed spike at 60 d after transplanting in ambient (purple box) or elevated (yellow box) CO_2 concentrations. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima (n=6). Each dot represents outliers.



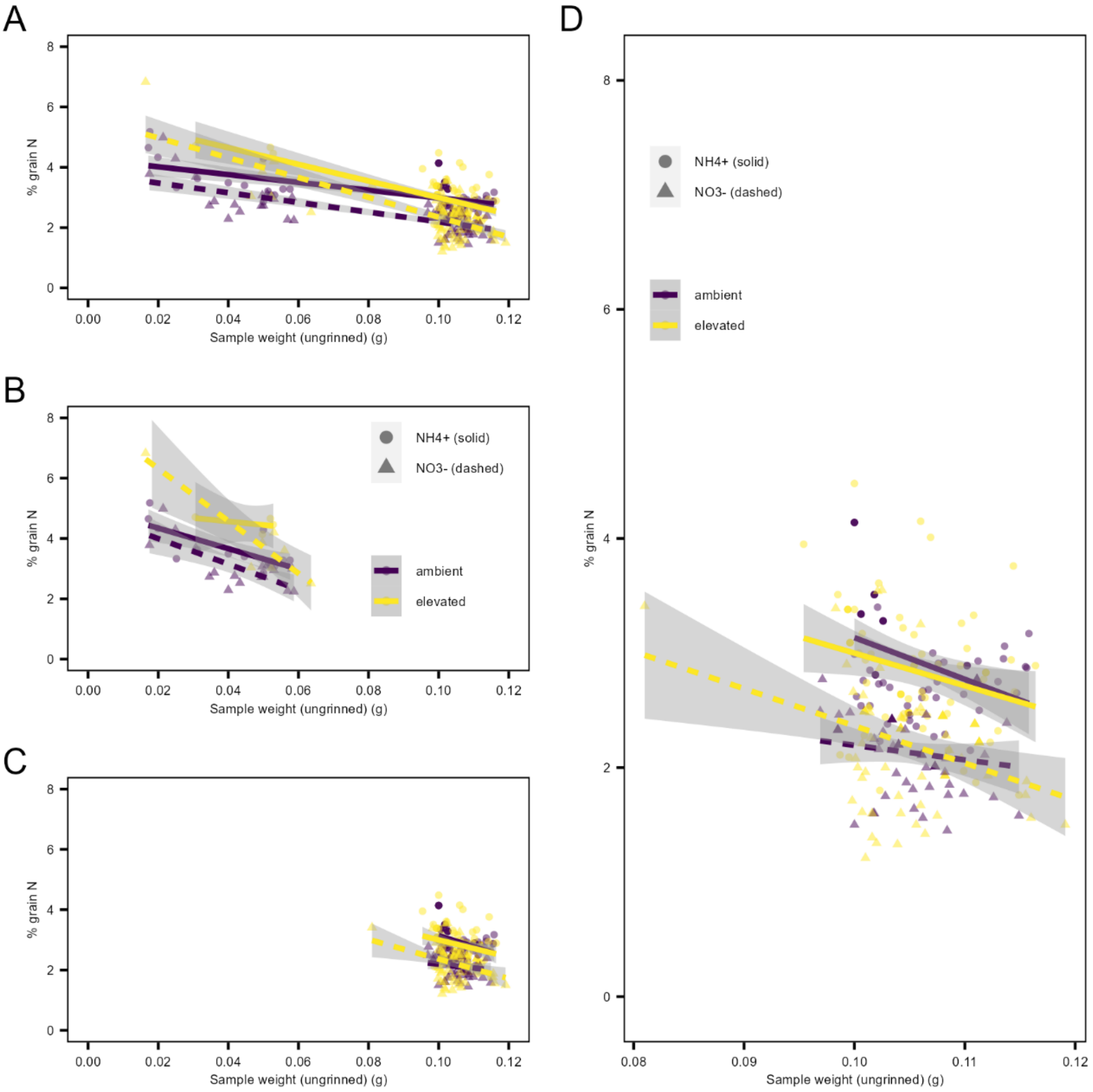
Supplementary Figure S7 Spike fertility

Distribution of spike fertility of the most (A) and least (B) developed spike at 60 d after transplanting in ambient (purple box) or elevated (yellow box) CO_2 concentrations. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima (n=6). Each dot represents outliers.



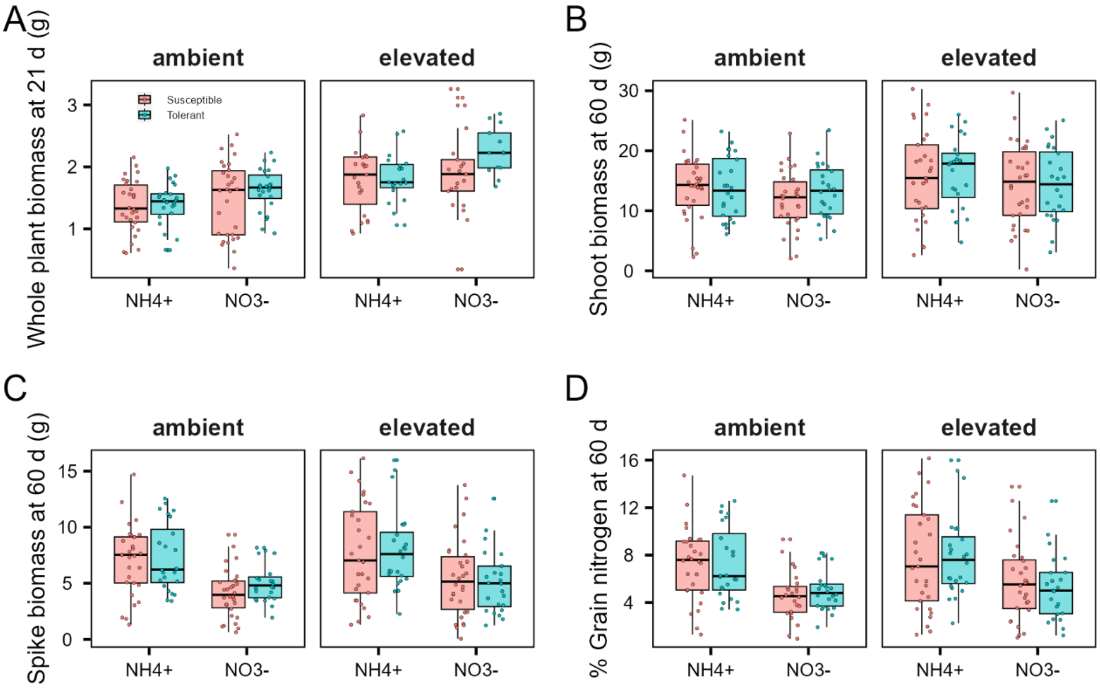
Supplementary Figure S8 Leaf biomass at 60 d varied with nitrogen forms, CO_2 levels and genotypes.

Distribution of leaf biomass of 12 wheat genotypes under NH_4^+ or NO_3^- as a sole nitrogen source at 21 d after transplanting in ambient (purple box) or elevated (yellow box) CO_2 concentrations. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima (n=6). Each dot represents outliers.



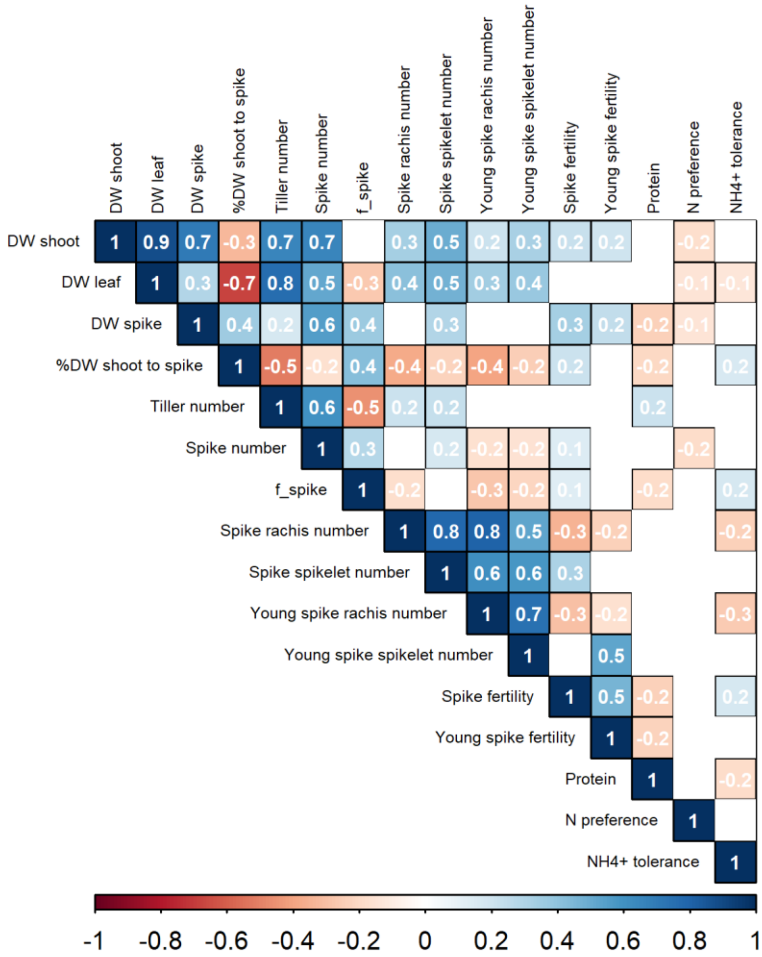
Supplementary Figure S9 Nitrogen concentrations of developing grains were generally higher under NH_4^+ nutrition, except for smaller grains of P10, P33 and P39.

Scatter plots and linear relationships between sample weight of all weights (A), only smaller than 0.075 g (B), and only larger than 0.075 g (C and D) across 10 wheat genotypes under NH_4^+ (purple line) or NO_3^- (yellow line) at ambient (circle, solid line) versus 750 ppm CO_2 (triangle, dashed line). Figure D is a close-up of figure C. We used sample weights as an indirect proxy for weight of developing grains because we directly use grain samples without grinding for the measurements. Gray areas represent 95% confidence intervals.



Supplementary Figure S10 The degree of tolerance to high NH_4^+ concentration was not associated with biomass production under the environments tested in this study.

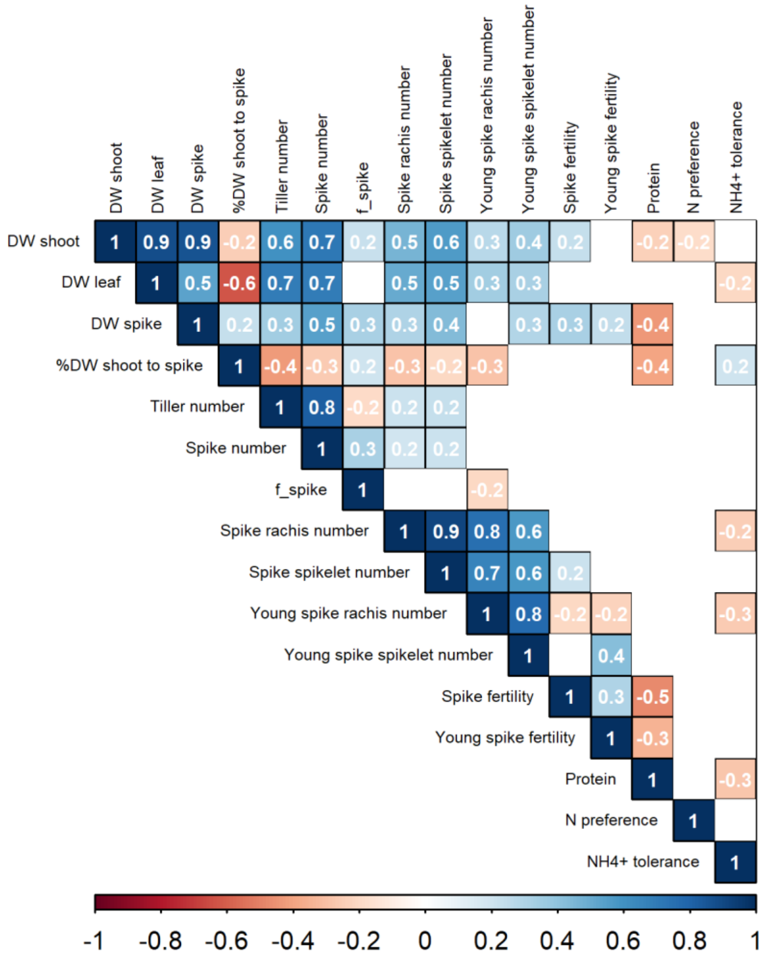
Distribution of whole plant biomass at 21 d (A), shoot biomass at 60 d (B), spike biomass at 60 d (C) and developing grain nitrogen concentration at 60 d (D) after transplanting of 9 wheat genotypes under NH_4^+ or NO_3^- as a sole nitrogen source in ambient or elevated CO_2 concentrations. The box colors denote classification of NH_4^+ tolerance as susceptible (pink box) or tolerant (blue box). The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima. Each dot represents each plant replicate across genotypes with the same nitrogen preference classifications.



	DW shoot	DW leaf	DW spike	%DW shoot to spike	Tiller number	Spike number	f_spike	Spike rachis number	Spike spikelet number	Young spike rachis number	Young spike spikelet number	Spike fertility	Young spike fertility	Protein	N preference	NH4+ tolerance
DW shoot	1	0.9	0.7	-0.3	0.7	0.7		0.3	0.5	0.2	0.3	0.2	0.2		-0.2	
DW leaf		1	0.3	-0.7	0.8	0.5	-0.3	0.4	0.5	0.3	0.4				-0.1	-0.1
DW spike			1	0.4	0.2	0.6	0.4		0.3			0.3	0.2	-0.2	-0.1	
%DW shoot to spike				1	-0.5	-0.2	0.4	-0.4	-0.2	-0.4	-0.2	0.2		-0.2		0.2
Tiller number					1	0.6	-0.5	0.2	0.2					0.2		
Spike number						1	0.3		0.2	-0.2	-0.2	0.1			-0.2	
f_spike							1	-0.2		-0.3	-0.2	0.1		-0.2		0.2
Spike rachis number								1	0.8	0.8	0.5	-0.3	-0.2			-0.2
Spike spikelet number									1	0.6	0.6	0.3				
Young spike rachis number										1	0.7	-0.3	-0.2			-0.3
Young spike spikelet number											1		0.5			
Spike fertility												1	0.5	-0.2		0.2
Young spike fertility													1	-0.2		
Protein														1		-0.2
N preference															1	
NH4+ tolerance																1

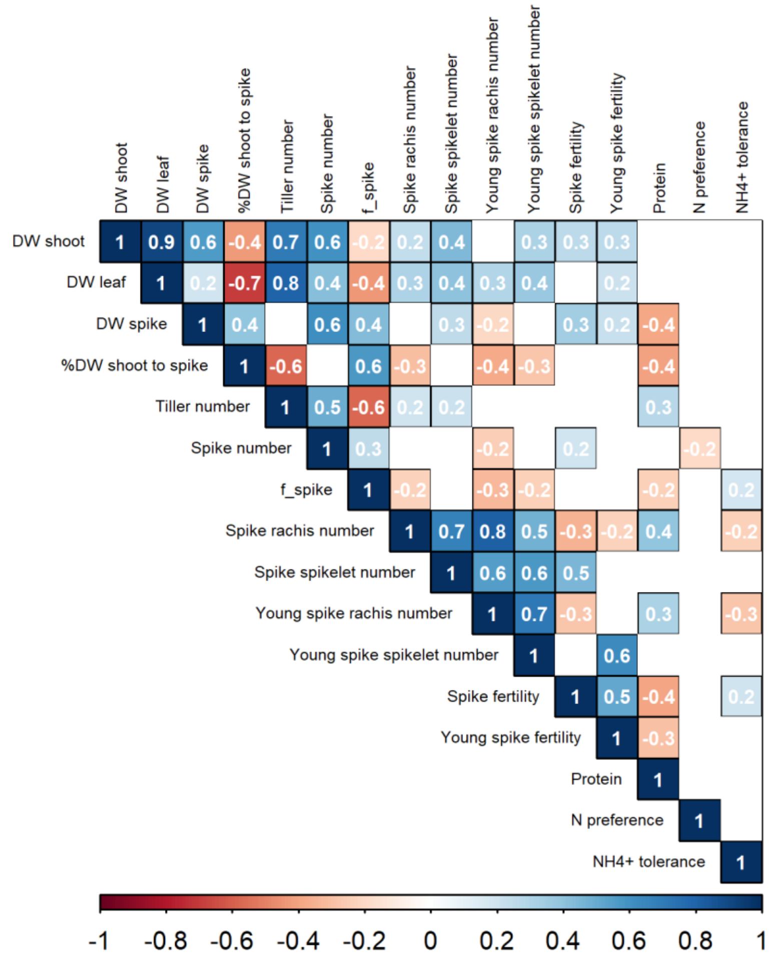
Supplementary Figure S11 Correlation of biomass traits

Pearson's correlation coefficients at p-value < 0.01 between biomass traits across the whole experiment. Insignificant correlations are not displayed. The horizontal bar displays reference color for negative correlations (red) to positive correlations (blue).



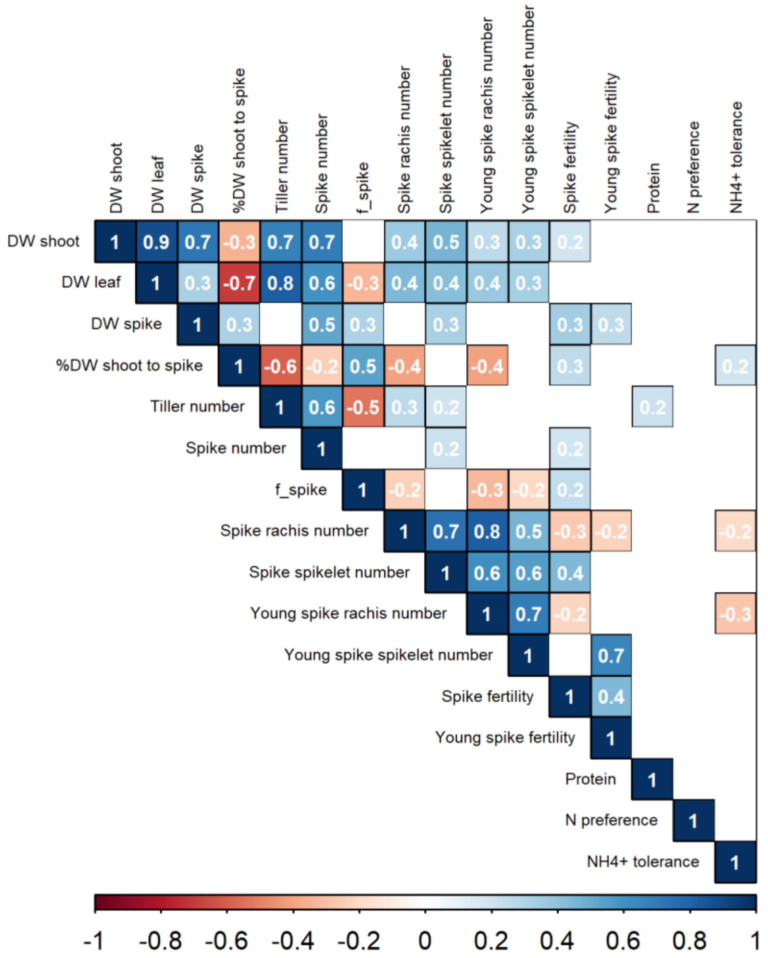
Supplementary Figure S12 Correlation of biomass traits of plants receiving NH_4^+

Pearson's correlation coefficients at p-value < 0.01 between biomass traits of only plants receiving NH_4^+. Insignificant correlations are not displayed. The horizontal bar displays reference color for negative correlations (red) to positive correlations (blue).



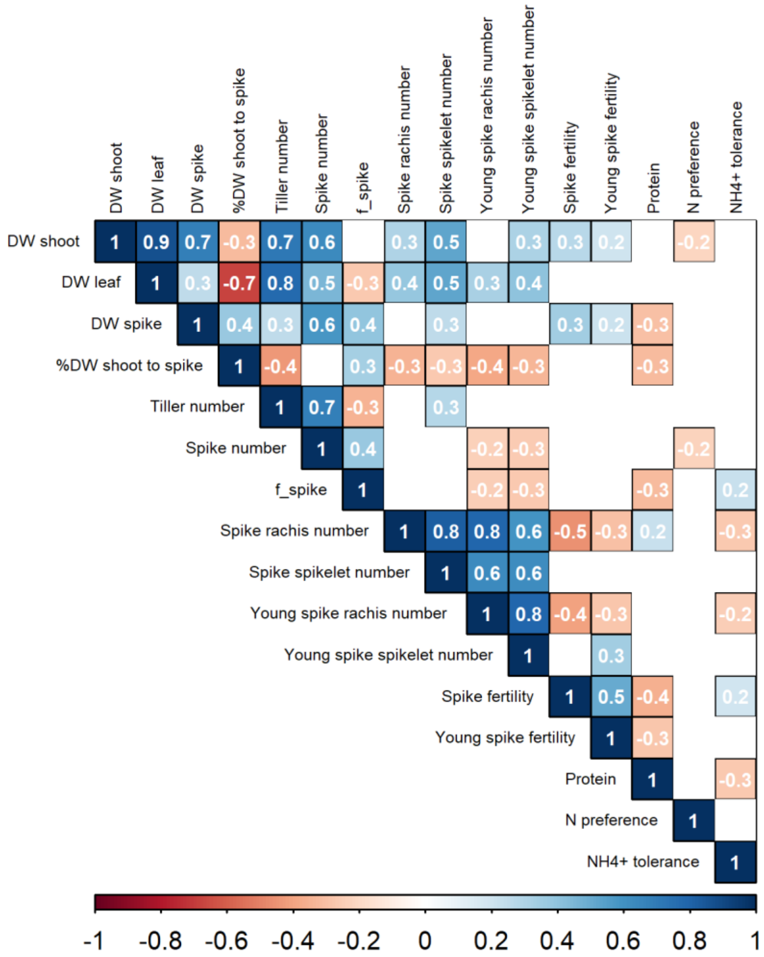
Supplementary Figure S13 Correlation of biomass traits of plants receiving NO_3^-

Pearson's correlation coefficients at p-value < 0.01 between biomass traits of only plants receiving NO_3^-. Insignificant correlations are not displayed. The horizontal bar displays reference color for negative correlations (red) to positive correlations (blue).



Supplementary Figure S14 Correlation of biomass traits of plants under the ambient CO_2 concentration

Pearson’s correlation coefficients at p-value < 0.01 between biomass traits of only plants under the ambient CO_2 concentration. Insignificant correlations are not displayed. The horizontal bar displays reference color for negative correlations (red) to positive correlations (blue).



Supplementary Figure S15 Correlation of biomass traits of plants under the elevated CO_2 concentration

Pearson's correlation coefficients at p-value < 0.01 between biomass traits of only plants under the elevated CO_2 concentration. Insignificant correlations are not displayed. The horizontal bar displays reference color for negative correlations (red) to positive correlations (blue).

Supplementary Table S3 Growth conditions of previous experiments using wheat cv Veery 10.

Study	µmol m^{-2} s^{-1} PPFD	Hours day/night	C/C Temperature	% Humidity	ppm CO_2	d Age	mM $[NO_3^-]$	mM $[NH_4^+]$	g $plant^{-1}$ or m^{-2} dry mass	Replication
(Smart et al., 1998)	1000	16/8, 18/6	22.5/19.5	65/85	360/1000	23	0.1-1		~1000-1200 m^{-2}	8 experiments
(Bloom et al., 2002)	700	16/8	25/15		360/700	14	0.2	0.2	0.15-0.55	10
(Bloom et al., 2010)					380/720	14	0.2,1		0.06 (shoot only)	6-12
(Bloom et al., 2012)	750	16/8	24/16	60/70	400/750	24	0.2	0.2		3-6
(Carlisle et al., 2012)	375	15/9	23/20	60/70	410/720		0.2	0.2	13.5-14.8	10
(Asensio et al., 2015)	500	15/9	25/16	70/60	390/720	10	0.2	0.2	0.15-0.2	10
(Rubio-Asensio & Bloom, 2016)					410/720	14,40	0.2	0.2	0.15-0.2	5-12
(Bloom et al., 2020)					380/720	21	0.2	0.2	0.6-0.9	10
This study	900	16/8	22/15	60/83	420/750	21	0.5	0.5	2.5	6
This study						60	0.5	0.5	8	12

References

Al-Hadeethi, I., Li, Y., Odhafa, A. K. H., Al-Hadeethi, H., Seneweera, S., & Lam, S. K. (2019). Assessment of grain quality in terms of functional group response to elevated [CO_2], water, and nitrogen using a meta-analysis: Grain protein, zinc, and iron under future climate. *Ecology and Evolution*, *9*(13), 7425–7437. https://doi.org/10.1002/ece3.5210

Asensio, J. S. R., Rachmilevitch, S., & Bloom, A. J. (2015). Responses of Arabidopsis and Wheat to Rising CO_2 Depend on Nitrogen Source and Nighttime CO_2 Levels. *Plant Physiology*, *168*(1), 156–163. https://doi.org/10.1104/pp.15.00110

Association of Official Analytical Chemists. (2000). *Food composition, additives, natural contaminants* (W. Horwitz, Ed.; 17th ed). AOAC International.

Backhaus, A. E., Griffiths, C., Vergara-Cruces, A., Simmonds, J., Lee, R., Morris, R. J., & Uauy, C. (2023). Delayed development of basal spikelets in wheat explains their increased floret abortion and rudimentary nature. *Journal of Experimental Botany*, erad233. https://doi.org/10.1093/jxb/erad233

Baxter, I., Brazelton, J. N., Yu, D., Huang, Y. S., Lahner, B., Yakubova, E., Li, Y., Bergelson, J., Borevitz, J. O., Nordborg, M., Vitek, O., & Salt, D. E. (2010). A Coastal Cline in Sodium Accumulation in Arabidopsis thaliana Is Driven by Natural Variation of the Sodium Transporter AtHKT1;1. *PLoS Genetics*, *6*(11), e1001193. https://doi.org/10.1371/journal.pgen.1001193

Bishop, D. L., & Bugbee, B. G. (1998). Photosynthetic capacity and dry mass partitioning in dwarf and semi-dwarf wheat (Triticum aestivum L.). *Journal of Plant Physiology*, *153*(5–6), 558–565. https://doi.org/10.1016/S0176-1617(98)80204-6

Blake, N. K., Pumphrey, M., Glover, K., Chao, S., Jordan, K., Jannick, J.-L., Akhunov, E. A., Dubcovsky, J., Bockelman, H., & Talbert, L. E. (2019). Registration of the Triticeae-CAP Spring Wheat Nested Association Mapping Population. *Journal of Plant Registrations*, *13*(2), 294–297. https://doi.org/10.3198/jpr2018.07.0052crmp

Bloom. (2015). The increasing importance of distinguishing among plant nitrogen sources. *Current Opinion in Plant Biology*, *25*, 10–16. http://dx.doi.org/10.1016/j.pbi.2015.03.002

Bloom, A. J., Asensio, J. S. R., Randall, L., Rachmilevitch, S., Cousins, A. B., & Carlisle, E. A. (2012). CO2 enrichment inhibits shoot nitrate assimilation in C3 but not C4 plants and slows growth under nitrate in C3 plants. *Ecology*, *93*(2), 355–367. https://doi.org/10.1890/11-0485.1

Bloom, A. J., Burger, M., Asensio, J. S. R., & Cousins, A. B. (2010). Carbon Dioxide Enrichment Inhibits Nitrate Assimilation in Wheat and Arabidopsis. *Science*, *328*(5980), 899–903. https://doi.org/10.1126/science.1186440

Bloom, A. J., Burger, M., Kimball, B. A., & Pinter, P. J. (2014). Nitrate assimilation is inhibited by elevated CO2 in field-grown wheat. *Nature Clim. Change*, *4*(6), 477–480. https://doi.org/10.1038/nclimate2183

http://www.nature.com/nclimate/journal/v4/n6/abs/nclimate2183.html#supplementary-information

Bloom, A. J., Kasemsap, P., & Rubio-Asensio, J. S. (2020). Rising atmospheric CO $_2$ concentration inhibits nitrate assimilation in shoots but enhances it in roots of C $_3$ plants. *Physiologia Plantarum*, *168*(4), 963–972. https://doi.org/10.1111/ppl.13040

Bloom, A. J., & Plant, R. E. (2021). Wheat grain yield decreased over the past 35 years, but protein content did not change. *Journal of Experimental Botany*, *72*(20), 6811–6821. https://doi.org/10.1093/jxb/erab343

Bloom, A. J., Smart, D. R., Nguyen, D. T., & Searles, P. S. (2002). Nitrogen assimilation and growth of wheat under elevated carbon dioxide. *Proceedings of the National Academy of Sciences*, *99*(3), 1730–1735. https://doi.org/10.1073/pnas.022627299

Britto, D. T., & Kronzucker, H. J. (2002). NH4+ toxicity in higher plants: A critical review. *J Plant Physiol*, *159*. https://doi.org/10.1078/0176-1617-0774

Britto, D. T., & Kronzucker, H. J. (2013). Ecological significance and complexity of N-source preference in plants. *Annals of Botany*, *112*(6), 957–963. https://doi.org/10.1093/aob/mct157

Britto, D. T., Siddiqi, M. Y., Glass, A. D. M., & Kronzucker, H. J. (2001). Futile transmembrane NH4+ cycling: A cellular hypothesis to explain ammonium toxicity in plants. *Proceedings of the National Academy of Sciences*, *98*(7), 4255–4258. https://doi.org/10.1073/pnas.061034698

Broberg, M., Högy, P., & Pleijel, H. (2017). CO2-Induced Changes in Wheat Grain Composition: Meta-Analysis and Response Functions. *Agronomy*, *7*(2), 32. https://doi.org/10.3390/agronomy7020032

Carlisle, E., Myers, S., Raboy, V., & Bloom, A. (2012). The Effects of Inorganic Nitrogen form and CO(2) Concentration on Wheat Yield and Nutrient Accumulation and Distribution. *Frontiers in Plant Science*, *3*, 195. https://doi.org/10.3389/fpls.2012.00195

Cheng, L., Booker, F. L., Tu, C., Burkey, K. O., Zhou, L., Shew, H. D., Rufty, T. W., & Hu, S. (2012). Arbuscular Mycorrhizal Fungi Increase Organic Carbon Decomposition Under Elevated CO2. *Science*, *337*(6098), 1084–1087. https://doi.org/10.1126/science.1224304

Dier, M., Meinen, R., Erbs, M., Kollhorst, L., Baillie, C.-K., Kaufholdt, D., Kücke, M., Weigel, H.-J., Zörb, C., Hänsch, R., & Manderscheid, R. (2018). Effects of free air carbon dioxide enrichment (FACE) on nitrogen assimilation and growth of winter wheat under nitrate and ammonium fertilization. *Global Change Biology*, *24*(1), e40–e54. https://doi.org/10.1111/gcb.13819

Epstein, E., & Bloom, A. J. (2005). *Mineral Nutrition of Plants: Principles and Perspectives*. Sinauer Associates, Incorporated.

Esteban, R., Ariz, I., Cruz, C., & Moran, J. F. (2016). Review: Mechanisms of ammonium toxicity and the quest for tolerance. *Plant Science*, *248*, 92–101. https://doi.org/10.1016/j.plantsci.2016.04.008

FAO. (2022, December 13). *FAOSTAT: Food Balances (2010-)* [Database]. FAOSTAT. https://www.fao.org/faostat/en/#data/FBS

Feng, Z., Rütting, T., Pleijel, H., Wallin, G., Reich, P. B., Kammann, C. I., Newton, P. C. D., Kobayashi, K., Luo, Y., & Uddling, J. (2015). Constraints to nitrogen acquisition of terrestrial plants under elevated CO2. *Global Change Biology*, *21*(8), 3152–3168. https://doi.org/10.1111/gcb.12938

Fernando, N., Hirotsu, N., Panozzo, J., Tausz, M., Norton, R. M., & Seneweera, S. (2017). Lower grain nitrogen content of wheat at elevated CO2 can be improved through post-anthesis NH4+ supplement. *Journal of Cereal Science*, *74*, 79–85. https://doi.org/10.1016/j.jcs.2017.01.009

Fischer, R. A., Moreno Ramos, O. H., Ortiz Monasterio, I., & Sayre, K. D. (2019). Yield response to plant density, row spacing and raised beds in low latitude spring wheat with ample soil resources: An update. *Field Crops Research*, *232*, 95–105. https://doi.org/10.1016/j.fcr.2018.12.011

Fox, J., & Weisberg, S. (2019). *An R companion to applied regression* (Third edition). SAGE.

Gaju, O., Allard, V., Martre, P., Le Gouis, J., Moreau, D., Bogard, M., Hubbart, S., & Foulkes, M. J. (2014). Nitrogen partitioning and remobilization in relation to leaf senescence, grain yield and grain nitrogen concentration in wheat cultivars. *Field Crops Research*, *155*, 213–223. https://doi.org/10.1016/j.fcr.2013.09.003

Gao, Y., & Cabrera Serrenho, A. (2023). Greenhouse gas emissions from nitrogen fertilizers could be reduced by up to one-fifth of current levels by 2050 with combined interventions. *Nature Food*, *4*(2), 170–178. https://doi.org/10.1038/s43016-023-00698-w

Gifford, R. M., Barrett, D. J., & Lutze, J. L. (2000). The effects of elevated [CO2] on the C:N and C:P mass ratios of plant tissues. *Plant and Soil*, *224*(1), 1–14. https://doi.org/10.1023/A:1004790612630

Gigon, A., & Rorison, I. H. (1972). The Response of Some Ecologically Distinct Plant Species to Nitrate- and to Ammonium-Nitrogen. *The Journal of Ecology*, *60*(1), 93. https://doi.org/10.2307/2258043

Hothorn, T., Bretz, F., & Westfall, P. (2008). *Simultaneous Inference in General Parametric Models*. *50*(3), 346–363.

Houlton, B. Z., Sigman, D. M., Schuur, E. A. G., & Hedin, L. O. (2007). A climate-driven switch in plant nitrogen acquisition within tropical forest communities. *Proceedings of the National Academy of Sciences*, *104*(21), 8902–8906. https://doi.org/10.1073/pnas.0609935104

IPCC. (2021). *Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* (V. Masson-

Delmotte, P. Zhai, A. Pirani, S. L. Connors, C. Péan, S. Berger, N. Caud, Y. Chen, L. Goldfarb, M. I. Gomis, M. Huang, K. Leitzell, E. Lonnoy, J. B. R. Matthews, T. K. Maycock, T. Waterfield, Ö. Yelekçi, R. Yu, & B. Zhou, Eds.). Cambridge University Press. https://doi.org/10.1017/9781009157896

Jackson, L. E., Schimel, J. P., & Firestone, M. K. (1989). Short-term partitioning of ammonium and nitrate between plants and microbes in an annual grassland. *Soil Biology and Biochemistry*, *21*(3), 409–415. https://doi.org/10.1016/0038-0717(89)90152-1

Jackson, R. B., & Caldwell, M. M. (1993). The Scale of Nutrient Heterogeneity Around Individual Plants and Its Quantification with Geostatistics. *Ecology*, *74*(2), 612–614. https://doi.org/10.2307/1939320

Kasemsap, P., & Bloom, A. J. (2022). Breeding for Higher Yields of Wheat and Rice through Modifying Nitrogen Metabolism. *Plants*, *12*(1), 85. https://doi.org/10.3390/plants12010085

Katz, E., Knapp, A., Lensink, M., Keller, C. K., Stefani, J., Li, J.-J., Shane, E., Tuermer-Lee, K., Bloom, A. J., & Kliebenstein, D. J. (2022). Genetic variation underlying differential ammonium and nitrate responses in *Arabidopsis thaliana*. *The Plant Cell*, *34*(12), 4696–4713. https://doi.org/10.1093/plcell/koac279

Langsrud, Ø. (2003). ANOVA for unbalanced data: Use Type II instead of Type III sums of squares. *Statistics and Computing*, *13*(2), 163–167. https://doi.org/10.1023/A:1023260610025

Lenth, R. V. (2023). *emmeans: Estimated Marginal Means, aka Least-Squares Means* (R package version 1.8.6) [Computer software]. https://CRAN.R-project.org/package=emmeans

Levene, H. (1960). Robust tests for equality of variances. In e a Ingram Olkin (Ed.), *Contributions to Probability and Statistic: Essays in honor of Harold Hotelling*.

Loladze, I. (2014). Hidden shift of the ionome of plants exposed to elevated CO2 depletes minerals at the base of human nutrition. *Elife*, *3*, e02245.

Maathuis, F. J. (2009). Physiological functions of mineral macronutrients. *Current Opinion in Plant Biology*, *12*(3), 250–258.

Maccaferri, M., Zhang, J., Bulli, P., Abate, Z., Chao, S., Cantu, D., Bossolini, E., Chen, X., Pumphrey, M., & Dubcovsky, J. (2015). A Genome-Wide Association Study of Resistance to Stripe Rust

(Puccinia striiformis f. Sp. Tritici) in a Worldwide Collection of Hexaploid Spring Wheat (Triticum aestivum L.). *G3: Genes|Genomes|Genetics*, *5*(3), 449–465. PMC. https://doi.org/10.1534/g3.114.014563

Mauro, A. A., & Ghalambor, C. K. (2020). Trade-offs, Pleiotropy, and Shared Molecular Pathways: A Unified View of Constraints on Adaptation. *Integrative and Comparative Biology*, *60*(2), 332–347. https://doi.org/10.1093/icb/icaa056

Miller, A. J., & Cramer, M. D. (2005). Root nitrogen acquisition and assimilation. In *Root Physiology: From Gene to Function* (pp. 1–36). Springer, Dordrecht. https://doi.org/10.1007/1-4020-4099-7_1

Myers, S. S., Zanobetti, A., Kloog, I., Huybers, P., Leakey, A. D. B., Bloom, A. J., Carlisle, E., Dietterich, L. H., Fitzgerald, G., Hasegawa, T., Holbrook, N. M., Nelson, R. L., Ottman, M. J., Raboy, V., Sakai, H., Sartor, K. A., Schwartz, J., Seneweera, S., Tausz, M., & Usui, Y. (2014). Increasing CO2 threatens human nutrition. *Nature*, *510*(7503), 139–142. https://doi.org/10.1038/nature13179

Nishina, K., Ito, A., Hanasaki, N., & Hayashi, S. (2017). Reconstruction of spatially detailed global map of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>−</sup> application in synthetic nitrogen fertilizer. *Earth System Science Data*, *9*(1), 149–162. https://doi.org/10.5194/essd-9-149-2017

Pleijel, H., Broberg, M. C., Högy, P., & Uddling, J. (2019). Nitrogen application is required to realize wheat yield stimulation by elevated CO $_2$ but will not remove the CO $_2$ -induced reduction in grain protein concentration. *Global Change Biology*, *25*(5), 1868–1876. https://doi.org/10.1111/gcb.14586

Raun, W. R., & Johnson, G. V. (1999). Improving Nitrogen Use Efficiency for Cereal Production. *Agronomy Journal*, *91*(3), 357–363. https://doi.org/10.2134/agronj1999.00021962009100030001x

Rawson, H. (1970). Spikelet Number, Its Control and Relation to Yield Per Ear in Wheat. *Australian Journal of Biological Sciences*, *23*(1), 1. https://doi.org/10.1071/BI9700001

Rivera-Amado, C., Trujillo-Negrellos, E., Molero, G., Reynolds, M. P., Sylvester-Bradley, R., & Foulkes, M. J. (2019). Optimizing dry-matter partitioning for increased spike growth, grain number and harvest index in spring wheat. *Field Crops Research*, *240*, 154–167. https://doi.org/10.1016/j.fcr.2019.04.016

Royston, J. P. (1982). An Extension of Shapiro and Wilk's W Test for Normality to Large Samples. *Applied Statistics*, *31*(2), 115. https://doi.org/10.2307/2347973

Rubio-Asensio, J. S., & Bloom, A. J. (2016). Inorganic nitrogen form: A major player in wheat and Arabidopsis responses to elevated CO2. *Journal of Experimental Botany*, erw465.

Salgó, A., & Gergely, S. (2012). Analysis of wheat grain development using NIR spectroscopy. *Journal of Cereal Science*, *56*(1), 31–38. https://doi.org/10.1016/j.jcs.2012.04.011

Seibert, R., Donath, T. W., Moser, G., Laser, H., Grünhage, L., Schmid, T., & Müller, C. (2021). Effects of long-term CO2 enrichment on forage quality of extensively managed temperate grassland. *Agriculture, Ecosystems & Environment*, *312*, 107347. https://doi.org/10.1016/j.agee.2021.107347

Sicher, R. C., & Bunce, J. A. (1997). Relationship of photosynthetic acclimation to changes of Rubisco activity in field-grown winter wheat and barley during growth in elevated carbon dioxide. *Photosynthesis Research*, *52*(1), 27–38. https://doi.org/10.1023/A:1005874932233

Sierra-Gonzalez, A., Molero, G., Rivera-Amado, C., Babar, M. A., Reynolds, M. P., & Foulkes, M. J. (2021). Exploring genetic diversity for grain partitioning traits to enhance yield in a high biomass spring wheat panel. *Field Crops Research*, *260*, 107979. https://doi.org/10.1016/j.fcr.2020.107979

Slafer, G. A., Elia, M., Savin, R., García, G. A., Terrile, I. I., Ferrante, A., Miralles, D. J., & González, F. G. (2015). Fruiting efficiency: An alternative trait to further rise wheat yield. *Food and Energy Security*, *4*(2), 92–109. https://doi.org/10.1002/fes3.59

Smart, D. R., Ritchie, K., Bloom, A. J., & Bugbee, B. B. (1998). Nitrogen balance for wheat canopies (*Triticum aestivum* cv. Veery 10) grown under elevated and ambient CO $_2$ concentrations. *Plant, Cell & Environment*, *21*(8), 753–763. https://doi.org/10.1046/j.1365-3040.1998.00315.x

Subbarao, G. V., Kishii, M., Bozal-Leorri, A., Ortiz-Monasterio, I., Gao, X., Ibba, M. I., Karwat, H., Gonzalez-Moro, M. B., Gonzalez-Murua, C., Yoshihashi, T., Tobita, S., Kommerell, V., Braun, H.-J., & Iwanaga, M. (2021). Enlisting wild grass genes to combat nitrification in wheat farming: A nature-based solution. *Proceedings of the National Academy of Sciences*, *118*(35), e2106595118. https://doi.org/10.1073/pnas.2106595118

Subbarao, G. V., & Searchinger, T. D. (2021). Opinion: A "more ammonium solution" to mitigate nitrogen pollution and boost crop yields. *Proceedings of the National Academy of Sciences*, *118*(22), e2107576118. https://doi.org/10.1073/pnas.2107576118

Taub, D. R., Miller, B., & Allen, H. (2008). Effects of elevated CO $_2$ on the protein concentration of food crops: A meta-analysis: ELEVATED CO $_2$ AND CROP PROTEIN CONCENTRATIONS. *Global Change Biology*, *14*(3), 565–575. https://doi.org/10.1111/j.1365-2486.2007.01511.x

Terés, J., Busoms, S., Perez Martín, L., Luís-Villarroya, A., Flis, P., Álvarez-Fernández, A., Tolrà, R., Salt, D. E., & Poschenrieder, C. (2019). Soil carbonate drives local adaptation in *ARABIDOPSIS THALIANA* . *Plant, Cell & Environment*, *42*(8), 2384–2398. https://doi.org/10.1111/pce.13567

Uddling, J., Broberg, M. C., Feng, Z., & Pleijel, H. (2018). Crop quality under rising atmospheric CO 2. *Current Opinion in Plant Biology*.

Waines, J. G., & Ehdaie, B. (2007). Domestication and Crop Physiology: Roots of Green-Revolution Wheat. *Annals of Botany*, *100*(5), 991–998. https://doi.org/10.1093/aob/mcm180

Wang, F., Gao, J., Yong, J. W. H., Wang, Q., Ma, J., & He, X. (2020). Higher Atmospheric CO2 Levels Favor C3 Plants Over C4 Plants in Utilizing Ammonium as a Nitrogen Source. *Frontiers in Plant Science*, *11*, 537443. https://doi.org/10.3389/fpls.2020.537443

Wujeska-Klause, A., Crous, K. Y., Ghannoum, O., & Ellsworth, D. S. (2019). Lower photorespiration in elevated CO $_2$ reduces leaf N concentrations in mature *Eucalyptus* trees in the field. *Global Change Biology*, *25*(4), 1282–1295. https://doi.org/10.1111/gcb.14555

Xie, Q., Mayes, S., & Sparkes, D. L. (2016). Early anthesis and delayed but fast leaf senescence contribute to individual grain dry matter and water accumulation in wheat. *Field Crops Research*, *187*, 24–34. https://doi.org/10.1016/j.fcr.2015.12.009

Xu, G., Fan, X., & Miller, A. J. (2012). Plant Nitrogen Assimilation and Use Efficiency. *Annual Review of Plant Biology*, *63*(1), 153–182. https://doi.org/10.1146/annurev-arplant-042811-105532

Zhang, J., Gizaw, S. A., Bossolini, E., Hegarty, J., Howell, T., Carter, A. H., Akhunov, E., & Dubcovsky, J. (2018). Identification and validation of QTL for grain yield and plant water status under contrasting water treatments in fall-sown spring wheats. *Theoretical and Applied Genetics*, *131*(8), 1741–1759. https://doi.org/10.1007/s00122-018-3111-9

General Discussion & Summary

Plants are major sources of protein in the human diets. Together, small cereal grains like wheat and rice, provide one third of human daily protein intake globally (FAO 2022). Developing countries, especially in Africa and Asia, rely more on plants as primary protein sources than developed countries (Awika 2011; Kasemsap and Bloom 2022). Furthermore, improving crop nitrogen use efficiency is a highly effective climate change mitigation strategy (Gao and Cabrera Serrenho 2023). To achieve sustainable global food systems in the face of climate change, we urgently need to improve the production of plant protein from the nitrogen fertilizer that we apply to meet the demands of the growing human population.

Here, I hypothesized that we may employ genetic adaptations to specific nitrogen forms, ammonium or nitrate, that allow different responses to environmental fluctuations to breed climate-resilient crops with improved nutritional values. Evaluating the body of existing literature and the global germplasms of wheat and rice, I have addressed three major research questions: (i) how have past breeding and genetic modifications for nitrogen metabolism improved grain protein concentration and yield? (Chapter 1), (ii) what are the natural variations in the ability to utilize different nitrogen forms for growth and development? (Chapter 2 and 3), (iii) how does adaptation to specific nitrogen sources influence responses to changing climates? (Chapter 3 and 4). The following paragraphs briefly summarize key insights and discuss their implications for each of the questions, and conclude with a remark on the potential impacts on the emerging alternative protein industry.

Firstly, I emphasized that breeding attempts to modify nitrogen metabolism for improved yield only have had limited success to date, despite the wide-scale use of synthetic nitrogen fertilizers for over a century. Any past successful applications have required concurrent improvement of both nitrogen and carbon assimilation throughout the growth period (Kasemsap and Bloom 2022). Furthermore, poor understanding of genetic mechanisms and their regulations across species, particularly the range of existing natural variations has limited applications to breeding food crops with improved yield and nutrition (Kasemsap and Bloom 2022). Therefore, the genetic and physiological insights derived from this dissertation should provide a critical foundation for subsequent research and breeding. Further functional validation and characterization are required to confirm the roles of the candidate genes identified in our study.

Secondly, comparisons of natural variations in growth under different nitrogen forms across species suggest that crop nitrogen responsiveness may be more plastic than previously thought. The nitrogen responsiveness ratio between two nitrogen forms in the model species *Arabidopsis* (Katz et al. 2022) and the two major food crops examined in this dissertation, rice (Chapter 2) (Kasemsap, Cohen, and Bloom 2024) and wheat (Chapter 3), confirm significant variations across the global germplasms of these species (Figure 1). Interestingly, most of the accessions within the germplasms seem to be a nitrogen “flexitarian”: that is, the growth responses of most genotypes to nitrogen provided at moderate concentrations below the toxicity threshold appeared to be comparable under both inorganic forms.

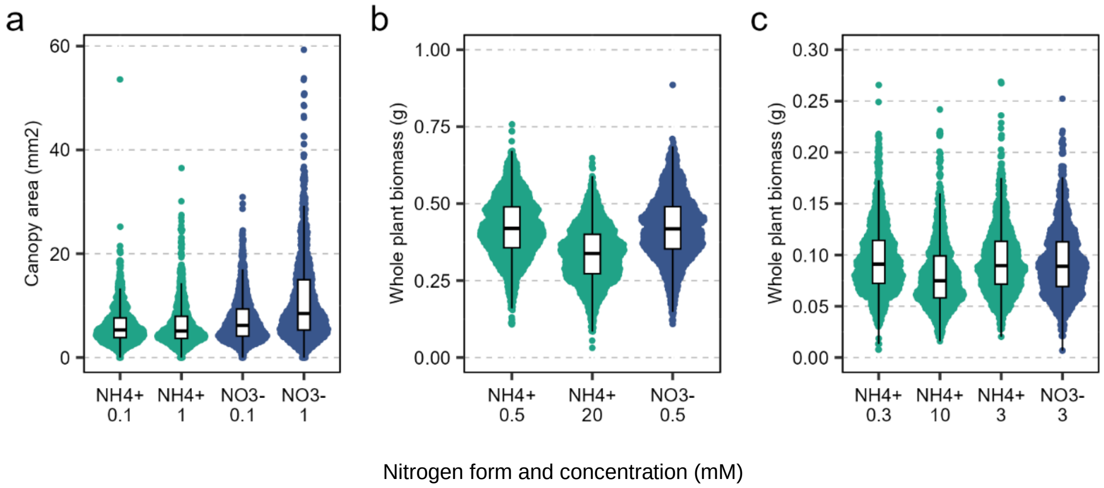


Figure 1 Significant variation in growth responses to different levels and sources of inorganic nitrogen. Distribution of growth responses to different concentrations of NH_4^+ (green) or NO_3^- (blue) in the global germplasms of (a) *Arabidopsis* (1092 accessions, canopy area), (b) bread wheat (875 accessions, whole plant biomass at 15 d after transplanting) and (c) rice (393 accessions, whole plant biomass at 21 d after transplanting. Each dot represents an individual accession. The box plots represent minima, 25th percentiles, medians, 75th percentiles and maxima.

The presence of both “generalists” and “specialists” adapted to specific forms of nitrogen prevalent in their natural habitats is expected in global germplasms. On one hand, flexibility in utilizing different forms should allow plants to swiftly make use of fluctuating soil resources. On the other hand, being adapted to certain forms further guarantee that they can make the most of the prevalent form in the soil at a given time, and potentially avoid suffering from toxicity symptoms from exposure to high concentration. Although understanding of the mechanisms behind tolerance to high nitrogen toxicity is beyond the scope of this dissertation, we identified tolerant genotypes and genetic information that may be useful for future studies. Understanding of genetic adaptations should enable breeders and farmers to better tailor nitrogen management to maximize the genetic potential of their plant materials.

Third, based on a subset of genotypes with constrasting nitrogen responses, we demonstrated that NH_4^+ nutrition can sustain wheat grain protein concentrations under elevated CO_2 levels (Kasemsap and Bloom 2023). Interestingly, wheat varieties that are either bred for or adapted to NH_4^+ nutrition also increased shoot and grain carbon accumulation, thereby producing higher grain yields without sacrificing nutritional quality (Kasemsap and Bloom 2023). As such, future breeding program may prioritize improved NH_4^+ adaptation as one of the key traits for both climate resilience and improved nutrition. The genetic markers identified in this study should inform selections of adapted genotypes for future investigations.

Our understanding of crop responses to nitrogen forms, however, remains far from complete. Based on the results in this dissertation, the missing knowledge gaps include the extent to which variations in genetic architectures of different plant species, production environments, and physiological mechanisms during grain development explain the differential responses to inorganic nitrogen forms. Fture research may focus on the following objectives and hypotheses:

- **Identify and compare homologs of major nitrogen use efficiency candidate genes across food crop species.** (Hypothesis: Major candidate genes are shared between diverged plant lineages, but each lineage depends on a large number of different sets of quantitative loci that contribute to variations in responses to nitrogen.)
- **Conduct meta-analysis to quantify the influence of nitrogen forms on crop yield and protein under heterogenous growing enviroments.** (Hypothesis: Production environments with high relative NH_4^+ availability to crops have higher crop yield and protein concentrations in harvestable parts.)
- **Characterize physiological and compositional changes during flower and grain development in responses to nitrogen source.** (Hypothesis: Higher grain protein concentration and yield under NH_4^+ than NO_3^- nutrition derived from accelerated

flowering time and longer grain filling period. Nitrogen forms do not affect grain protein quality.)

As climate changes, food production will require alternative approaches to meet growing demand. The success in rapid adoption and deployment of the alternative protein industry is likely to come from combining different technologies and building upon existing infrastructures in the food systems (Anon n.d.). In addition to providing a significant portion of the global food protein intake (FAO 2022; Kasemsap and Bloom 2022), major food crops also currently serve as a main ingredient in plant-based alternative protein products. By employing multiple synergistic approaches, we have a higher chance of meeting the food and nutritional demand of the rising human population within the boundaries of the environmental constraints. Therefore, this doctoral dissertation not only addressed the current challenges in the conventional plant and food production, but also provided key insights that may help accelerate plant-based protein production as an alternative protein source.

References

Anon. n.d. “Deep Dive: Plant-Based Meat Crop Development | GFI.” Retrieved February 5, 2024 (https://gfi.org/science/the-science-of-plant-based-meat/deep-dive-plant-based-meat-crop-development/).

Awika, Joseph M. 2011. “Major Cereal Grains Production and Use around the World.” Pp. 1–13 in *ACS Symposium Series*. Vol. 1089, edited by J. M. Awika, V. Piironen, and S. Bean. Washington, DC: American Chemical Society.

FAO. 2022. “FAOSTAT: Food Balances (2010-).” *FAOSTAT*. Retrieved December 13, 2022 (https://www.fao.org/faostat/en/#data/FBS).

Gao, Yunhu, and André Cabrera Serrenho. 2023. “Greenhouse Gas Emissions from Nitrogen Fertilizers Could Be Reduced by up to One-Fifth of Current Levels by 2050 with Combined Interventions.” *Nature Food* 4(2):170–78. doi: 10.1038/s43016-023-00698-w.

Kasemsap, Pornpipat, and Arnold J. Bloom. 2022. “Breeding for Higher Yields of Wheat and Rice through Modifying Nitrogen Metabolism.” *Plants* 12(1):85. doi: 10.3390/plants12010085.

Kasemsap, Pornpipat, and Arnold J. Bloom. 2023. *Genetic Adaptation to Ammonium Sustains Wheat Grain Quality and Alleviates Acclimation to CO* $_2$ *Enrichment*. *preprint*. Plant Biology. doi: 10.1101/2023.11.21.568194.

Kasemsap, Pornpipat, Itay Cohen, and Arnold J. Bloom. 2024. “Genome-Wide Association Study of Rice Vegetative Biomass under Different Inorganic Nitrogen Forms — Ammonium or Nitrate.”

Katz, Ella, Anna Knapp, Mariele Lensink, Caroline Kaley Keller, Jordan Stefani, Jia-Jie Li, Emily Shane, Kaelyn Tuermer-Lee, Arnold J. Bloom, and Daniel J. Kliebenstein. 2022. “Genetic Variation Underlying Differential Ammonium and Nitrate Responses in *Arabidopsis Thaliana*.” *The Plant Cell* 34(12):4696–4713. doi: 10.1093/plcell/koac279.

Bibliography

Alexandrov, N. N., Brover, V. V., Freidin, S., Troukhan, M. E., Tatarinova, T. V., Zhang, H., Swaller, T. J., Lu, Y.-P., Bouck, J., Flavell, R. B., & Feldmann, K. A. (2009). Insights into corn genes derived from large-scale cDNA sequencing. *Plant Molecular Biology*, *69*(1–2), 179–194. https://doi.org/10.1007/s11103-008-9415-4

Al-Hadeethi, I., Li, Y., Odhafa, A. K. H., Al-Hadeethi, H., Seneweera, S., & Lam, S. K. (2019). Assessment of grain quality in terms of functional group response to elevated [CO_2], water, and nitrogen using a meta-analysis: Grain protein, zinc, and iron under future climate. *Ecology and Evolution*, *9*(13), 7425–7437. https://doi.org/10.1002/ece3.5210

Araus, V., Swift, J., Alvarez, J. M., Henry, A., & Coruzzi, G. M. (2020). A balancing act: How plants integrate nitrogen and water signals. *Journal of Experimental Botany*, *71*(15), 4442–4451. https://doi.org/10.1093/jxb/eraa054

Arends, D., Prins, P., Jansen, R. C., & Broman, K. W. (2010). R/qtl: High-throughput multiple QTL mapping: Fig. 1. *Bioinformatics*, *26*(23), 2990–2992. https://doi.org/10.1093/bioinformatics/btq565

Asensio, J. S. R., Rachmilevitch, S., & Bloom, A. J. (2015). Responses of Arabidopsis and Wheat to Rising CO_2 Depend on Nitrogen Source and Nighttime CO_2 Levels. *Plant Physiology*, *168*(1), 156–163. https://doi.org/10.1104/pp.15.00110

Association of Official Analytical Chemists. (2000). *Food composition, additives, natural contaminants* (W. Horwitz, Ed.; 17th ed). AOAC International.

Awika, J. M. (2011). Major Cereal Grains Production and Use around the World. In J. M. Awika, V. Piironen, & S. Bean (Eds.), *ACS Symposium Series* (Vol. 1089, pp. 1–13). American Chemical Society. https://doi.org/10.1021/bk-2011-1089.ch001

Backhaus, A. E., Griffiths, C., Vergara-Cruces, A., Simmonds, J., Lee, R., Morris, R. J., & Uauy, C. (2023). Delayed development of basal spikelets in wheat explains their increased floret abortion and rudimentary nature. *Journal of Experimental Botany*, erad233. https://doi.org/10.1093/jxb/erad233

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using **lme4**. *Journal of Statistical Software*, *67*(1). https://doi.org/10.18637/jss.v067.i01

Baxter, I., Brazelton, J. N., Yu, D., Huang, Y. S., Lahner, B., Yakubova, E., Li, Y., Bergelson, J., Borevitz, J. O., Nordborg, M., Vitek, O., & Salt, D. E. (2010). A Coastal Cline in Sodium Accumulation in Arabidopsis thaliana Is Driven by Natural Variation of the Sodium Transporter AtHKT1;1. *PLoS Genetics*, *6*(11), e1001193. https://doi.org/10.1371/journal.pgen.1001193

Bishop, D. L., & Bugbee, B. G. (1998). Photosynthetic capacity and dry mass partitioning in dwarf and semi-dwarf wheat (Triticum aestivum L.). *Journal of Plant Physiology*, *153*(5–6), 558–565. https://doi.org/10.1016/S0176-1617(98)80204-6

Blake, N. K., Pumphrey, M., Glover, K., Chao, S., Jordan, K., Jannick, J.-L., Akhunov, E. A., Dubcovsky, J., Bockelman, H., & Talbert, L. E. (2019). Registration of the Triticeae-CAP Spring Wheat Nested Association Mapping Population. *Journal of Plant Registrations*, *13*(2), 294–297. https://doi.org/10.3198/jpr2018.07.0052crmp

Bloom. (2015). The increasing importance of distinguishing among plant nitrogen sources. *Current Opinion in Plant Biology*, *25*, 10–16. http://dx.doi.org/10.1016/j.pbi.2015.03.002

Bloom, A. J. (2010). Energetics of Nitrogen Acquisition. In *Annual Plant Reviews Volume 42* (pp. 63–81). Wiley-Blackwell.

Bloom, A. J., Asensio, J. S. R., Randall, L., Rachmilevitch, S., Cousins, A. B., & Carlisle, E. A. (2012). CO2 enrichment inhibits shoot nitrate assimilation in C3 but not C4 plants and slows growth under nitrate in C3 plants. *Ecology*, *93*(2), 355–367. https://doi.org/10.1890/11-0485.1

Bloom, A. J., Burger, M., Asensio, J. S. R., & Cousins, A. B. (2010a). Carbon Dioxide Enrichment Inhibits Nitrate Assimilation in Wheat and Arabidopsis. *Science*, *328*(5980), 899–903. https://doi.org/10.1126/science.1186440

Bloom, A. J., Burger, M., Asensio, J. S. R., & Cousins, A. B. (2010b). Carbon Dioxide Enrichment Inhibits Nitrate Assimilation in Wheat and Arabidopsis. *Science*, *328*(5980), 899–903. https://doi.org/10.1126/science.1186440

Bloom, A. J., Burger, M., Kimball, B. A., & Pinter, P. J. (2014). Nitrate assimilation is inhibited by elevated CO2 in field-grown wheat. *Nature Clim. Change*, *4*(6), 477–480. https://doi.org/10.1038/nclimate2183 http://www.nature.com/nclimate/journal/v4/n6/abs/nclimate2183.html#supplementary-information

Bloom, A. J., Chapin, F. S., & Mooney, H. A. (1985). Resource Limitation in Plants-An Economic Analogy. *Annual Review of Ecology and Systematics*, *16*(1), 363–392. https://doi.org/10.1146/annurev.es.16.110185.002051

Bloom, A. J., Kasemsap, P., & Rubio-Asensio, J. S. (2020). Rising atmospheric CO $_2$ concentration inhibits nitrate assimilation in shoots but enhances it in roots of C $_3$ plants. *Physiologia Plantarum*, *168*(4), 963–972. https://doi.org/10.1111/ppl.13040

Bloom, A. J., & Plant, R. E. (2021). Wheat grain yield decreased over the past 35 years, but protein content did not change. *Journal of Experimental Botany*, *72*(20), 6811–6821. https://doi.org/10.1093/jxb/erab343

Bloom, A. J., Smart, D. R., Nguyen, D. T., & Searles, P. S. (2002). Nitrogen assimilation and growth of wheat under elevated carbon dioxide. *Proceedings of the National Academy of Sciences*, *99*(3), 1730–1735. https://doi.org/10.1073/pnas.022627299

Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., & Buckler, E. S. (2007). TASSEL: Software for association mapping of complex traits in diverse samples. *Bioinformatics*, *23*(19), 2633–2635. https://doi.org/10.1093/bioinformatics/btm308

Britto, D. T., & Kronzucker, H. J. (2002a). NH4+ toxicity in higher plants: A critical review. *Journal of Plant Physiology*, *159*(6), 567–584. http://dx.doi.org/10.1078/0176-1617-0774

Britto, D. T., & Kronzucker, H. J. (2002b). NH4+ toxicity in higher plants: A critical review. *J Plant Physiol*, *159*. https://doi.org/10.1078/0176-1617-0774

Britto, D. T., & Kronzucker, H. J. (2013a). Ecological significance and complexity of N-source preference in plants. *Annals of Botany*, *112*(6), 957–963. https://doi.org/10.1093/aob/mct157

Britto, D. T., & Kronzucker, H. J. (2013b). Ecological significance and complexity of N-source preference in plants. *Annals of Botany*, *112*(6), 957–963. https://doi.org/10.1093/aob/mct157

Britto, D. T., & Kronzucker, H. J. (2013c). Ecological significance and complexity of N-source preference in plants. *Annals of Botany*, *112*(6), 957–963. https://doi.org/10.1093/aob/mct157

Britto, D. T., Siddiqi, M. Y., Glass, A. D. M., & Kronzucker, H. J. (2001). Futile transmembrane NH4+ cycling: A cellular hypothesis to explain ammonium toxicity in plants. *Proceedings of the National Academy of Sciences*, *98*(7), 4255–4258. https://doi.org/10.1073/pnas.061034698

Broberg, M., Högy, P., & Pleijel, H. (2017). CO2-Induced Changes in Wheat Grain Composition: Meta-Analysis and Response Functions. *Agronomy*, *7*(2), 32. https://doi.org/10.3390/agronomy7020032

Carlisle, E., Myers, S., Raboy, V., & Bloom, A. (2012). The Effects of Inorganic Nitrogen form and CO(2) Concentration on Wheat Yield and Nutrient Accumulation and Distribution. *Frontiers in Plant Science*, *3*, 195. https://doi.org/10.3389/fpls.2012.00195

Cassan, O., Pimparé, L.-L., Mozzanino, T., Fizames, C., Devidal, S., Roux, F., Milcu, A., Lèbre, S., Gojon, A., & Martin, A. (2023). *Natural genetic variation underlying the negative effect of elevated CO $_2$ on ionome composition in* Arabidopsis thaliana [Preprint]. Plant Biology. https://doi.org/10.1101/2023.06.05.543678

Chen, G., Guo, S., Kronzucker, H. J., & Shi, W. (2013). Nitrogen use efficiency (NUE) in rice links to NH4 + toxicity and futile NH4 + cycling in roots. *Plant and Soil*, *369*(1), 351–363. https://doi.org/10.1007/s11104-012-1575-y

Cheng, L., Booker, F. L., Tu, C., Burkey, K. O., Zhou, L., Shew, H. D., Rufty, T. W., & Hu, S. (2012). Arbuscular Mycorrhizal Fungi Increase Organic Carbon Decomposition Under Elevated CO2. *Science*, *337*(6098), 1084–1087. https://doi.org/10.1126/science.1224304

Clarke, E., Sherrill-Mix, S., & Dawson, C. (2023). *ggbeeswarm: Categorical Scatter (Violin Point) Plots* (Version R package version 0.7.2) [Computer software]. https://CRAN.R-project.org/package=ggbeeswarm

Cormier, F., Faure, S., Dubreuil, P., Heumez, E., Beauchêne, K., Lafarge, S., Praud, S., & Le Gouis, J. (2013). A multi-environmental study of recent breeding progress on nitrogen use efficiency in wheat (Triticum aestivum L.). *Theoretical and Applied Genetics*, *126*(12), 3035–3048. https://doi.org/10.1007/s00122-013-2191-9

Cormier, F., Foulkes, J., Hirel, B., Gouache, D., Moënne-Loccoz, Y., & Le Gouis, J. (2016). Breeding for increased nitrogen-use efficiency: A review for wheat (*T. aestivum* L.). *Plant Breeding*, *135*(3), 255–278. https://doi.org/10.1111/pbr.12371

Cormier, F., Le Gouis, J., Dubreuil, P., Lafarge, S., & Praud, S. (2014). A genome-wide identification of chromosomal regions determining nitrogen use efficiency components in wheat (Triticum aestivum L.). *Theoretical and Applied Genetics*, *127*(12), 2679–2693.

Cox, W. J., & Reisenauer, H. M. (1973). Growth and ion uptake by wheat supplied nitrogen as nitrate, or ammonium, or both. *Plant and Soil*, *38*(2), 363–380. https://doi.org/10.1007/BF00779019

Crowell, S., Korniliev, P., Falcão, A., Ismail, A., Gregorio, G., Mezey, J., & McCouch, S. (2016). Genome-wide association and high-resolution phenotyping link Oryza sativa panicle traits to numerous trait-specific QTL clusters. *Nature Communications*, *7*(1), 10527. https://doi.org/10.1038/ncomms10527

Deep dive: Plant-based meat crop development | GFI. (n.d.). Retrieved February 5, 2024, from https://gfi.org/science/the-science-of-plant-based-meat/deep-dive-plant-based-meat-crop-development/

Devienne-Barret, F. (2000). Integrated Control of Nitrate Uptake by Crop Growth Rate and Soil Nitrate Availability under Field Conditions. *Annals of Botany*, *86*(5), 995–1005. https://doi.org/10.1006/anbo.2000.1264

Di, D.-W., Sun, L., Zhang, X., Li, G., Kronzucker, H. J., & Shi, W. (2018). Involvement of auxin in the regulation of ammonium tolerance in rice (Oryza sativa L.). *Plant and Soil*, *432*(1–2), 373–387. https://doi.org/10.1007/s11104-018-3813-4

Dier, M., Meinen, R., Erbs, M., Kollhorst, L., Baillie, C.-K., Kaufholdt, D., Kücke, M., Weigel, H.-J., Zörb, C., Hänsch, R., & Manderscheid, R. (2018). Effects of free air carbon dioxide enrichment

(FACE) on nitrogen assimilation and growth of winter wheat under nitrate and ammonium fertilization. *Global Change Biology*, *24*(1), e40–e54. https://doi.org/10.1111/gcb.13819

Dowle, M., & Srinivasan, A. (2023). *data.table: Extension of `data.frame`* [Computer software]. https://r-datatable.com, https://Rdatatable.gitlab.io/data.table, https://github.com/Rdatatable/data.table

Edwards, J. A., Santos-Medellín, C. M., Liechty, Z. S., Nguyen, B., Lurie, E., Eason, S., Phillips, G., & Sundaresan, V. (2018). Compositional shifts in root-associated bacterial and archaeal microbiota track the plant life cycle in field-grown rice. *PLOS Biology*, *16*(2), e2003862. https://doi.org/10.1371/journal.pbio.2003862

Edwards, J., Santos-Medellín, C., Nguyen, B., Kilmer, J., Liechty, Z., Veliz, E., Ni, J., Phillips, G., & Sundaresan, V. (2019). Soil domestication by rice cultivation results in plant-soil feedback through shifts in soil microbiota. *Genome Biology*, *20*(1), 221. https://doi.org/10.1186/s13059-019-1825-x

Eizenga, G. C., Ali, Md. L., Bryant, R. J., Yeater, K. M., McClung, A. M., & McCouch, S. R. (2014). Registration of the Rice Diversity Panel 1 for Genomewide Association Studies. *Journal of Plant Registrations*, *8*(1), 109–116. https://doi.org/10.3198/jpr2013.03.0013crmp

Epstein, E., & Bloom, A. J. (2005). *Mineral Nutrition of Plants: Principles and Perspectives*. Sinauer Associates, Incorporated.

Erisman, J. W. (2021). How ammonia feeds and pollutes the world. *Science*, *374*(6568), 685–686. https://doi.org/10.1126/science.abm3492

Erisman, J. W., Sutton, M. A., Galloway, J., Klimont, Z., & Winiwarter, W. (2008). How a century of ammonia synthesis changed the world. *Nature Geoscience*, *1*(10), 636–639. https://doi.org/10.1038/ngeo325

Esteban, R., Ariz, I., Cruz, C., & Moran, J. F. (2016). Review: Mechanisms of ammonium toxicity and the quest for tolerance. *Plant Science*, *248*, 92–101. https://doi.org/10.1016/j.plantsci.2016.04.008

Fageria, N. K. (2007). Yield Physiology of Rice. *Journal of Plant Nutrition*, *30*(6), 843–879. https://doi.org/10.1080/15226510701374831

Fageria, N. K., & Baligar, V. C. (2003). Methodology for Evaluation of Lowland Rice Genotypes for Nitrogen Use Efficiency. *Journal of Plant Nutrition*, *26*(6), 1315–1333. https://doi.org/10.1081/PLN-120020373

Fageria, N. K., Moreira, A., & Coelho, A. M. (2011). YIELD AND YIELD COMPONENTS OF UPLAND RICE AS INFLUENCED BY NITROGEN SOURCES. *Journal of Plant Nutrition*, *34*(3), 361–370. https://doi.org/10.1080/01904167.2011.536878

FAO. (2022, December 13). *FAOSTAT: Food Balances (2010-)* [Database]. FAOSTAT. https://www.fao.org/faostat/en/#data/FBS

FAOSTAT. (2022). *Food Supply: Crops Primary Equivalent.*

Feng, Z., Rütting, T., Pleijel, H., Wallin, G., Reich, P. B., Kammann, C. I., Newton, P. C. D., Kobayashi, K., Luo, Y., & Uddling, J. (2015). Constraints to nitrogen acquisition of terrestrial plants under elevated CO2. *Global Change Biology*, *21*(8), 3152–3168. https://doi.org/10.1111/gcb.12938

Fernando, N., Hirotsu, N., Panozzo, J., Tausz, M., Norton, R. M., & Seneweera, S. (2017). Lower grain nitrogen content of wheat at elevated CO2 can be improved through post-anthesis NH4+ supplement. *Journal of Cereal Science*, *74*, 79–85. https://doi.org/10.1016/j.jcs.2017.01.009

Fischer, R. A., Moreno Ramos, O. H., Ortiz Monasterio, I., & Sayre, K. D. (2019). Yield response to plant density, row spacing and raised beds in low latitude spring wheat with ample soil resources: An update. *Field Crops Research*, *232*, 95–105. https://doi.org/10.1016/j.fcr.2018.12.011

Fox, J., & Weisberg, S. (2019). *An R companion to applied regression* (Third edition). SAGE.

Fuertes-Mendizábal, T., González-Torralba, J., Arregui, L. M., González-Murua, C., González-Moro, M. B., & Estavillo, J. M. (2013). Ammonium as sole N source improves grain quality in wheat. *Journal of the Science of Food and Agriculture*, *93*(9), 2162–2171. https://doi.org/10.1002/jsfa.6022

Gaju, O., Allard, V., Martre, P., Le Gouis, J., Moreau, D., Bogard, M., Hubbart, S., & Foulkes, M. J. (2014). Nitrogen partitioning and remobilization in relation to leaf senescence, grain yield and grain nitrogen concentration in wheat cultivars. *Field Crops Research*, *155*, 213–223. https://doi.org/10.1016/j.fcr.2013.09.003

Galloway, J. N., Bleeker, A., & Erisman, J. W. (2021). The Human Creation and Use of Reactive Nitrogen: A Global and Regional Perspective. *Annual Review of Environment and Resources*, *46*(1), 255–288. https://doi.org/10.1146/annurev-environ-012420-045120

Gao, Y., & Cabrera Serrenho, A. (2023). Greenhouse gas emissions from nitrogen fertilizers could be reduced by up to one-fifth of current levels by 2050 with combined interventions. *Nature Food*, *4*(2), 170–178. https://doi.org/10.1038/s43016-023-00698-w

Gao, Z., Wang, Y., Chen, G., Zhang, A., Yang, S., Shang, L., Wang, D., Ruan, B., Liu, C., Jiang, H., Dong, G., Zhu, L., Hu, J., Zhang, G., Zeng, D., Guo, L., Xu, G., Teng, S., Harberd, N. P., & Qian, Q. (2019). The indica nitrate reductase gene OsNR2 allele enhances rice yield potential and nitrogen use efficiency. *Nature Communications*, *10*(1), 5207. https://doi.org/10.1038/s41467-019-13110-8

George, T., Ladha, J. K., Buresh, R. J., & Garrity, D. P. (1993). Nitrate Dynamics during the Aerobic Soil Phase in Lowland Rice-Based Cropping Systems. *Soil Science Society of America Journal*, *57*(6), 1526–1532. https://doi.org/10.2136/sssaj1993.03615995005700060022x

Ghasemi, A., & Zahediasl, S. (2012). Normality Tests for Statistical Analysis: A Guide for Non-Statisticians. *International Journal of Endocrinology and Metabolism*, *10*(2), 486–489. https://doi.org/10.5812/ijem.3505

Ghosh, P., & Kashyap, A. K. (2003). Effect of rice cultivars on rate of N-mineralization, nitrification and nitrifier population size in an irrigated rice ecosystem. *Applied Soil Ecology*, *24*(1), 27–41. https://doi.org/10.1016/S0929-1393(03)00068-4

Gifford, R. M., Barrett, D. J., & Lutze, J. L. (2000). The effects of elevated [CO2] on the C:N and C:P mass ratios of plant tissues. *Plant and Soil*, *224*(1), 1–14. https://doi.org/10.1023/A:1004790612630

Gigon, A., & Rorison, I. H. (1972). The Response of Some Ecologically Distinct Plant Species to Nitrate- and to Ammonium-Nitrogen. *The Journal of Ecology*, *60*(1), 93. https://doi.org/10.2307/2258043

Gonzalez-Dugo, V., Durand, J.-L., & Gastal, F. (2010). Water deficit and nitrogen nutrition of crops. A review. *Agronomy for Sustainable Development*, *30*(3), 529–544. https://doi.org/10.1051/agro/2009059

Gooding, M. J., Addisu, M., Uppal, R. K., Snape, J. W., & Jones, H. E. (2012). Effect of wheat dwarfing genes on nitrogen-use efficiency. *The Journal of Agricultural Science*, *150*(1), 3–22. https://doi.org/10.1017/S0021859611000414

Hawkesford, M. J. (2014). Reducing the reliance on nitrogen fertilizer for wheat production. *Journal of Cereal Science*, *59*(3), 276–283. https://doi.org/10.1016/j.jcs.2013.12.001

He, X., Chi, Q., Meng, L., Zhao, C., He, M., Dan, X., Huang, X., Zhao, J., Cai, Z., Zhang, J., & Müller, C. (2022). Plants with nitrate preference can regulate nitrification to meet their nitrate demand. *Soil Biology and Biochemistry*, *165*, 108516. https://doi.org/10.1016/j.soilbio.2021.108516

Hothorn, T., Bretz, F., & Westfall, P. (2008). *Simultaneous Inference in General Parametric Models*. *50*(3), 346–363.

Houlton, B. Z., Sigman, D. M., Schuur, E. A. G., & Hedin, L. O. (2007). A climate-driven switch in plant nitrogen acquisition within tropical forest communities. *Proceedings of the National Academy of Sciences*, *104*(21), 8902–8906. https://doi.org/10.1073/pnas.0609935104

Hu, B., Wang, W., Ou, S., Tang, J., Li, H., Che, R., Zhang, Z., Chai, X., Wang, H., Wang, Y., Liang, C., Liu, L., Piao, Z., Deng, Q., Deng, K., Xu, C., Liang, Y., Zhang, L., Li, L., & Chu, C. (2015). Variation in NRT1.1B contributes to nitrate-use divergence between rice subspecies. *Nature Genetics*, *47*(7), 834–838. https://doi.org/10.1038/ng.3337

Huang, M., Liu, X., Zhou, Y., Summers, R. M., & Zhang, Z. (2019). BLINK: A package for the next level of genome-wide association studies with both individuals and markers in the millions. *GigaScience*, *8*(2). https://doi.org/10.1093/gigascience/giy154

Huang, X., & Han, B. (2014). Natural Variations and Genome-Wide Association Studies in Crop Plants. *Annual Review of Plant Biology*, *65*(1), 531–551. https://doi.org/10.1146/annurev-arplant-050213-035715

IPCC. (2021). *Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* (V. Masson-Delmotte, P. Zhai, A. Pirani, S. L. Connors, C. Péan, S. Berger, N. Caud, Y. Chen, L. Goldfarb, M. I. Gomis, M. Huang, K. Leitzell, E. Lonnoy, J. B. R. Matthews, T. K. Maycock, T. Waterfield, Ö. Yelekçi, R. Yu, & B. Zhou, Eds.). Cambridge University Press. https://doi.org/10.1017/9781009157896

Jackson, L. E., Schimel, J. P., & Firestone, M. K. (1989). Short-term partitioning of ammonium and nitrate between plants and microbes in an annual grassland. *Soil Biology and Biochemistry*, *21*(3), 409–415. https://doi.org/10.1016/0038-0717(89)90152-1

Jackson, R. B., & Caldwell, M. M. (1993). The Scale of Nutrient Heterogeneity Around Individual Plants and Its Quantification with Geostatistics. *Ecology*, *74*(2), 612–614. https://doi.org/10.2307/1939320

Jia, L., Xie, Y., Wang, Z., Luo, L., Zhang, C., Pélissier, P., Parizot, B., Qi, W., Zhang, J., Hu, Z., Motte, H., Luo, L., Xu, G., Beeckman, T., & Xuan, W. (2020). Rice plants respond to ammonium stress by adopting a helical root growth pattern. *The Plant Journal*, *104*(4), 1023–1037. https://doi.org/10.1111/tpj.14978

Kasemsap, P., & Bloom, A. J. (2022). Breeding for Higher Yields of Wheat and Rice through Modifying Nitrogen Metabolism. *Plants*, *12*(1), 85. https://doi.org/10.3390/plants12010085

Kasemsap, P., & Bloom, A. J. (2023). *Genetic adaptation to ammonium sustains wheat grain quality and alleviates acclimation to CO $_2$ enrichment* [Preprint]. Plant Biology. https://doi.org/10.1101/2023.11.21.568194

Kasemsap, P., Cohen, I., & Bloom, A. J. (2024). *Genome-wide Association Study of Rice Vegetative Biomass under Different Inorganic Nitrogen Forms—Ammonium or Nitrate*. https://doi.org/10.1101/2024.08.12.607622

Katz, E., Knapp, A., Lensink, M., Keller, C. K., Stefani, J., Li, J.-J., Shane, E., Tuermer-Lee, K., Bloom, A. J., & Kliebenstein, D. J. (2022). Genetic variation underlying differential ammonium and nitrate responses in *Arabidopsis thaliana*. *The Plant Cell*, *34*(12), 4696–4713. https://doi.org/10.1093/plcell/koac279

Kawahara, Y., de la Bastide, M., Hamilton, J. P., Kanamori, H., McCombie, W. R., Ouyang, S., Schwartz, D. C., Tanaka, T., Wu, J., Zhou, S., Childs, K. L., Davidson, R. M., Lin, H., Quesada-Ocampo, L., Vaillancourt, B., Sakai, H., Lee, S. S., Kim, J., Numa, H., … Matsumoto, T. (2013). Improvement of the Oryza sativa Nipponbare reference genome using next generation sequence and optical map data. *Rice*, *6*(1), 4. https://doi.org/10.1186/1939-8433-6-4

Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M., & Haussler, and D. (2002). The Human Genome Browser at UCSC. *Genome Research*, *12*(6), 996–1006. https://doi.org/10.1101/gr.229102

Kirk, G. J. D., & Kronzucker, H. J. (2005). The Potential for Nitrification and Nitrate Uptake in the Rhizosphere of Wetland Plants: A Modelling Study. *Annals of Botany*, *96*(4), 639–646. https://doi.org/10.1093/aob/mci216

Kong, L., Xie, Y., Hu, L., Feng, B., & Li, S. (2016). Remobilization of vegetative nitrogen to developing grain in wheat (Triticum aestivum L.). *Field Crops Research*, *196*, 134–144. https://doi.org/10.1016/j.fcr.2016.06.015

Korte, A., & Farlow, A. (2013). The advantages and limitations of trait analysis with GWAS: A review. *Plant Methods*, *9*(1), 29. https://doi.org/10.1186/1746-4811-9-29

Kronzucker, H. J., Glass, A. D. M., Siddiqi, M. Y., & Kirk, G. J. D. (2000). Comparative kinetic analysis of ammonium and nitrate acquisition by tropical lowland rice: Implications for rice cultivation and yield potential: Nitrate acquisition by rice. *New Phytologist*, *145*(3), 471–476. https://doi.org/10.1046/j.1469-8137.2000.00606.x

Kumar, A., Verulkar, S., Dixit, S., Chauhan, B., Bernier, J., Venuprasad, R., Zhao, D., & Shrivastava, M. N. (2009). Yield and yield-attributing traits of rice (Oryza sativa L.) under lowland drought and suitability of early vigor as a selection criterion. *Field Crops Research*, *114*(1), 99–107. https://doi.org/10.1016/j.fcr.2009.07.010

Ladha, J. K., Tirol-Padre, A., Reddy, C. K., Cassman, K. G., Verma, S., Powlson, D. S., van Kessel, C., de B. Richter, D., Chakraborty, D., & Pathak, H. (2016). Global nitrogen budgets in cereals: A 50-year assessment for maize, rice and wheat production systems. *Scientific Reports*, *6*(1), 19355. https://doi.org/10.1038/srep19355

Lammerts Van Bueren, E. T., & Struik, P. C. (2017). Diverse concepts of breeding for nitrogen use efficiency. A review. *Agronomy for Sustainable Development*, *37*(5), 50. https://doi.org/10.1007/s13593-017-0457-3

Langsrud, Ø. (2003). ANOVA for unbalanced data: Use Type II instead of Type III sums of squares. *Statistics and Computing*, *13*(2), 163–167. https://doi.org/10.1023/A:1023260610025

Lenth, R. V. (2023). *emmeans: Estimated Marginal Means, aka Least-Squares Means* (Version R package version 1.8.6) [Computer software]. https://CRAN.R-project.org/package=emmeans

Levene, H. (1960). Robust tests for equality of variances. In e a Ingram Olkin (Ed.), *Contributions to Probability and Statistic: Essays in honor of Harold Hotelling*.

Li, B., Zhang, Y., Mohammadi, S. A., Huai, D., Zhou, Y., & Kliebenstein, D. J. (2016). An Integrative Genetic Study of Rice Metabolism, Growth and Stochastic Variation Reveals Potential C/N Partitioning Loci. *Scientific Reports*, *6*(1), 30143. https://doi.org/10.1038/srep30143

Li, S., Tian, Y., Wu, K., Ye, Y., Yu, J., Zhang, J., Liu, Q., Hu, M., Li, H., Tong, Y., Harberd, N. P., & Fu, X. (2018). Modulating plant growth–metabolism coordination for sustainable agriculture. *Nature*, *560*(7720), 595–600. https://doi.org/10.1038/s41586-018-0415-5

Li, Y. L., Fan, X. R., & Shen, Q. R. (2007). The relationship between rhizosphere nitrification and nitrogen-use efficiency in rice plants. *Plant, Cell & Environment*, *0*(0), 071018035446001-??? https://doi.org/10.1111/j.1365-3040.2007.01737.x

Li, Y. L., Zhang, Y. L., Hu, J., & Shen, Q. R. (2007). Contribution of nitrification happened in rhizospheric soil growing with different rice cultivars to N nutrition. *Biology and Fertility of Soils*, *43*(4), 417–425. https://doi.org/10.1007/s00374-006-0119-0

Lipka, A. E., Tian, F., Wang, Q., Peiffer, J., Li, M., Bradbury, P. J., Gore, M. A., Buckler, E. S., & Zhang, Z. (2012). GAPIT: Genome association and prediction integrated tool. *Bioinformatics*, *28*(18), 2397–2399. https://doi.org/10.1093/bioinformatics/bts444

Liu, S., Chi, Q., Cheng, Y., Zhu, B., Li, W., Zhang, X., Huang, Y., Müller, C., Cai, Z., & Zhang, J. (2019). Importance of matching soil N transformations, crop N form preference, and climate to enhance crop yield and reducing N loss. *Science of The Total Environment*, *657*, 1265–1273. https://doi.org/10.1016/j.scitotenv.2018.12.100

Liu, X., Huang, M., Fan, B., Buckler, E. S., & Zhang, Z. (2016). Iterative Usage of Fixed and Random Effect Models for Powerful and Efficient Genome-Wide Association Studies. *PLOS Genetics*, *12*(2), e1005767. https://doi.org/10.1371/journal.pgen.1005767

Liu, Y., Wang, H., Jiang, Z., Wang, W., Xu, R., Wang, Q., Zhang, Z., Li, A., Liang, Y., Ou, S., Liu, X., Cao, S., Tong, H., Wang, Y., Zhou, F., Liao, H., Hu, B., & Chu, C. (2021). Genomic basis of geographical adaptation to soil nitrogen in rice. *Nature*. https://doi.org/10.1038/s41586-020-03091-w

Loladze, I. (2014). Hidden shift of the ionome of plants exposed to elevated CO2 depletes minerals at the base of human nutrition. *Elife*, *3*, e02245.

Lu, K., Wu, B., Wang, J., Zhu, W., Nie, H., Qian, J., Huang, W., & Fang, Z. (2018). Blocking amino acid transporter *Os AAP 3* improves grain yield by promoting outgrowth buds and increasing tiller number in rice. *Plant Biotechnology Journal*, *16*(10), 1710–1722. https://doi.org/10.1111/pbi.12907

Lüdecke, D., Patil, I., Ben-Shachar, M., Wiernik, B., Waggoner, P., & Makowski, D. (2021). see: An R Package for Visualizing Statistical Models. *Journal of Open Source Software*, *6*(64), 3393. https://doi.org/10.21105/joss.03393

Lumley, T., Diehr, P., Emerson, S., & Chen, L. (2002). The Importance of the Normality Assumption in Large Public Health Data Sets. *Annual Review of Public Health*, *23*(1), 151–169. https://doi.org/10.1146/annurev.publhealth.23.100901.140546

Luo, L., Zhu, M., Jia, L., Xie, Y., Wang, Z., & Xuan, W. (2022). Ammonium transporters cooperatively regulate rice crown root formation responding to ammonium nitrogen. *Journal of Experimental Botany*, erac059. https://doi.org/10.1093/jxb/erac059

Maathuis, F. J. (2009). Physiological functions of mineral macronutrients. *Current Opinion in Plant Biology*, *12*(3), 250–258.

Maccaferri, M., Zhang, J., Bulli, P., Abate, Z., Chao, S., Cantu, D., Bossolini, E., Chen, X., Pumphrey, M., & Dubcovsky, J. (2015). A Genome-Wide Association Study of Resistance to Stripe Rust (Puccinia striiformis f. Sp. Tritici) in a Worldwide Collection of Hexaploid Spring Wheat (Triticum aestivum L.). *G3: Genes|Genomes|Genetics*, *5*(3), 449–465. PMC. https://doi.org/10.1534/g3.114.014563

Magwa, R. A., Zhao, H., & Xing, Y. (2016). Genome-wide association mapping revealed a diverse genetic basis of seed dormancy across subpopulations in rice (Oryza sativa L.). *BMC Genetics*, *17*(1), 28. https://doi.org/10.1186/s12863-016-0340-2

Matassa, S., Boeckx, P., Boere, J., Erisman, J. W., Guo, M., Manzo, R., Meerburg, F., Papirio, S., Pikaar, I., Rabaey, K., Rousseau, D., Schnoor, J., Smith, P., Smolders, E., Wuertz, S., & Verstraete, W. (2023). How can we possibly resolve the planet’s nitrogen dilemma? *Microbial Biotechnology*, *16*(1), 15–27. https://doi.org/10.1111/1751-7915.14159

Mauro, A. A., & Ghalambor, C. K. (2020). Trade-offs, Pleiotropy, and Shared Molecular Pathways: A Unified View of Constraints on Adaptation. *Integrative and Comparative Biology*, *60*(2), 332–347. https://doi.org/10.1093/icb/icaa056

McCarthy, M. I., & Hirschhorn, J. N. (2008). Genome-wide association studies: Potential next steps on a genetic journey. *Human Molecular Genetics*, *17*(R2), R156–R165. https://doi.org/10.1093/hmg/ddn289

McCaw, Z. R., Lane, J. M., Saxena, R., Redline, S., & Lin, X. (2020). Operating characteristics of the rank-based inverse normal transformation for quantitative trait analysis in genome-wide association studies. *Biometrics*, *76*(4), 1262–1272. https://doi.org/10.1111/biom.13214

McCouch, S. R., Wright, M. H., Tung, C.-W., Maron, L. G., McNally, K. L., Fitzgerald, M., Singh, N., DeClerck, G., Agosto-Perez, F., Korniliev, P., Greenberg, A. J., Naredo, Ma. E. B., Mercado, S. M. Q., Harrington, S. E., Shi, Y., Branchini, D. A., Kuser-Falcão, P. R., Leung, H., Ebana, K., … Mezey, J. (2016). Open access resources for genome-wide association mapping in rice. *Nature Communications*, *7*(1), 10532. https://doi.org/10.1038/ncomms10532

McMullen, M. D., Kresovich, S., Villeda, H. S., Bradbury, P., Li, H., Sun, Q., Flint-Garcia, S., Thornsberry, J., Acharya, C., Bottoms, C., Brown, P., Browne, C., Eller, M., Guill, K., Harjes, C., Kroon, D., Lepak, N., Mitchell, S. E., Peterson, B., … Ware, D. (2009). Genetic properties of the maize nested association mapping population. *Science*, *325*. https://doi.org/10.1126/science.1174320

Melillo, E. D. (2012). The First Green Revolution: Debt Peonage and the Making of the Nitrogen Fertilizer Trade, 1840–1930. *The American Historical Review*, *117*(4), 1028–1060. https://doi.org/10.1093/ahr/117.4.1028

Miller, A. J., & Cramer, M. D. (2005). Root nitrogen acquisition and assimilation. In *Root Physiology: From Gene to Function* (pp. 1–36). Springer, Dordrecht. https://doi.org/10.1007/1-4020-4099-7_1

Molero, G., Joynson, R., Pinera-Chavez, F. J., Gardiner, L., Rivera-Amado, C., Hall, A., & Reynolds, M. P. (2019). Elucidating the genetic basis of biomass accumulation and radiation use efficiency in spring wheat and its role in yield potential. *Plant Biotechnology Journal*, *17*(7), 1276–1288. https://doi.org/10.1111/pbi.13052

Myers, S. S., Zanobetti, A., Kloog, I., Huybers, P., Leakey, A. D. B., Bloom, A. J., Carlisle, E., Dietterich, L. H., Fitzgerald, G., Hasegawa, T., Holbrook, N. M., Nelson, R. L., Ottman, M. J., Raboy, V., Sakai, H., Sartor, K. A., Schwartz, J., Seneweera, S., Tausz, M., & Usui, Y. (2014). Increasing CO2 threatens human nutrition. *Nature*, *510*(7503), 139–142. https://doi.org/10.1038/nature13179

Namuco, O. S., Cairns, J. E., & Johnson, D. E. (2009). Investigating early vigour in upland rice (Oryza sativa L.): Part I. Seedling growth and grain yield in competition with weeds. *Field Crops Research*, *113*(3), 197–206. https://doi.org/10.1016/j.fcr.2009.05.008

Näsholm, T., Kielland, K., & Ganeteg, U. (2009). Uptake of organic nitrogen by plants: Tansley review. *New Phytologist*, *182*(1), 31–48. https://doi.org/10.1111/j.1469-8137.2008.02751.x

Nishina, K., Ito, A., Hanasaki, N., & Hayashi, S. (2017). Reconstruction of spatially detailed global map of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>−</sup> application in synthetic nitrogen fertilizer. *Earth System Science Data*, *9*(1), 149–162. https://doi.org/10.5194/essd-9-149-2017

Norton, J. M. (2015). Nitrification in Agricultural Soils. In J. S. Schepers & W. R. Raun (Eds.), *Agronomy Monographs* (pp. 173–199). American Society of Agronomy, Crop Science Society of America, Soil Science Society of America. https://doi.org/10.2134/agronmonogr49.c6

Norton, J., & Ouyang, Y. (2019). Controls and Adaptive Management of Nitrification in Agricultural Soils. *Frontiers in Microbiology*, *10*. https://www.frontiersin.org/article/10.3389/fmicb.2019.01931

O'Sullivan, C. A., Fillery, I. R. P., Roper, M. M., & Richards, R. A. (2016). Identification of several wheat landraces with biological nitrification inhibition capacity. *Plant and Soil*, *404*(1–2), 61–74. https://doi.org/10.1007/s11104-016-2822-4

O'Sullivan, C. A., Whisson, K., Treble, K., Roper, M. M., Micin, S. F., & Ward, P. R. (2017). Biological nitrification inhibition by weeds: Wild radish, brome grass, wild oats and annual ryegrass decrease nitrification rates in their rhizospheres. *Crop and Pasture Science*, *68*(8), 798. https://doi.org/10.1071/CP17243

O'Sullivan, C., Palta, J., Farrell, M., & Treble, K. (2016). Genotypic variability in wheat for preference of NH4 + , NO3—Or NH4 + NO3. *Proceedings of the 2016 International Nitrogen Initiative Conference*. 7th International Nitrogen Initiative Conference, Melbourne, 4th to 8th Dec 2016. http://www.ini2016.com/pdf-papers/INI2016_Osullivan_Cathryn.pdf

Pan, W. L., Madsen, I. J., Bolton, R. P., Graves, L., & Sistrunk, T. (2016). Ammonia/Ammonium Toxicity Root Symptoms Induced by Inorganic and Organic Fertilizers and Placement. *Agronomy Journal*, *108*(6), 2485–2492. https://doi.org/10.2134/agronj2016.02.0122

Paterson, A. H., Bowers, J. E., Bruggmann, R., Dubchak, I., Grimwood, J., Gundlach, H., Haberer, G., Hellsten, U., Mitros, T., Poliakov, A., Schmutz, J., Spannagl, M., Tang, H., Wang, X., Wicker, T., Bharti, A. K., Chapman, J., Feltus, F. A., Gowik, U., … Rokhsar, D. S. (2009). The Sorghum bicolor genome and the diversification of grasses. *Nature*, *457*(7229), 551–556. https://doi.org/10.1038/nature07723

Pirinen, M. (2023, March 2). *GWAS 1: What is a GWAS?* GWAS 1: What Is a GWAS? https://www.mv.helsinki.fi/home/mjxpirin/GWAS_course/material/GWAS1.html

Pleijel, H., Broberg, M. C., Högy, P., & Uddling, J. (2019). Nitrogen application is required to realize wheat yield stimulation by elevated CO $_2$ but will not remove the CO $_2$ -induced reduction in grain protein concentration. *Global Change Biology*, *25*(5), 1868–1876. https://doi.org/10.1111/gcb.14586

Price, A. L., Patterson, N. J., Plenge, R. M., Weinblatt, M. E., Shadick, N. A., & Reich, D. (2006). Principal components analysis corrects for stratification in genome-wide association studies. *Nature Genetics*, *38*(8), 904–909. https://doi.org/10.1038/ng1847

Qi, X., Wu, W., Shah, F., Peng, S., Huang, J., Cui, K., Liu, H., & Nie, L. (2012). Ammonia Volatilization from Urea-Application Influenced Germination and Early Seedling Growth of Dry Direct-Seeded Rice. *The Scientific World Journal*, *2012*, 1–7. https://doi.org/10.1100/2012/857472

R Core Team,. (2023). *R: A Language and Environment for Statistical Computing* [Computer software]. R Foundation for Statistical Computing. https://www.R-project.org/

Raun, W. R., & Johnson, G. V. (1999). Improving Nitrogen Use Efficiency for Cereal Production. *Agronomy Journal*, *91*(3), 357–363. https://doi.org/10.2134/agronj1999.00021962009100030001x

Rawson, H. (1970). Spikelet Number, Its Control and Relation to Yield Per Ear in Wheat. *Australian Journal of Biological Sciences*, *23*(1), 1. https://doi.org/10.1071/BI9700001

Ren, C., Zhang, X., Reis, S., Wang, S., Jin, J., Xu, J., & Gu, B. (2023). Climate change unequally affects nitrogen use and losses in global croplands. *Nature Food*. https://doi.org/10.1038/s43016-023-00730-z

Rivera-Amado, C., Trujillo-Negrellos, E., Molero, G., Reynolds, M. P., Sylvester-Bradley, R., & Foulkes, M. J. (2019). Optimizing dry-matter partitioning for increased spike growth, grain number and harvest index in spring wheat. *Field Crops Research*, *240*, 154–167. https://doi.org/10.1016/j.fcr.2019.04.016

Royston, J. P. (1982). An Extension of Shapiro and Wilk's W Test for Normality to Large Samples. *Applied Statistics*, *31*(2), 115. https://doi.org/10.2307/2347973

RStudio Team. (2020). *RStudio: Integrated Development for R* [Computer software]. http://www.rstudio.com/.

Rubio-Asensio, J. S., & Bloom, A. J. (2016). Inorganic nitrogen form: A major player in wheat and Arabidopsis responses to elevated CO2. *Journal of Experimental Botany*, erw465.

Saini, D. K., Chopra, Y., Singh, J., Sandhu, K. S., Kumar, A., Bazzer, S., & Srivastava, P. (2022). Comprehensive evaluation of mapping complex traits in wheat using genome-wide association studies. *Molecular Breeding*, *42*(1), 1. https://doi.org/10.1007/s11032-021-01272-7

Salgó, A., & Gergely, S. (2012). Analysis of wheat grain development using NIR spectroscopy. *Journal of Cereal Science*, *56*(1), 31–38. https://doi.org/10.1016/j.jcs.2012.04.011

Sasakawa, H., & Yamamoto, Y. (1978). Comparison of the Uptake of Nitrate and Ammonium by Rice Seedlings: Influences of Light, Temperature, Oxygen Concentration, Exogenous Sucrose, and Metabolic Inhibitors. *Plant Physiology*, *62*(4), 665–669. https://doi.org/10.1104/pp.62.4.665

Segura, V., Vilhjálmsson, B. J., Platt, A., Korte, A., Seren, Ü., Long, Q., & Nordborg, M. (2012). An efficient multi-locus mixed-model approach for genome-wide association studies in structured populations. *Nature Genetics*, *44*(7), 825–830. https://doi.org/10.1038/ng.2314

Seibert, R., Donath, T. W., Moser, G., Laser, H., Grünhage, L., Schmid, T., & Müller, C. (2021). Effects of long-term CO2 enrichment on forage quality of extensively managed temperate grassland. *Agriculture, Ecosystems & Environment*, *312*, 107347. https://doi.org/10.1016/j.agee.2021.107347

Shakiba, E., Edwards, J. D., Jodari, F., Duke, S. E., Baldo, A. M., Korniliev, P., McCouch, S. R., & Eizenga, G. C. (2017). Genetic architecture of cold tolerance in rice (Oryza sativa) determined through high resolution genome-wide analysis. *PLOS ONE*, *12*(3), e0172133. https://doi.org/10.1371/journal.pone.0172133

Sicher, R. C., & Bunce, J. A. (1997). Relationship of photosynthetic acclimation to changes of Rubisco activity in field-grown winter wheat and barley during growth in elevated carbon dioxide. *Photosynthesis Research*, *52*(1), 27–38. https://doi.org/10.1023/A:1005874932233

Sierra-Gonzalez, A., Molero, G., Rivera-Amado, C., Babar, M. A., Reynolds, M. P., & Foulkes, M. J. (2021). Exploring genetic diversity for grain partitioning traits to enhance yield in a high biomass spring wheat panel. *Field Crops Research*, *260*, 107979. https://doi.org/10.1016/j.fcr.2020.107979

Slafer, G. A., Elia, M., Savin, R., García, G. A., Terrile, I. I., Ferrante, A., Miralles, D. J., & González, F. G. (2015). Fruiting efficiency: An alternative trait to further rise wheat yield. *Food and Energy Security*, *4*(2), 92–109. https://doi.org/10.1002/fes3.59

Smart, D. R., Ritchie, K., Bloom, A. J., & Bugbee, B. B. (1998). Nitrogen balance for wheat canopies (*Triticum aestivum* cv. Veery 10) grown under elevated and ambient CO $_2$ concentrations. *Plant, Cell & Environment*, *21*(8), 753–763. https://doi.org/10.1046/j.1365-3040.1998.00315.x

Subbarao, G. V., Kishii, M., Bozal-Leorri, A., Ortiz-Monasterio, I., Gao, X., Ibba, M. I., Karwat, H., Gonzalez-Moro, M. B., Gonzalez-Murua, C., Yoshihashi, T., Tobita, S., Kommerell, V., Braun, H.-J., & Iwanaga, M. (2021). Enlisting wild grass genes to combat nitrification in wheat farming: A nature-based solution. *Proceedings of the National Academy of Sciences*, *118*(35), e2106595118. https://doi.org/10.1073/pnas.2106595118

Subbarao, G. V., & Searchinger, T. D. (2021). Opinion: A “more ammonium solution” to mitigate nitrogen pollution and boost crop yields. *Proceedings of the National Academy of Sciences*, *118*(22), e2107576118. https://doi.org/10.1073/pnas.2107576118

Sun, J., Guo, Y., Zhang, G., Gao, M., Zhang, G., Kong, F., Zhao, Y., & Li, S. (2013). QTL mapping for seedling traits under different nitrogen forms in wheat. *Euphytica*, *191*(3), 317–331. https://doi.org/10.1007/s10681-012-0834-6

Sun, L., Lu, Y., Yu, F., Kronzucker, H. J., & Shi, W. (2016). Biological nitrification inhibition by rice root exudates and its relationship with nitrogen-use efficiency. *New Phytologist*, *212*(3), 646–656. https://doi.org/10.1111/nph.14057

Tabuchi, M., Abiko, T., & Yamaya, T. (2007). Assimilation of ammonium ions and reutilization of nitrogen in rice (Oryza sativa L.). *Journal of Experimental Botany*, *58*(9), 2319–2327. https://doi.org/10.1093/jxb/erm016

Taub, D. R., Miller, B., & Allen, H. (2008). Effects of elevated CO $_2$ on the protein concentration of food crops: A meta-analysis: ELEVATED CO $_2$ AND CROP PROTEIN CONCENTRATIONS. *Global Change Biology*, *14*(3), 565–575. https://doi.org/10.1111/j.1365-2486.2007.01511.x

Terés, J., Busoms, S., Perez Martín, L., Luís-Villarroya, A., Flis, P., Álvarez-Fernández, A., Tolrà, R., Salt, D. E., & Poschenrieder, C. (2019). Soil carbonate drives local adaptation in *ARABIDOPSIS THALIANA* . *Plant, Cell & Environment*, *42*(8), 2384–2398. https://doi.org/10.1111/pce.13567

Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z., Xu, W., & Su, Z. (2017). agriGO v2.0: A GO analysis toolkit for the agricultural community, 2017 update. *Nucleic Acids Research*, *45*(W1), W122–W129. https://doi.org/10.1093/nar/gkx382

Tilman, D., Balzer, C., Hill, J., & Befort, B. L. (2011). Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences*, *108*(50), 20260–20264. https://doi.org/10.1073/pnas.1116437108

Uddling, J., Broberg, M. C., Feng, Z., & Pleijel, H. (2018). Crop quality under rising atmospheric CO 2. *Current Opinion in Plant Biology*.

Ueda, Y., Ohtsuki, N., Kadota, K., Tezuka, A., Nagano, A. J., Kadowaki, T., Kim, Y., Miyao, M., & Yanagisawa, S. (2020). Gene regulatory network and its constituent transcription factors that control nitrogen-deficiency responses in rice. *New Phytologist*, *227*(5), 1434–1452. https://doi.org/10.1111/nph.16627

Waines, J. G., & Ehdaie, B. (2007). Domestication and Crop Physiology: Roots of Green-Revolution Wheat. *Annals of Botany*, *100*(5), 991–998. https://doi.org/10.1093/aob/mcm180

Walker, A. P., De Kauwe, M. G., Bastos, A., Belmecheri, S., Georgiou, K., Keeling, R. F., McMahon, S. M., Medlyn, B. E., Moore, D. J. P., Norby, R. J., Zaehle, S., Anderson-Teixeira, K. J., Battipaglia, G., Brienen, R. J. W., Cabugao, K. G., Cailleret, M., Campbell, E., Canadell, J. G., Ciais, P., … Zuidema, P. A. (2021). Integrating the evidence for a terrestrial carbon sink caused by increasing atmospheric CO2. *New Phytologist*, *229*(5), 2413–2445. https://doi.org/10.1111/nph.16866

Wang, F., Gao, J., Yong, J. W. H., Wang, Q., Ma, J., & He, X. (2020). Higher Atmospheric CO2 Levels Favor C3 Plants Over C4 Plants in Utilizing Ammonium as a Nitrogen Source. *Frontiers in Plant Science*, *11*, 537443. https://doi.org/10.3389/fpls.2020.537443

Wang, J., Wu, B., Lu, K., Wei, Q., Qian, J., Chen, Y., & Fang, Z. (2019). The Amino Acid Permease 5 (OsAAP5) Regulates Tiller Number and Grain Yield in Rice. *Plant Physiology*, *180*(2), 1031–1045. https://doi.org/10.1104/pp.19.00034

Wang, J., & Zhang, Z. (2020). *GAPIT Version 3: Boosting Power and Accuracy for Genomic Association and Prediction* [Preprint]. Bioinformatics. https://doi.org/10.1101/2020.11.29.403170

Wang, Q., Nian, J., Xie, X., Yu, H., Zhang, J., Bai, J., Dong, G., Hu, J., Bai, B., Chen, L., Xie, Q., Feng, J., Yang, X., Peng, J., Chen, F., Qian, Q., Li, J., & Zuo, J. (2018). Genetic variations in ARE1 mediate grain yield by modulating nitrogen utilization in rice. *Nature Communications*, *9*(1), 735. https://doi.org/10.1038/s41467-017-02781-w

Wang, W., Hu, B., Yuan, D., Liu, Y., Che, R., Hu, Y., Ou, S., Liu, Y., Zhang, Z., Wang, H., Li, H., Jiang, Z., Zhang, Z., Gao, X., Qiu, Y., Meng, X., Liu, Y., Bai, Y., Liang, Y., … Chu, C. (2018).

Expression of the Nitrate Transporter Gene *OsNRT1.1A/OsNPF6.3* Confers High Yield and Early Maturation in Rice. *The Plant Cell*, *30*(3), 638–651. https://doi.org/10.1105/tpc.17.00809

Wang, X., Mao, W., Wang, Y., Lou, H., Guan, P., Chen, Y., Peng, H., & Wang, J. (2023). Breeding design in wheat by combining the QTL information in a GWAS panel with a general genetic map and computer simulation. *The Crop Journal*, S2214514123001368. https://doi.org/10.1016/j.cj.2023.10.001

Wei, T., & Simko, V. (2021). *R package "corrplot": Visualization of a Correlation* (Version Version 0.92) [Computer software]. https://github.com/taiyun/corrplot

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis* (2nd ed. 2016). Springer International Publishing : Imprint: Springer. https://doi.org/10.1007/978-3-319-24277-4

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T., Miller, E., Bache, S., Müller, K., Ooms, J., Robinson, D., Seidel, D., Spinu, V., … Yutani, H. (2019). Welcome to the Tidyverse. *Journal of Open Source Software*, *4*(43), 1686. https://doi.org/10.21105/joss.01686

Wujeska-Klause, A., Crous, K. Y., Ghannoum, O., & Ellsworth, D. S. (2019). Lower photorespiration in elevated CO $_2$ reduces leaf N concentrations in mature *Eucalyptus* trees in the field. *Global Change Biology*, *25*(4), 1282–1295. https://doi.org/10.1111/gcb.14555

Xiao, M., Zang, H., Liu, S., Ye, R., Zhu, Z., Su, Y., Wu, J., & Ge, T. (2019). Nitrogen fertilization alters the distribution and fates of photosynthesized carbon in rice–soil systems: A 13C-CO2 pulse labeling study. *Plant and Soil*, *445*(1–2), 101–112. https://doi.org/10.1007/s11104-019-04030-z

Xie, Q., Mayes, S., & Sparkes, D. L. (2016). Early anthesis and delayed but fast leaf senescence contribute to individual grain dry matter and water accumulation in wheat. *Field Crops Research*, *187*, 24–34. https://doi.org/10.1016/j.fcr.2015.12.009

Xu, F., Bao, J., He, Q., & Park, Y.-J. (2016). Genome-wide association study of eating and cooking qualities in different subpopulations of rice (Oryza sativa L.). *BMC Genomics*, *17*(1), 663. https://doi.org/10.1186/s12864-016-3000-z

Xu, G., Fan, X., & Miller, A. J. (2012). Plant Nitrogen Assimilation and Use Efficiency. *Annual Review of Plant Biology, 63*(1), 153–182. https://doi.org/10.1146/annurev-arplant-042811-105532

Yan, M., Fan, X., Feng, H., Miller, A. J., Shen, Q., & Xu, G. (2011). Rice OsNAR2.1 interacts with OsNRT2.1, OsNRT2.2 and OsNRT2.3a nitrate transporters to provide uptake over high and low concentration ranges: Rice two component nitrate transport. *Plant, Cell & Environment, 34*(8), 1360–1372. https://doi.org/10.1111/j.1365-3040.2011.02335.x

Yang, H.-C., Kan, C.-C., Hung, T.-H., Hsieh, P.-H., Wang, S.-Y., Hsieh, W.-Y., & Hsieh, M.-H. (2017). Identification of early ammonium nitrate-responsive genes in rice roots. *Scientific Reports, 7*(1), 16885. https://doi.org/10.1038/s41598-017-17173-9

Yang, Y., Zhang, J., & Cai, Z. (2016). Nitrification activities and N mineralization in paddy soils are insensitive to oxygen concentration. *Acta Agriculturae Scandinavica, Section B — Soil & Plant Science, 66*(3), 272–281. https://doi.org/10.1080/09064710.2015.1093653

Yin, L., Zhang, H., Tang, Z., Xu, J., Yin, D., Zhang, Z., Yuan, X., Zhu, M., Zhao, S., Li, X., & Liu, X. (2020). *rMVP: A Memory-efficient, Visualization-enhanced, and Parallel-accelerated tool for Genome-Wide Association Study* [Preprint]. Genetics. https://doi.org/10.1101/2020.08.20.258491

Yu, J., Holland, J. B., McMullen, M. D., & Buckler, E. S. (2008). Genetic Design and Statistical Power of Nested Association Mapping in Maize. *Genetics, 178*(1), 539–551. https://doi.org/10.1534/genetics.107.074245

Yu, J., Pressoir, G., Briggs, W. H., Vroh Bi, I., Yamasaki, M., Doebley, J. F., McMullen, M. D., Gaut, B. S., Nielsen, D. M., Holland, J. B., Kresovich, S., & Buckler, E. S. (2006). A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. *Nature Genetics, 38*(2), 203–208. https://doi.org/10.1038/ng1702

Zhang, J., Gizaw, S. A., Bossolini, E., Hegarty, J., Howell, T., Carter, A. H., Akhunov, E., & Dubcovsky, J. (2018). Identification and validation of QTL for grain yield and plant water status under contrasting water treatments in fall-sown spring wheats. *Theoretical and Applied Genetics, 131*(8), 1741–1759. https://doi.org/10.1007/s00122-018-3111-9

Zhang, J., Liu, Y.-X., Zhang, N., Hu, B., Jin, T., Xu, H., Qin, Y., Yan, P., Zhang, X., Guo, X., Hui, J., Cao, S., Wang, X., Wang, C., Wang, H., Qu, B., Fan, G., Yuan, L., Garrido-Oter, R., … Bai, Y. (2019). NRT1.1B is associated with root microbiota composition and nitrogen use in field-grown rice. *Nature Biotechnology*, *37*(6), 676–684. https://doi.org/10.1038/s41587-019-0104-4

Zhang, X., Lu, Y., Yang, T., Kronzucker, H. J., & Shi, W. (2019). Factors influencing the release of the biological nitrification inhibitor 1,9-decanediol from rice (Oryza sativa L.) roots. *Plant and Soil*, *436*(1–2), 253–265. https://doi.org/10.1007/s11104-019-03933-1

Zhang, X., Zou, T., Lassaletta, L., Mueller, N. D., Tubiello, F. N., Lisk, M. D., Lu, C., Conant, R. T., Dorich, C. D., Gerber, J., Tian, H., Bruulsema, T., Maaz, T. M., Nishina, K., Bodirsky, B. L., Popp, A., Bouwman, L., Beusen, A., Chang, J., … Davidson, E. A. (2021). Quantification of global and national nitrogen budgets for crop production. *Nature Food*, *2*(7), 529–540. https://doi.org/10.1038/s43016-021-00318-5

Zhang, Z., & Chu, C. (2020). Nitrogen-Use Divergence Between Indica and Japonica Rice: Variation at Nitrate Assimilation. *Molecular Plant*, *13*(1), 6–7. https://doi.org/10.1016/j.molp.2019.11.011

Zhang, Z., Ersoz, E., Lai, C.-Q., Todhunter, R. J., Tiwari, H. K., Gore, M. A., Bradbury, P. J., Yu, J., Arnett, D. K., Ordovas, J. M., & Buckler, E. S. (2010). Mixed linear model approach adapted for genome-wide association studies. *Nature Genetics*, *42*(4), 355–360. https://doi.org/10.1038/ng.546

Zhang, Z., Gao, S., & Chu, C. (2020). Improvement of nutrient use efficiency in rice: Current toolbox and future perspectives. *Theoretical and Applied Genetics*, *133*(5), 1365–1384. https://doi.org/10.1007/s00122-019-03527-6

Zhao, K., Tung, C.-W., Eizenga, G. C., Wright, M. H., Ali, M. L., Price, A. H., Norton, G. J., Islam, M. R., Reynolds, A., Mezey, J., McClung, A. M., Bustamante, C. D., & McCouch, S. R. (2011). Genome-wide association mapping reveals a rich genetic architecture of complex traits in Oryza sativa. *Nature Communications*, *2*(1), 467. https://doi.org/10.1038/ncomms1467

Zhong, Y., Hu, J., Xia, Q., Zhang, S., Li, X., Pan, X., Zhao, R., Wang, R., Yan, W., Shangguan, Z., Hu, F., Yang, C., & Wang, W. (2020). Soil microbial mechanisms promoting ultrahigh rice yield. *Soil Biology and Biochemistry*, *143*, 107741. https://doi.org/10.1016/j.soilbio.2020.107741