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Evolutionary Systems Biology Identifies Genetic Trade-offs in Rice Defense against Aboveground and Belowground Attackers

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Like other plants, wild and domesticated rice species (*Oryza nivara*, *O. rufipogon*, and *O. sativa*) evolve in environments with various biotic and abiotic stresses that fluctuate in intensity through space and time. Microbial pathogens and invertebrate herbivores such as plant-parasitic nematodes and caterpillars show geographical and temporal variation in activity patterns and may respond differently to certain plant-defensive mechanisms. As such, plant interactions with multiple community members may result in conflicting selection pressures on genetic polymorphisms. Here, through assays with different aboveground and belowground herbivores, the fall armyworm (*Spodoptera frugiperda*) and the southern root-knot nematode (*Meloidogyne incognita*), and comparison with rice responses to microbial pathogens, we identify potential genetic trade-offs at the *KSL8* and *MG1* loci on chromosome 11. *KSL8* encodes the first committed step toward the biosynthesis of either stemarane-type or stemodane-type diterpenoids through the japonica (*KSL8-jap*) or indica (*KSL8-ind*) allele. Knocking out *KSL8-jap* and *CPS4*, encoding an enzyme that acts upstream in diterpenoid synthesis, in japonica rice cultivars increased resistance to *S. frugiperda* and decreased resistance to *M. incognita*. Furthermore, *MG1* resides in a haplotype that provided resistance to *M. incognita*, while alternative haplotypes are involved in mediating resistance to the rice blast fungus *Magnaporthe oryzae* and other pests and pathogens. Finally, *KSL8* and *MG1* alleles are located within trans-species polymorphic haplotypes and may be evolving under long-term balancing selection. Our data are consistent with a hypothesis that polymorphisms at *KSL8* and *MG1* may be maintained through complex and diffuse community interactions.

Keywords: Balancing selection • Diterpenoids • Herbivore • *Oryza sativa* • Pathogen • Polymorphism

Introduction

Rice is consumed by approximately half of the global population and is an important staple crop for securing the global food supply (Wing et al. 2018). Yet, elite rice varieties are profoundly vulnerable to both pathogens and invertebrate herbivore pests. As it stands, rice is more sensitive to such attackers than other staple crops (Savary et al. 2019). Moreover, these negative agronomic impacts of pests and pathogens are likely to deteriorate with the increased frequency of weather extremes and anticipated decreases in water availability associated with climate change (Tonnang et al. 2022, Wang et al. 2022a, Dutta and Phani 2023). This concern has become even more critical with the global push for direct-seeded rice cultivation. Direct-seeded rice cultivation reduces the amount of water, labor and greenhouse gas emissions from current rice production methods (Wing et al. 2018). However, it will not be possible without resistance to pests and pathogens. Foundational research of molecular resistance mechanisms is essential for developing tools and novel approaches for pest and pathogen management in rice production.

Our approach is to harness the natural defense strategies that wild and domesticated rice evolved for crop improvement. We use the extensive genetic diversity of rice to discover resistance to biotic stressors, in particular to herbivorous pests. Traditional rice cultivars have locally adapted to diverse agroecosystems (e.g. irrigated and rainfed lowland as well as rainfed upland). Rainfed cultivars generally evolved more potent stress-resistance mechanisms than irrigated cultivars as many stressors tend to be more prevalent agents of selection in rainfed agroecosystems (Wing et al. 2018). Furthermore, rice cultivars are classified into varietal groups based on the evolutionary history of domesticated rice and their wild relatives (temperate japonica, tropical japonica, 'circum'-basmati, 'circum'-aus and indica).

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Temperate japonica is least similar to the other varietal groups in terms of cultivation environment. Temperate japonica is typically only grown in irrigated lowland systems at higher latitudes, whereas the other varietal groups are cultivated in a range of agroecosystems, often in (sub)tropical regions with more stable and intense herbivore activity (Wing et al. 2018). Varietal groups other than temperate japonica are therefore more likely to have evolved potent anti-herbivore resistance mechanisms. This presents an opportunity to use a comparative framework for investigation. Differences in resistance between rice cultivars can be exploited to reveal the genetic basis of rice responses to herbivorous pests.

Revealing regulatory mechanisms of complex traits such as anti-herbivore resistance has the potential to provide insights into major biological properties of plants that promote translational research (Jung et al. 2008, Mochida and Shinozaki 2011). For example, we can determine suites of genes that are functioning in tandem under specific conditions to achieve a precise phenotype. Furthermore, regulatory networks provide measurements of the plasticity of molecular mechanisms under fluctuating environmental stressors (Rivera et al. 2021). Thus, we are using evolutionary systems biology to identify molecular resistance mechanisms to herbivory in rice.

Plants are susceptible to aboveground and belowground herbivory on shoots and roots, respectively. This presents an opportunity for diverging selection pressures on rice defenses from one herbivore to another. Plant-parasitic root-knot nematodes (RKNs) of the genus *Meloidogyne* are important belowground herbivores in tropical rice agroecosystems (Dutta and Phani 2023). RKNs are obligate sedentary endoparasites and cause characteristic root swellings, commonly called knots or galls, which are symptomatic of successfully established RKN feeding sites. In rice production, nematodes cause patchiness in fields and severe yield loss (Mantelin et al. 2017). With regard to variability in infection intensities among rice cultivars, temperate japonica cultivars tend to be more susceptible to RKNs than cultivars from other varietal groups (Dimkpa et al. 2016, Zhan et al. 2018).

An emerging aboveground herbivore found in Asian rice agroecosystems is *Spodoptera frugiperda* or the fall armyworm (FAW). Its larvae are caterpillars that act as leaf-chewing herbivores, and these have an affinity for grass crops. Levels of *S. frugiperda* infestation are linked linearly to increases in rice defoliation, reductions in plant and panicle densities, and reductions in rice yields (Pantoja et al. 1986). Temperate japonica cultivars also tend to be more susceptible to FAW and other chewing insect herbivores than cultivars from other rice varietal groups (Heinrichs 1986, Wang et al. 2022b). FAW is present yearround in tropical regions, but its presence elsewhere is restricted by coldest annual temperature (Early et al. 2018).

In tropical rainfed lowland rice environments, RKN and FAW infestations often occur after standing water has evaporated from paddies during periods of drought, whereas rainfed upland environments are not only natural habitats for RKNs but also experience increased outbreaks of FAW when periods of drought are followed by rains (Bridge et al. 2005, Tay et al. 2023).

Rice is equipped with molecular defense mechanisms against herbivory. The ability of plants to launch an induced defense response relies on the recognition of an attacker. An important recognition mechanism is through intracellular nucleotide-binding leucine-rich repeat (NLR) immune receptors that evolve under species-specific selection. Following recognition, signaling cascades are activated that regulate a hypersensitive resistance response, which includes phytoalexin accumulation, cell wall restructuring, deposition of waxes and suberin, protein reinforcements and production of reactive oxygen species (Bernaola et al. 2021, Groen et al. 2016a; as reviewed in Qi et al. 2018). Phytoalexin accumulation further serves as a major line of constitutive defense. Rice produces two major types of phytoalexins, phenolics (e.g. flavonoids and phenylamides) and diterpenoids (e.g. momilactones, oryzalexins, and phytocassanes). Interestingly, diterpenoid synthase genes are known to be upregulated in response to various stressors, including herbivory (Schmelz et al. 2014), and rice displays inter-cultivar variation in the amount and diversity of diterpenoid molecules produced (Gu et al. 2019, Kariya et al. 2019, 2020, 2023, 2024).

Here, we leverage leaf and root gene coexpression networks constructed from a rice diversity panel that consisted of aus, indica and japonica cultivars from irrigated and rainfed agroecosystems; these cultivars were grown in wet and dry field conditions where plants were naturally subjected to biotic factors that included leaf-chewing herbivores and RKNs (Sandhu et al. 2015, Xu et al. 2021, Groen et al. 2022). Combining these networks with existing genome-wide gene expression datasets from plants that were experimentally subjected to individual attacks by FAWs and RKNs, we identify modules of coexpressed genes that are enriched for herbivore-responsive genes and functionally test candidate genes for their role in defense against aboveground and belowground herbivores.

Results

Evolutionary systems biology identifies diterpenoids as anti-herbivore defenses in rice

We identified herbivore-responsive genes that significantly overlapped with fitness-linked modules of a gene coexpression network constructed from a rice diversity panel grown in wet and dry field environments in which plants experienced variable herbivory by RKNs and leaf-chewing herbivores (Sandhu et al. 2015, Xu et al. 2021, Groen et al. 2022). From publicly available rice herbivore-response transcriptomic datasets (Zhou et al. 2020, cv. Nipponbare and *Meloidogyne incognita*; Kyndt et al. 2012, cv. Nipponbare and *M. graminicola*; Leclerc et al. 2024, cv. Kitaake and *Spodoptera frugiperda*; Venu et al. 2010, cv. Nipponbare and *S. exigua*), we assigned each differentially expressed transcript to a coexpression module from the root and shoot transcript networks described by Groen et al. (2022), respectively (**Supplementary Tables S1–S4**).

For both aboveground and belowground tissues, we found enrichment of herbivore-responsive transcripts in several coexpression modules [root network (**Supplementary Table S5**): distribution of *M. incognita*, **Fig. 1**, and *M. graminicola*, **Supplementary Fig. S1**; shoot network (**Supplementary Table S6**): distribution of *S. frugiperda*, **Fig. 2**, and *S. exigua*, **Supplementary Fig. S2**].

From our RKN analysis, we identified four modules from the root coexpression network that are enriched with RKN-responsive transcripts, specifically *M. incognita*-responsive transcripts (**Fig. 1A**). Of the total 971 differentially expressed transcripts reported, we found 145 in module 1 ($P = 1.97 \times 10^{-4}$), 75 in module 8 ($P = 2.46 \times 10^{-14}$), 37 in module 11 ($P = 5.86 \times 10^{-5}$) and 21 in module 38 [$P = 3.39 \times 10^{-5}$; *P*-values are false discovery rate (FDR) adjusted for a Fisher's exact test on count data]. For each of these gene sets, we performed gene set enrichment analysis (GSEA) to categorize any Gene Ontology (GO) biological processes or Kyoto Encyclopedia of Genes and Genomes pathways of significance. Enrichment of root modules 8 and 38 included defense-related processes and pathways, including ones related to cell wall restructuring, biosynthesis of pathogenesis-related proteins, stress signaling, and secondary metabolite synthesis (*M. incognita*, **Fig. 1B, C**, complete list in **Supplementary Table S7**; *M. graminicola*, **Supplementary Fig. S1B, C**, complete list in **Supplementary Table S8**). Strongly represented among the genes related to secondary metabolites are those involved in diterpenoid metabolism (Os02g0569000, Os04g0179700, Os07g0190000, Os12g0491800, and Os06g0568600). The gene sets of root modules 1 and 11 were not significant for any enrichment of processes or pathways.

From our FAW analysis, we identified one module from the shoot coexpression network that is enriched with FAW-responsive transcripts, specifically *S. frugiperda*–responsive transcripts (**Fig. 2A**). Of the 2,638 differentially expressed transcripts that were assigned to a module in the shoot network, only module 8 was enriched for FAW-responsive transcripts ($n = 165$, $P = 4.88 \times 10^{-12}$; *P*-values are FDR adjusted for a Fisher's exact test on count data). GSEA on this gene set showed enrichment for processes and pathways related to photosynthesis, generation of reactive oxygen species, hormone signal transduction, and secondary metabolite synthesis. Interestingly,

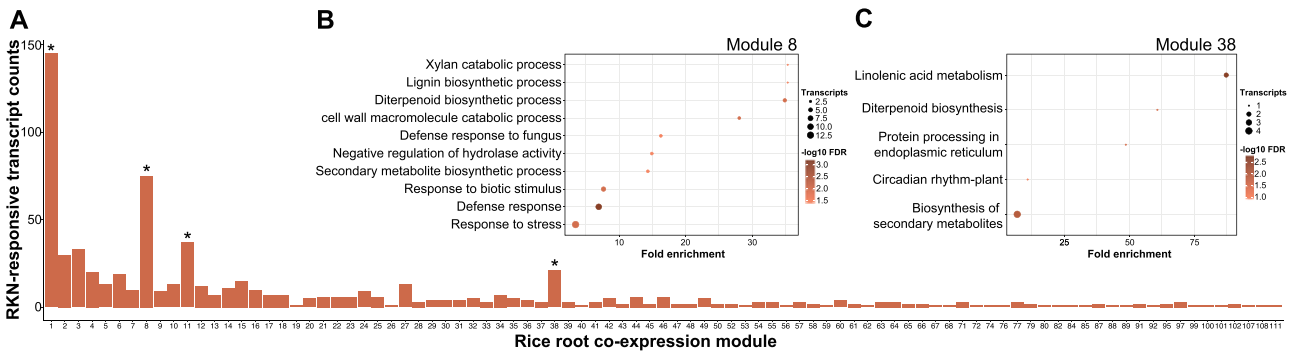

Fig. 1 Distribution and gene set enrichment of RKN-responsive transcripts across the rice root coexpression network. (A) Overlay of *M. incognita*-induced transcripts, at 6 days after infection, on the rice root coexpression network. Asterisk (*), FDR-adjusted *P*-value <0.001 for a Fisher's exact test on count data. (B, C) Enrichment of defense-related processes as shown by GO biological process enrichment analysis on root modules 8 and 38, respectively.

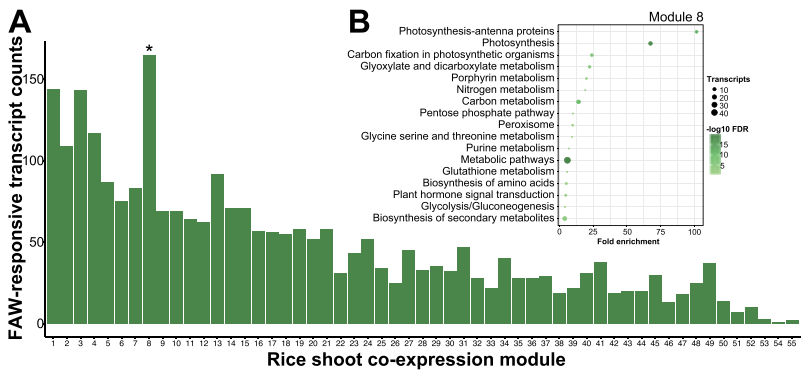

Fig. 2 Distribution and gene set enrichment of FAW-responsive transcripts across the rice shoot coexpression network. (A) Overlay of *S. frugiperda*-induced transcripts, at 2 hours after infestation, on the rice shoot coexpression network. Asterisk (*), FDR-adjusted *P*-value <0.001 for a Fisher's exact test on count data. (B) Enrichment of defense-related processes as shown by GO biological process enrichment analysis on shoot module 8.

shoot module 8 is enriched for genes involved in isopentenyl diphosphate (IPP) biosynthesis (*S. frugiperda*, **Fig. 2B**, complete list in **Supplementary Table S9**; *S. exigua*, **Supplementary Fig. S2B**, complete list in **Supplementary Table S10**; Groen et al. 2022). IPP and its isomer dimethylallyl pyrophosphate are isoprenoid precursors, which in turn can form building blocks for diterpenoid biosynthesis (Murphy and Zerbe 2020).

These findings suggest a role for diterpenoid metabolism as having potential roles in anti-herbivore defenses in both belowground and aboveground tissues. We therefore further investigated the expression patterns of genes known to be involved in the biosynthesis of diterpenoid phytoalexins.

Expression of diterpenoid metabolism genes responds to herbivory

To further investigate expression of genes underlying diterpenoid metabolism, we explored the complete gene sets of root coexpression modules 8 and 38 (Groen et al. 2022). We discovered 15 diterpene synthase genes that are within these two root modules. These include ones encoding enzymes such as copalyl diphosphate (CPP) synthases (CPSs), kaurene-synthase-like enzymes (KSLs) and cytochrome P450 monooxygenases that span the biosynthesis of all four major classes of rice diterpenoids: oryzalexins (including oryzalexin S), phytocassanes, oryzalides and momilactones (**Fig. 3**). This evidence suggests diterpenoid metabolism genes as being coexpressed in rice roots.

The majority of genes responsible for production of phytocassanes A–E, oryzalides A–C and oryzalexins A–F was downregulated upon RKN herbivory (**Fig. 3**; **Supplementary Table S11**). For RKNs, the same was true for rice genes involved in production of momilactones, although expression of some of these genes was upregulated in response to FAW feeding. Interestingly, however, among all the KSLs, which represent different branching points in the diterpenoid production pathway, expression of *OsKSL8* was unique in being upregulated in response to both RKN and FAW herbivory (**Fig. 3**).

KSL8 is located on chromosome 11, and two alleles are known to segregate at this locus, *KSL8-jap* and *KSL8-ind*, with the former being prevalent in temperate japonica and the wild relative and ancestor of all japonica rice cultivars, *Oryza rufipogon*, and the latter being prevalent in tropical japonica (through introgression) and cultivars belonging to the indica and aus varietal groups as well as their respective wild relative and ancestor, *Oryza nivara* (Zhao et al. 2023, Kariya et al. 2024). KSL8-jap and -ind both act downstream of CPS4, with the *KSL8-jap* allele encoding the first committed step in the synthesis of stemarane-type diterpenoids, including oryzalexin S, and the *KSL8-ind* allele encoding a stemod-13-ene synthase that does not engender oryzalexin S production and rather represents the committed step in the production of stemodane-type diterpenoids (Zhao et al. 2023, Kariya et al. 2024). We hypothesized that *KSL8* could partially underlie previously observed differences in the general level of anti-herbivore resistance of temperate and tropical rice cultivars (Heinrichs 1986, Dimkpa et al. 2016, Zhan et al. 2018, Wang et al. 2022b).

KSL8-jap positively mediates defense against belowground herbivores

Based on these analyses, we tested if KSL8-jap could play a role in defending rice plants against herbivory by the RKN *M. incognita*. To identify time points that reflect early rice defense against RKNs and subsequent long-term establishment of RKNs in rice roots, we surveyed *M. incognita* life cycle patterns in cv. Kitaake (**Supplementary Fig. S3**). The developmental speed of *M. incognita* in our conditions was similar to previously reported developmental speeds of this RKN on temperate japonica rice (Nguyễn et al. 2014), and from the patterns, we decided to focus on 3 days after infestation (3DAI) and 10DAI as representative time points. At 3DAI, *ksl8-jap* knockout mutant plants had attracted significantly more second-stage juvenile nematodes (J2s) of *M. incognita* than wild-type and *cps4* knockout mutant plants of cv. Kitaake (χ^2 tests, $P \leq 0.00640$ and $P = 0.218$, respectively; **Fig. 4A**). These patterns persisted at 10DAI, and once J2s had infected plants, they progressed significantly faster through development in *ksl8-jap* knockout plants than in wild-type and *cps4* knockout plants of cv. Kitaake (χ^2 tests, $P \leq 2.00 \times 10^{-5}$ and $P = 0.275$, respectively; **Fig. 4B**).

Since previous work has shown enhanced susceptibility of *cps4* knockout mutants of cv. Kitaake to the RKN *M. graminicola* at 14DAI (Desmedt et al. 2022), and since we did not observe significant differences at the earlier time points of 3DAI and 10DAI, we decided to test a *cps4* knockout mutant of cv. Nipponbare, a cultivar that is known to accumulate higher diterpenoid levels than cv. Kitaake and contains the same allele at *KSL8*, *KSL8-jap* (Zhang et al. 2021). Indeed, at 10DAI, *cps4* knockout mutants of cv. Nipponbare showed significantly enhanced susceptibility to RKNs compared to wild-type plants (χ^2 test, $P = 1.13 \times 10^{-10}$; **Fig. 4B**).

These results are in contrast with the hypothesis that allelic variation at *KSL8* may underpin observed general differences between temperate and tropical rice cultivars in their resistance to RKNs, since the temperate japonica allele *KSL8-jap* contributes to rice immunity. However, the effects of KSL8 can be overridden in the presence of the immune receptor MG1, which is segregating among tropical japonica, indica and aus cultivars but not temperate japonica cultivars (Dimkpa et al. 2016, Wang et al. 2023). This intracellular NLR immune receptor was previously shown to confer strong resistance to the RKNs *M. graminicola* and *M. javanica* upon recognition of nematode attack through a hypersensitive response (Lahari et al. 2020, Wang et al. 2023). We found that rice cultivars carrying *MG1* are strongly resistant to *M. incognita* as well, with nearly zero galling in cultivars LD24 and Khao Pahk Maw (*t*-test relative to cv. Kitaake, $P = 1.7 \times 10^{-4}$ and $P = 4.23 \times 10^{-4}$, respectively; **Fig. 5A**) along with the inability of juveniles to reach reproductive stages (in both comparisons: χ^2 test,

$P = 9.46 \times 10^{-48}$; **Fig. 5B**). Perhaps coincidentally, at least one cultivar carrying *MG1*, Zhonghua 11, also accumulates much higher levels of diterpenoids than temperate japonica cultivars (Zhang et al. 2021).

KSL8-jap negatively mediates defense against aboveground herbivores

Next, we tested rice resistance to leaf feeding by FAW caterpillars. At 2DAI, compared to wild-type plants, *cps4* knockout

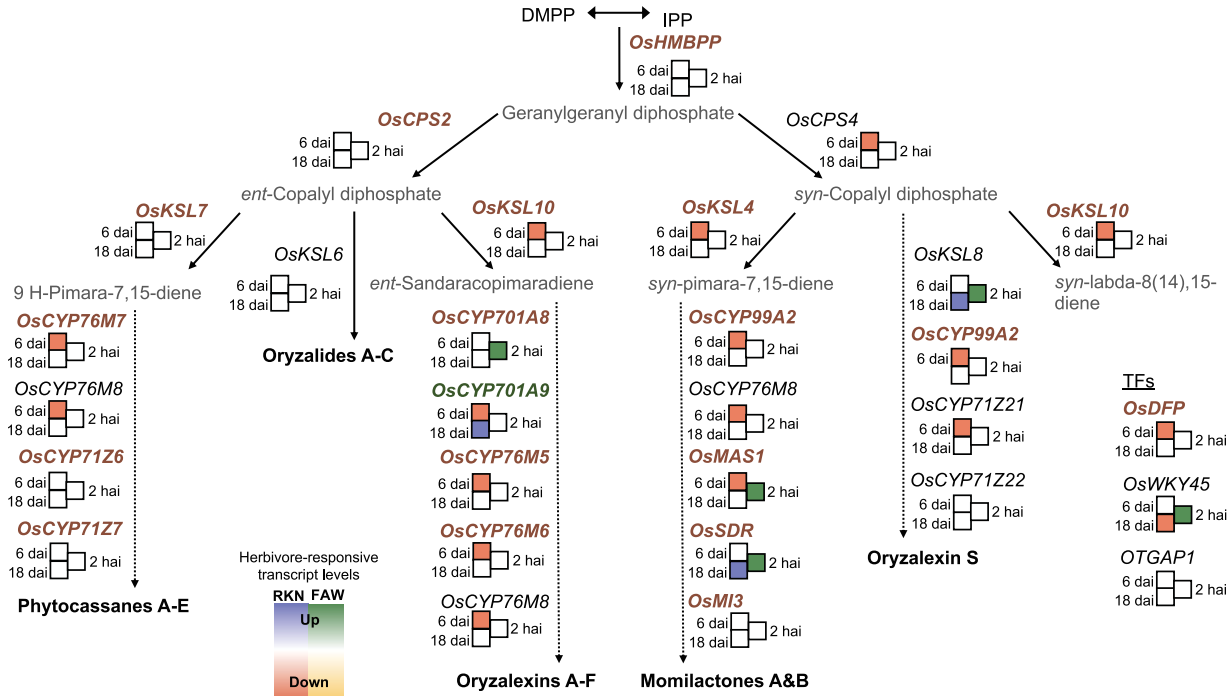

Fig. 3 Reported herbivore-responsive expression levels of genes underlying the diterpenoid biosynthetic pathway as found to be coexpressed in rice roots and leaves. Genes in bold and brown belong to rice root coexpression module 8 (Groen et al. 2022). The relative expression levels of diterpene synthase genes in response to RKNs (Zhou et al. 2020; *M. incognita* on cv. Nipponbare) and FAWs (Leclerc et al. 2024; *S. frugiperda* on cv. Kitaake) are integrated with the diterpenoid pathway. RKN-responsive transcript levels: blue, upregulated; orange, downregulated; dai is days after infection. FAW-responsive transcript levels: green, upregulated; yellow, downregulated; hai is hours after infestation. White boxes indicate no differential expression.

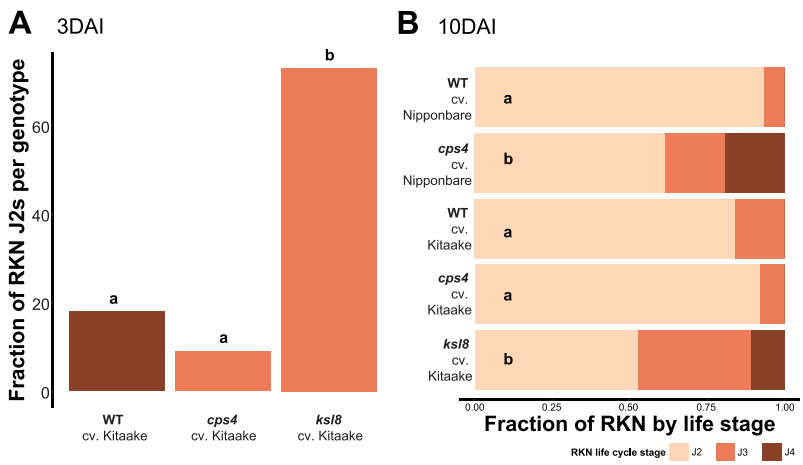

Fig. 4 Changes in susceptibility of rice to RKNs on *cps4* and *ksl8-jap* knockout mutant lines. (A) Percent of total J2s able to infect root systems present in *cps4* and *ksl8* knockouts compared to wild-type (WT) cv. Kitaake at 3DAI (*N* per genotype was 6–14 plants). (B) Fraction of RKNs by life stage in the root systems of *cps4* and *ksl8* cv. Nipponbare and Kitaake knockouts compared to their wild-type counterparts at 10DAI (*N* per genotype was 5–11 plants).

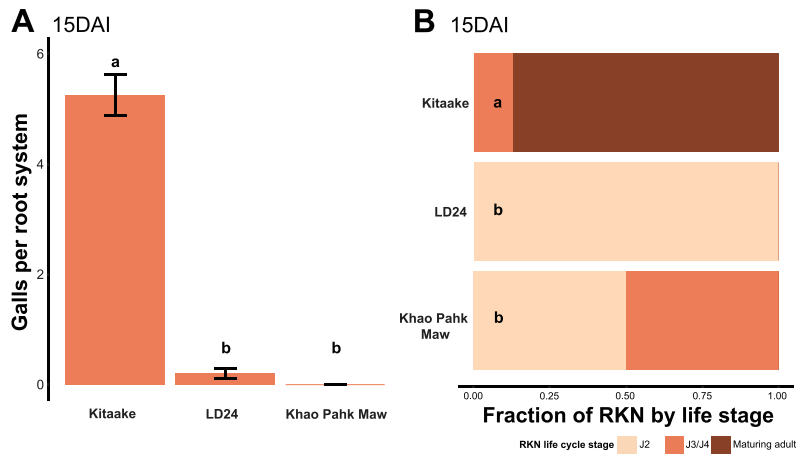

Fig. 5 Effects of MG1 on infection rates of rice by *M. incognita*. (A) Average number of galls per root system for cultivars Kitaake, LD24, and Khao Pahk Maw. (B) Fraction of RKNs by life stage in the root systems of cultivars Kitaake, LD24, and Khao Pahk Maw. Phenotyping occurred at 15DAI (*N* per genotype was 4–5 plants).

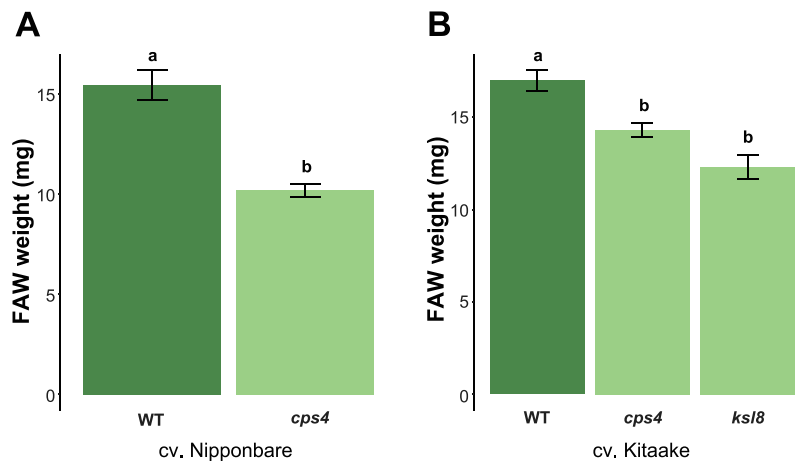

Fig. 6 Changes in FAW weight from feeding on *cps4* and *ksl8-jap* knockout lines of rice. (A) Weight of FAWs after feeding for 2 continuous days on *cps4* and wild-type (WT) cv. Nipponbare leaves (*N* per genotype was 14–15 plants). (B) Weight of FAWs after feeding for 2 continuous days on *cps4*, *ksl8*, and wild-type cv. Kitaake leaves (*N* per genotype was 11–23 plants).

mutants of cv. Nipponbare showed enhanced resistance to feeding by second-instar FAW caterpillars, with the caterpillars gaining less weight on leaves of the mutant (*t*-test, $P = 0.0252$; **Fig. 6A**). As for RKN herbivory, also for FAW herbivory, the difference between *cps4* knockout mutants and wild-type plants was less obvious for cv. Kitaake than for cv. Nipponbare, but the data did confirm a pattern of increased resistance to caterpillar feeding for the *cps4* knockout line of cv. Kitaake as well (*t*-test, $P = 0.0657$; **Fig. 6B**).

Like the *cps4* mutant, also the *ksl8-jap* knockout mutant in cv. Kitaake showed an opposite pattern for resistance against FAWs compared to resistance against RKNs: caterpillars gained significantly less weight on leaves of *ksl8-jap* mutants than on those from wild-type plants (*t*-test, $P = 0.0367$; **Fig. 6B**). The results for the cv. Kitaake knockout mutants *cps4* and *ksl8-jap* were confirmed in a second experiment, with FAW caterpillars gaining significantly less weight on leaves of either mutant compared to wild-type leaves (*t*-tests, $P = 0.00401$ for *cps4* and $P = 0.0302$ for *ksl8-jap*; **Supplementary Fig. S4**).

Since tropical rice cultivars from the tropical japonica, indica, and aus varietal groups are known to carry an alternative allele (*KSL8-ind*) at the *KSL8* locus that, similar to knocking out *KSL8-jap*, prevents production of stemarane-type diterpenoids such as oryzalexin S, we decided to test if tropical rice cultivars would be more resistant to FAW caterpillars than temperate cultivars, which was indeed the case (*t*-tests, $P \leq 0.0131$ relative to cv. Kitaake and $P \leq 0.0887$ relative to cv. Nipponbare; **Fig. 7**).

Discussion

We identified potential anti-herbivore defense mechanisms in both aboveground and belowground rice tissues. Our GSEA for herbivore-responsive transcripts that are significantly enriched in gene coexpression modules linked with rice fitness in wet

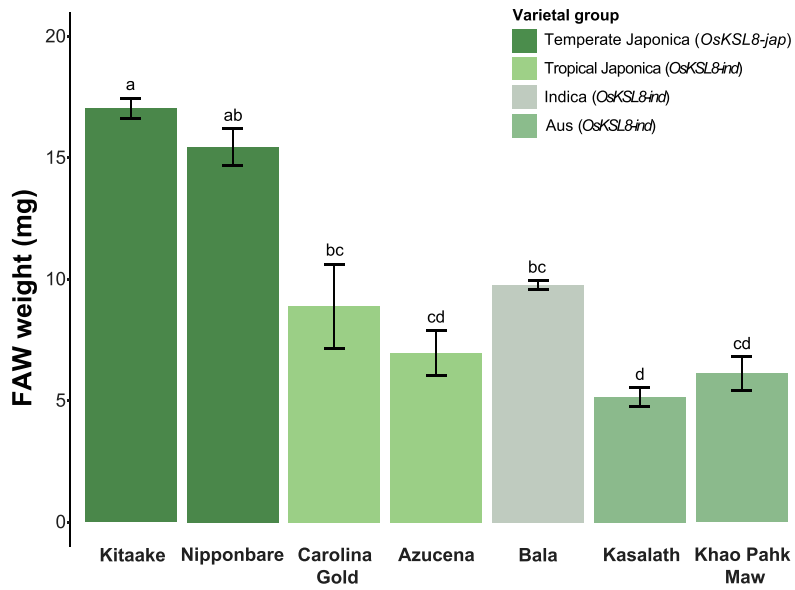

Fig. 7 Effects of the *KSL8-ind* and *KSL8-jap* alleles on feeding rates of FAW on rice leaves. Weight of FAWs after feeding for 2 continuous days on leaves of cultivars Kitaake, Nipponbare, Carolina Gold, Azucena, Bala, Kasalath, and Khao Pahk Maw (*N* per genotype was 3–23 plants).

and dry field environments revealed processes related to phytoalexin biosynthesis, cell wall restructuring, and immune responses. In this study, we investigated genes underlying production of phytoalexins, specifically diterpenoids, for their role in anti-herbivore defenses in rice.

It was evident from our analyses that genes underlying diterpenoid production are coregulated. Of the estimated 23 genes involved in diterpenoid biosynthesis, 14 belong to a single root gene coexpression module (module 8), including the major regulator transcription factor *DITERPENOID PHYTOALEXIN FACTOR*. Furthermore, we know that two major gene clusters of diterpene synthases are located on chromosomes 2 and 4 (Miyamoto et al. 2016). Although expression of most of these genes is downregulated in response to RKNs, expression of *KSL8* forms an exception, and diterpenoid biosynthesis is upregulated in response to several stressors and elicitors (e.g. Kato-Noguchi 2009, Schmelz et al. 2014, Desmedt et al. 2022, and Liu et al. 2024). For example, rice plants treated with exogenous phenylalanine ammonia-lyase inhibitor (AIP) display induced transcription of diterpene synthase genes and a high accumulation of phytocassanes, momilactones and oryzalexins 3 days post-treatment. Interestingly, rice plants treated with AIP also show increased resistance to *M. graminicola* (Liu et al. 2024). Furthermore, a RKN-resistant mutant indica line showed upregulation of the diterpenoid synthesis pathway upon early recognition of *M. graminicola* (Dash et al. 2021). Previous studies have demonstrated diterpenoid production to be inducible by jasmonic acid (JA), a hormone that regulates anti-herbivore and anti-fungal defenses (Shimizu et al. 2013). RKNs and FAWs appear to avoid or prevent induction of JA-regulated immune responses because, although exogenous application of methyl jasmonate increases diterpenoid production and resistance to *M. graminicola* and *S. frugiperda* in rice (Stout et al. 2009, Nahar et al. 2011), the expression of many genes underlying diterpenoid synthesis is downregulated upon herbivory. RKNs and potentially FAWs may avoid inducing most branches of diterpenoid metabolism. Other studies have shown that expression of diterpenoid biosynthesis genes and diterpenoid accumulation are inducible by drought (Zhang et al. 2021). Since herbivory by RKNs and chewing herbivores also induces plant compensatory responses for water loss and osmotic stress mitigation (Reymond et al. 2000, Guarneri et al. 2024), this may to some extent explain why we observe an overlap between herbivore-responsive genes and gene coexpression modules from rice plants growing in field environments with variable water availabilities and interactions with herbivores, pointing to integrated plant transcriptional and metabolic responses to combinations of biotic and abiotic stresses (Groen et al. 2022).

Rice diterpenoids are diverse and have distinct functions (Li et al. 2021, Rayee et al. 2024). In our study, we found distinct and conflicting roles for the diterpenoid biosynthetic enzymes CPS4 and KSL8 in mediating defenses against RKN and FAW herbivory. A similar pattern has been observed for aboveground and belowground microbial pathogens. CPS4 has opposite effects on rice resistance to bacterial and fungal pathogens, functioning negatively in host resistance to the leaf bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* and positively in non-host resistance to the root fungal pathogen *Magnaporthe poae* but not in host resistance to the related fungal pathogen *Magnaporthe oryzae* (Lu et al. 2018). It was found previously that RKN herbivory increases for *M. graminicola* when *CPS4* is knocked out in cv. Kitaake (Desmedt et al. 2022). Our findings for cv. Kitaake infected by *M. incognita* showed a similar trend, but this was not significant. This may have been due to the earlier time points we chose to assess

nematode numbers at because we identified a significantly enhanced susceptibility of *cps4* knockout mutants of cv. Nipponbare, a cultivar that is known to accumulate higher levels of diterpenoids than cv. Kitaake (Zhang et al. 2021). Furthermore, as was previously observed for *X. oryzae* pv. *oryzae*, we identified increased resistance to FAW in *cps4* knockouts. This opposing pattern of herbivore performance for RKNs and FAWs on *cps4* knockout lines was also visible for our *ksl8-jap* knockout line.

As to an explanation of why rice resistance to RKNs was significantly reduced relative to wild-type cv. Kitaake in the *ksl8-jap* knockout line, but not the *cps4* knockout line, we can only speculate. One reason might be that knocking out the function of the syn-CPP synthase CPS4 directly affects a wider range of diterpenoids than knocking out the function of downstream KSL enzymes. Downstream of CPS4, KSL8 competes with KSL4 and KSL10 for substrates, and, therefore, knocking out KSL8 will only affect a subset of substrates (Lu et al. 2018, Zhang et al. 2021). It has been hypothesized that knocking out the function of enzymes upstream in a biosynthetic pathway may have more profound effects on altering flux balances through alternative branches than knocking out the function of more downstream enzymes (Olson-Manning et al. 2013). In keeping with the concept of altering flux balances, *cps4* knockout mutants of rice are known to produce constitutively higher levels of CPS2-dependent ent-CPP–derived diterpenoids, including phytocassanes (Lu et al. 2018, Zhang et al. 2021). Higher accumulation of CPS2-dependent diterpenoids can lead to increased rice resistance to RKN herbivory (Desmedt et al. 2022). While we do not know how knocking out KSL8 function influences flux balances through alternative branches of the diterpenoid biosynthetic pathway at present, it is likely to have a much smaller effect than knocking out CPS4. In support of this explanation, knocking out the function of KSL4 (which directs the metabolic flux of syn-CPP–derived diterpenoids toward momilactone biosynthesis in a branch alternative to the one in which KSL8 is active) has much stronger effects on rice allelopathic interactions with weeds than knocking out CPS4, which may be because elevated phytocassane levels in *cps4* knockout mutants could also have allelopathic effects (Xu et al. 2012, Kong 2007). This observation is analogous to our observations on how knocking out KSL8 and CPS4 influences rice interactions with RKNs. More experimentation in future studies will be needed to resolve this question.

Temperate and tropical rice cultivars are known to segregate for a polymorphism in *OsKSL8*, with temperate japonica cultivars possessing the *KSL8-jap* allele that encodes a stemar-13-ene synthase, which engenders the production of oryzalexin S, and tropical japonica, indica, and aus cultivars carrying the *KSL8-ind* allele that encodes a stemod-13-ene synthase, which does not engender oryzalexin S production but rather represents the committed step in the production of diterpenoids of the stemodane family (Zhao et al. 2023, Kariya et al. 2024). Although some of the stemodane family diterpenoids possess antiviral activity (Morrone et al. 2006), it is unclear whether they could affect invertebrate herbivores. While our results suggest they might, more experimental work will be needed to verify this. Our results further point to a potential role for oryzalexin S in rice defense against RKNs, but this also requires further experimental corroboration.

O. sativa ssp. japonica was domesticated from the (sub)tropical perennial *O. rufipogon* (Choi et al. 2017), which contains the *KSL8-jap* allele that contributes to anti-RKN defenses. It may be especially important for perennial herbaceous plants to protect their root systems from nematode herbivory since the roots persist in the soil for years. However, as japonica rice was domesticated and became an annual crop and as cultivation practices developed that avoid or resist prolonged exposure to active RKNs—e.g. through slash-and-burn agriculture, crop rotation, and flood irrigation—the increased RKN resistance that the *KSL8-jap* allele confers may have become less important. This could have facilitated the current situation in which the *KSL8-ind* allele has become the most prevalent allele in tropical japonica, indica, and aus cultivars after originating from the wild relative and ancestor of indica and aus cultivars, *O. nivara*, and subsequent introgression into tropical japonica cultivars (Zhao et al. 2023, Kariya et al. 2024).

Moreover, KSL8-mediated resistance to RKNs can be overridden in tropical rice cultivars by hypersensitive resistance through the NLR immune receptor MG1. The *MG1* gene resides in a NLR gene–rich genome region on chromosome 11 that contains trans-species haplotypes (Zhou et al. 2021). Certain NLR alleles in this region are known to confer resistance to the fungus *M. oryzae* (Ma et al. 2022), whereas another encodes the MG1 immune receptor that recognizes RKN attack (Wang et al. 2023).

Long-term balancing selection provides an advantage for adaptation in plants by maintaining genetic diversity within a population or species. We hypothesize that long-term balancing selection acts at the *MG1* and *KSL8* loci in *O. sativa* cultivars and contributes to maintaining the trans-species allelic polymorphisms found at these loci (Zhou et al. 2021, Zhao et al. 2023, Kariya et al. 2024). Several studies in *Brassica* species have shown long-term balancing selection in maintaining polymorphisms for immunity genes, such as NLRs, which span spatial and temporal scales (Kroymann et al. 2003, Bakker et al. 2006, Clark et al. 2007, Wu et al. 2017, Koenig et al. 2019, Wang et al. 2019). In *Boechera stricta*, an enrichment of NLRs was found within genomic regions under balancing selection (Wang et al. 2019). These regions are syntenic with *Arabidopsis thaliana* regions that also contain NLRs under balancing selection (Clark et al. 2007), suggesting the maintenance of ancient NLR gene polymorphisms by balancing selection. Moreover, trans-species polymorphisms under balancing selection in *Arabidopsis* and *Capsella* are linked to genes related to biotic and abiotic stress responses (Wu et al. 2017).

The sessile nature of plants poses opportunities for dynamic and complex interactions with their environment, including

synchronous and asynchronous temporal and spatial fluctuations in various biotic and abiotic factors. Such dynamic conditions and diffuse multispecies interactions can contribute to the maintenance of ancient polymorphisms in defense genes through balancing selection (Karasov et al. 2014, Groen et al. 2016b). It has been demonstrated in *B. stricta* that balancing selection maintains complex trait variation in the chemical profiles of leaves to withstand varying ecological factors, specifically herbivory and drought (Carley et al. 2021). It is well established that diterpenoids function to combat biotic and abiotic stresses (Schmelz et al. 2014, Zhao et al. 2018). As such, long-term balancing selection at the *KSL8* locus may contribute to maintaining polymorphisms and provide resilience against fluctuating combinations of stressors.

Materials and Methods

Plant material and growth conditions

Rice plants [*O. sativa* ssp. *japonica* cultivars Kitaake, KitaakeX, Nipponbare, and knockout lines (**Supplementary Table S12**)] were grown under supplemental 16/8–hours light/dark conditions at 28°C in a greenhouse at the University of California Riverside's Plant Research 1 facility. Rice seed preparation included a 3-day heat treatment at 50°C and sterilization (15% bleach for 5 mins followed by sterile water washes). We germinated seeds on sterile wet paper for 6 days at 30°C in the dark. We transplanted seedlings into cone pots containing 68 g of autoclaved soil composed of 70% sand and 30% organics. Seedlings were fertilized at 4, 7, 10, and 14 days after transplanting with Peter's solution (24-8-16; 10 g/l) and Sequestrene 330 10% Fe chelate (1 g/l).

Confirmation of rice knockout lines

To confirm that the rice knockout mutant lines we tested are true knockouts for the targeted genes, we performed reverse transcription PCR (RT-PCR) (**Supplementary Figs. S5 and S6**). For this analysis, root tissue was flash frozen and ground to a fine powder. We isolated total RNA using the RNeasy Plant Mini Kit (Qiagen, Germantown, MD) following the manufacturer's protocol. Residual DNA was removed using the TURBO DNA-*free* kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. From our RNA template, we produced cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA) following the manufacturer's protocol. We then performed RT-PCR to check for the presence of transcripts. *OsACTIN* was used as a control. Primers used in our analysis are listed in **Supplementary Table S13**.

RKN and FAW husbandry

RKNs of *M. incognita* population 'Project 77' (race 3) were cultured on tomato plants (*Solanum lycopersicum* cv. Moneymaker). To isolate RKN eggs, rice root systems were centrifuged for 3 mins in a 10% bleach solution. From the suspension, eggs were collected using a 500-mesh sieve (Godinez-Vidal et al. 2024). The RKN eggs were left for hatching on a filter paper for 3 days in the dark at 24°C. We collected freshly hatched J2s for the inoculum.

Second-instar FAW caterpillars (*S. frugiperda*; corn-strain) were purchased commercially (Benzon Research; Carlisle, PA).

Coexpression enrichment analyses of herbivore-responsive genes

Herbivore-responsive genes were analyzed for enrichment in modules of a rice coexpression network (Groen et al. 2022). We surveyed the differentially expressed genes (DEGs) identified from the following datasets: Venu et al. (2010), Kyndt et al. (2012), Zhou et al. (2020), Leclerc et al. (2024), and Xue et al. (2024). RKN-responsive DEGs were assigned to root coexpression modules, and FAW-responsive DEGs were assigned to shoot coexpression modules accordingly. We used a Fisher's exact test designed for comparing count data to test for enrichment of DEGs within each module with the R package 'stats' (R Core Team 2022). This was followed by a post-hoc test, the Benjamini–Hochberg test or FDR, to adjust *P*-values for multiple comparison testing. Gene sets from significantly enriched modules underwent GSEA using ShinyGo v0.80 (Ge et al. 2020).

Root herbivory assessment

For RKN infection assays on rice diterpenoid mutant lines and wild-type plants, individually potted 2-week-old seedlings in 70-ml cones, containing approximately 68 g of soil composed of 30% organic matter and 70% sand, were inoculated with 350 J2s per seedling (approximately five J2s/1 g of soil) and maintained in the growth conditions described earlier. After 3 days and 10 days, root growth of the rice knockout mutant lines tested was similar to their respective wild-type and controls (**Supplementary Fig. S7**). At these time points, the rice plants were further evaluated for the number of RKNs present inside their roots, separating nematodes by life stage. For RKN infection and galling assays on rice cultivars with and without the *MG1* haplotype, individually potted 2-week-old seedlings in 70-ml cones, containing approximately 68 g of soil composed of 30% organic matter and 70% sand, were inoculated with 500 J2s per seedling (approximately seven J2s/1 g soil) and maintained in the growth conditions described earlier. After 15 days, the root systems were collected for evaluations of gall and nematode presence. For acid fuchsin staining, the roots were treated with 10% bleach for 5 mins, washed thoroughly with water and boiled for 10 s in acid fuchsin solution (3.5% acid fuchsin in 25% acetic acid). After letting the solution cool down to room temperature, the roots were transferred to a destaining solution (1: 1: 1, acetic acid: glycerol: H_2O) before the number of nematodes that had entered the roots was evaluated using a dissecting microscope.

Leaf herbivory assessment

Caterpillar weight gain assays were performed as previously described (Groen et al. 2013). Individual second-instar caterpillars were transferred to leaves of each experimental plant using a fine paintbrush. The leaves with caterpillars were kept individually in plastic boxes with insect-proof lids, and the caterpillars were weighed to the nearest 0.1 mg using a microbalance (Mettler Toledo; Columbus, OH) before and after feeding for 2 continuous days.

Supplementary Data

Supplementary data are available at *PCP* online.

Data Availability

Raw RNA sequence data from Groen et al. (2022) that some of our analyses rely upon have been deposited as part of SRA BioProject PRJNA564338. Processed RNA expression counts, alongside a key to the RNA sequence data in SRA BioProject PRJNA564338, and the sample metadata were deposited in Zenodo under DOI 10.5281/zenodo.4779049. Normalized count data and further information on root and shoot gene coexpression module compositions and characteristics can be found in the Supplementary material of Groen et al. (2022).

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Disclosures

The authors have no conflicts of interest to declare.

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