UNIVERSITY OF CALIFORNIA SAN DIEGO

Identification of Transcription Factors Regulating Stress in *Arabidopsis thaliana*

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The thesis of Rhys Newcomb is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

Identification of Transcription Factors Regulating Stress in *Arabidopsis thaliana*

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Transcription factors are a vital class of genes in eukaryotes that regulate almost every aspect of organism development, function, and physiology. Characterizing the function of transcription factors in plants, and the regulatory networks that they make up, becomes crucial as we search for ways to adapt our food sources to the rapidly changing climate. In this thesis, we

start by creating a resource of transgenic lines to aid in the identification and characterization of *Arabidopsis thaliana* transcription factors. We then develop several methods of testing stress-response phenotype in *A. thaliana*, and use the initial state of this resource to identify transcription factors that are likely to regulate stress response. The resource and methods outlined in this thesis can be developed over time to become a useful pipeline for the identification of transcription factors involved in the regulation of any plant function of interest.

Introduction

In natural habitats, plants face a wide range of potential biotic and abiotic stresses, such as drought, salinity, high or low temperatures, herbivory, disease, and more. Any stress, applied to a plant at a high enough level or for a long enough time, is capable of irreversibly damaging a plant beyond repair, whether it inhibits plant growth, prevents reproduction, or kills the plant outright. Due to their sessile nature, plants have had to adapt over millennia to build up certain defenses against external stresses so that they can continue to flourish even under adverse conditions. Whether these be structural changes (such as thicker cuticles or hardy roots) or biochemical ones (such as the inactivation of toxic heavy metals), plants have developed a diverse array of responses to keep up with issues they may face in the wild (He et al. 2018).

One of the largest problems facing us this century is a limited, declining supply of available arable land. With a global population that is expected to reach 9 billion people by 2050, as well as a rapidly growing biofuel sector, we are seeing issues with finding enough cropland to grow food while minimizing destruction of natural ecosystems (Kehoe et al. 2017). One of the ways that we can address this issue is not by further expanding land usage, but instead by minimizing crop loss to environmental factors like weather and disease. Plants that are poorer at responding to stress will grow slower and smaller, and produce fewer fruits or seeds, leading to massive losses in food production if many fields in the area experience the same stress at once. Additionally, global climate change is placing additional pressure on many areas to expand crop production due to losses from rising temperatures and widespread drought (Hu & Xiong 2014). For example, global wheat production is estimated to be reduced by 6%, or 42 metric tons, for each 1°C increase in mean temperature (Asseng 2014). If we understand the biochemical mechanisms behind plant stress response, we could engineer crops that would better withstand

potential sources of stress in the field. We could even engineer crops to withstand future stresses due to climate change such as heat and drought, in anticipation of the changing environment.

One major component of the stress-response pathway in plants is the generation of reactive oxygen species (ROS) in excess. ROS are present at a low level in plant tissues as a byproduct of normal metabolic processes like photosynthesis and respiration, and have crucial signaling functions, particularly related to protection from harsh light (high concentrations of electrons) (Edreva 2005). However, ROS can react with many different biomolecules present in plant cells, especially chlorophyll, membrane lipids, and nucleic acids, and cause irreversible cellular damage (Sharma et al. 2012). Thus, plants have had to evolve mechanisms to regulate the production of ROS as well as scavenging mechanisms to prevent ROS from accumulating in excess. Under abiotic stress conditions, ROS production becomes elevated due to limited carbon intake leading to changes in the photosynthesis electron transport chain, and ROS rapidly accumulates, causing oxidative damage to plant tissues (Apel & Hirt 2004). Plants with better regulation of ROS production or more efficient scavenging mechanisms are able to resist oxidative damage, conferring increased resistance to abiotic stress. For example, the *Arabidopsis* gene *HDG11*, which increases ROS scavenging rate when highly expressed, confers increased drought tolerance in crops such as rice (Yu et al. 2013). As all types of abiotic stress eventually lead to oxidative stress, a key area of research for stress response in plants is to characterize mechanisms of ROS production and removal. This would allow us to understand how plants could be made to be more resistant to oxidative stress, and in turn, stress in general.

Plants are able to respond to environmental cues, including stress, through the use of regulatory genes called transcription factors (TFs). In all living organisms, transcription factors are fundamental in regulating which genes are expressed, when they are expressed, where they

are expressed and by how much they are expressed. A single TF can work alone or with other TFs to regulate multitudes of other genes, including other TFs, which creates complex regulatory networks and pathways inside every organism. TFs are a large category of genes in eukaryotes, making up approximately 3.5% of genes in *Drosophila melanogaster*, 4% in *Saccharomyces cerevisiae*, and 8% in humans (Lambert et al. 2018, Rhee et al. 2014, Shokri et al. 2019). In *Arabidopsis thaliana*, comprehensive estimates of the number of TFs range to about 2500, or 9.5% of the total gene pool (Pruneda-Paz et al. 2014). However, despite their important biological function, the roles of only a few TFs in plants are fully understood at the molecular level. This makes them a promising group to search for key genes that are part of the stress response pathway.

One major difficulty emerges when studying TF function, especially in plants. For one, TFs in plants are more likely to be duplicated within the genome and create large gene families with overlapping, redundant functions (Shiu et al. 2005). The widespread existence of semi-redundant TFs make the use of forward genetics — that is, identification of gene function through mutagenesis screens — more difficult, because TFs that have been knocked out may have their role filled by a related gene (Pruneda-Paz & Kay 2010). In this project, we develop a resource to aid in the functional study of *A. thaliana* transcription factors, in which we overexpress (OX) each TF in a transgenic *A. thaliana* line. This method allows us to overcome the redundancy issues that would be present in a loss-of-function/knock-out approach. As a proof of concept, we also use this resource to identify novel regulators of stress response in *A. thaliana*.

Results

Creation of transcription factor overexpression Arabidopsis lines

In order to create the library of transcription factor (TF) overexpression lines, a multi-stage method was devised. Protein coding sequences for *Arabidopsis* TFs were obtained from a comprehensive, sequence-validated clonal library containing 78.5% of all potential *Arabidopsis* TFs (Pruneda-Paz et al. 2014). The desired TF coding sequence was then inserted into a binary vector (able to replicate in both *E. coli* and *A. tumefaciens*) holding a cassette with the *35S* cauliflower mosaic virus promoter, designed to constitutively overexpress (OX) the attached TF, as well as a glufosinate ammonium resistance gene for selection purposes (Figure 1A). We then transformed the engineered cassette into *Arabidopsis* plants of the *Col-0* ecotype via *Agrobacterium tumefaciens*-mediated transformation (Zhang et al. 2006). This resulted in a small number of *Arabidopsis* progeny having integrated the cassette into a random position in their genome, thus overexpressing the chosen TF. As expression levels of the *35S::TF* construct can vary depending on its location of insertion into the genome (Pérez-González & Caro 2016), and because the random nature of *Agrobacterium*-mediated transformation comes with a risk of disrupting native gene function, we aimed to create four independently transformed OX lines for each TF. Multiple lines for each TF enhances the chances to obtain at least one line with a sufficiently high expression level for the gene in question, with limited disruption of native genes.

After *Agrobacterium* transformation, we screened T_1 progeny for glufosinate resistance in order to identify transgenic plants carrying the TF-overexpression construct (Figure 1B). Next, we identified lines which had integrated the insertion at only one location in its genome, as opposed to two or more locations. Having a mutation at only one location in the genome makes it

simpler to create homozygous lines and to facilitate future genetic crosses using these transgenic lines. In order to screen out lines carrying two or more insertions, the T_2 progeny generated from the T_1 transgenic plants selected in the previous step were grown in glufosinate-containing medium. Lines that resulted in a roughly 3:1 ratio of resistant to non-resistant plants were favorably chosen over lines that generated a 15:1 ratio (pointing to the existence of two inheritable OX genes). We then grew chosen lines and harvested the T_3 progeny for selecting lines homozygous for the insertion, by choosing lines where all T_3 progeny carried the selection marker. Using this method, we aimed to create 4 independent-insertion, homozygous OX lines for each chosen TF for use in future screens.

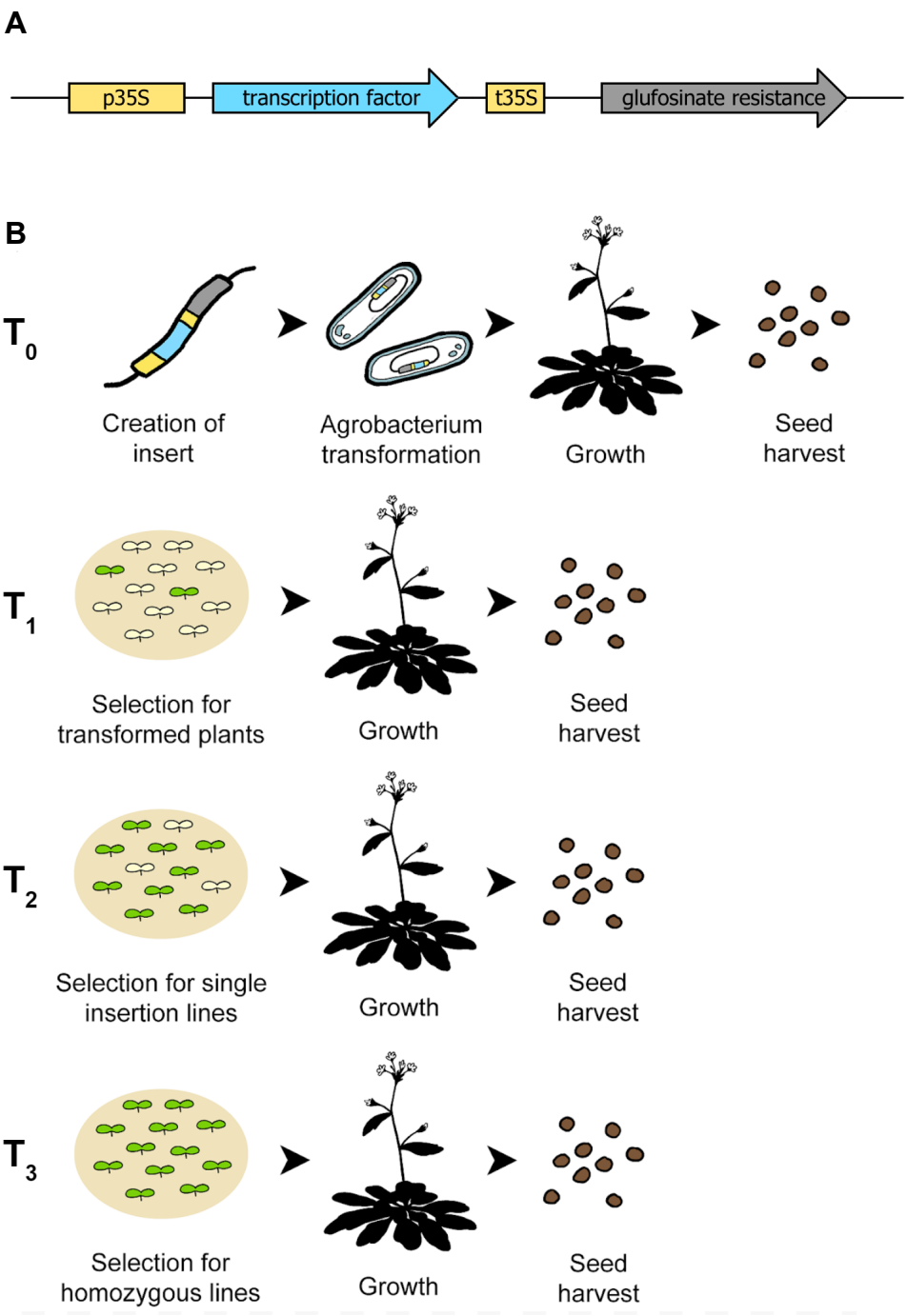


Figure 1. Creation of the TF-OX library. A) Schematic of the cassette inserted into *A. thaliana*. The cauliflower mosaic virus 35S promoter is attached to the coding sequence of a chosen transcription factor gene. *BAR*, a *Streptomyces hygroscopicus* gene, confers resistance to glufosinate ammonium when expressed in plants, and acts as the selection marker. **B)** Flowchart of line creation process at each generation.

A subset of 400 TFs previously shown to be differentially expressed under stress conditions (personal correspondence from Dr. Katherine Denby) was initially chosen to be the first batch of TFs used to create OX lines for this library (Table 1). We anticipated that generating 1600 OX lines (400 TFs with 4 lines each) would take an immense amount of time, so we decided to start by transforming TF-OX constructs into plants in smaller subsets. OX constructs for each TF were transformed to Arabidopsis Col-0 plants and grown onto glufosinate for transgenic selection. We obtained T_1 insertion lines for 131 TFs, and followed the outlined procedure (Figure 1B) to generate single-insertion, homozygous lines. Currently, we have finalized (brought to homozygosity) 176 lines, encompassing 60 different TFs (Table 1). For 21 TFs, we have created a full set of four independent insertion lines (84 total lines). For 39 TFs, we have finalized between 1-3 lines, and the lines needed to complete the set are in progress (at the T_2 stage, see Table 1). Only 4 of 131 TFs have shown consistent issues with bringing OX lines to homozygosity (Table 2), suggesting that it may not be possible to include these TFs in the final collection and that other means will be required to establish their function in planta.

TABLE 1 - Number of transcription factors at each stage of the project.

Category	# of TFs	# of Lines
Identified in *A. thaliana* genome	2492	
In clone library	1956	
Selected for first stage of OX library creation	400	
Transformed into plants via the OX system	131	
Has 1 finalized OX line	3	3
Has 2 finalized OX lines	19	38
Has 3 finalized OX lines	17	51
Has 4 finalized OX lines	21	84

TABLE 2 - TF-OX lines with issues prohibiting line finalization.

Gene	AGI Code	Issue	Collection Code
HB30	AT5G15210.1	Senescing before maturity	U05-H07
MYB20	AT1G66230.1	No germination in T_3 generation	U06-C08
WRKY18	AT4G31800.1	Senescing before maturity	U04-A06
ZFP8	AT2G41940.1	Senescing during early vegetative phase	U03-F12

Developing a phenotype-based screen to identify regulators of plant stress responses

Once we had created a substantial number of TF OX lines, we sought to use them in screens to quickly identify TFs that may play a role in stress response pathways. TFs that directly affect stress response should, when overexpressed, cause plants to exhibit an abnormal phenotype under stress conditions compared to wild-type *Arabidopsis*. To screen for abnormal stress response phenotypes, we devised several different stress treatment protocols that could be easily scaled to test hundreds of plant lines at once, as was necessary for the large number of transgenic plant lines we were working with. We sought to find a single protocol that would be easy to perform, produce results quickly, and be sufficiently reproducible. Given that all forms of abiotic stress eventually lead to oxidative stress, we chose to induce oxidative stress in our different OX lines to measure stress response. For that, we tested hydrogen peroxide (H_2O_2), a ROS which decomposes into hydroxyl radicals inside plant tissues, as a potential treatment. H_2O_2 (1-400 mM) was applied to *Col-0 Arabidopsis* seedlings via aerosol spray at 9 days of age, and plants were then monitored for 7 days to visualize phenotypic differences compared to a control group (treated with H_2O). Using this treatment protocol, phenotypic differences were not observed under any of the treatment conditions tested (data not shown). We hypothesized that the plants were too old at the time of treatment to be affected by the concentrations used. We also decided that delivering the treatment manually via a spray was not optimal for homogenizing treatment across groups, as it was slow and delivered varying amounts depending on angle and distance of the applicator.

We decided to instead test a different protocol where plants would be grown for 10 days and then transferred into a medium containing H_2O_2, rather than spraying them (Figure 2A). Using this treatment protocol, we tested a range of H_2O_2 concentrations from 5-400 mM and

evaluated phenotypic differences for 3 days after transfer to the treatment plate. We found that phenotypic differences were visible in H_2O_2-treated plants compared to a mock-treated control group at concentrations higher than 50 mM H_2O_2. In order to quantify these phenotypes, we decided to measure hue of the plant as a visual indicator of chlorosis (loss of chlorophyll), as chlorosis is a direct indicator of ROS oxidative damage to plant tissue, and using a visual metric would simplify the data collection in such a large screen. Hue of each treatment group was quantified and then normalized to get a value of "relative greenness" (RG), plotted along a scale of "fully bleached" (0.0) to "fully healthy" (1.0) (Figure 2B). Results of the hue analysis indicated that plants treated with 50 mM or more displayed a quantifiable phenotypic difference (not shown). Given that the ideal set-up for a stress screen would allow us to easily identify lines that were either more or less resistant to the treatment compared to *Col-0*, we chose to use 100 mM H_2O_2 as the treatment concentration, for it resulted in an RG value near the middle of the scale (0.40-0.50).

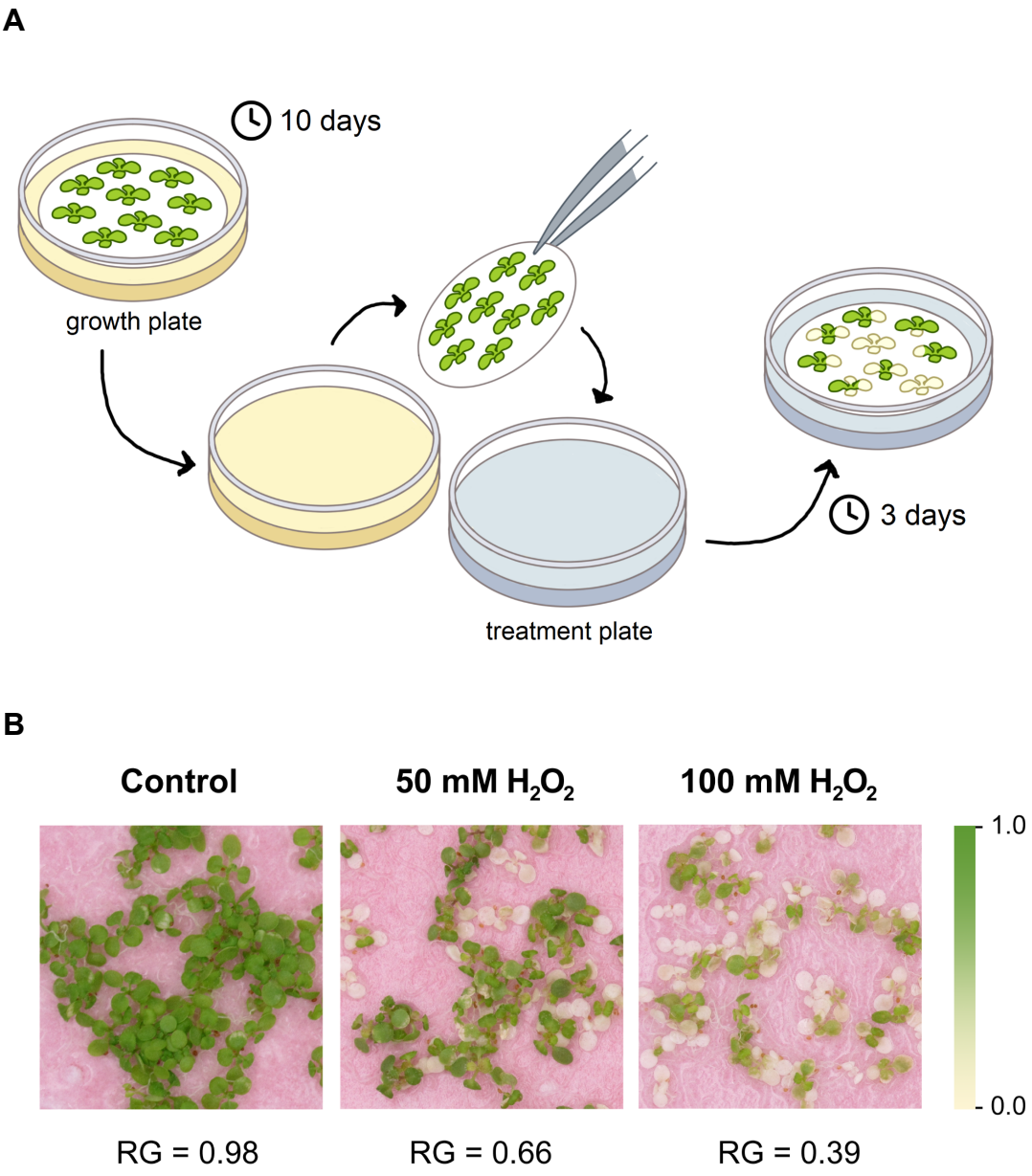


Figure 2. A hydrogen peroxide screen to measure *Arabidopsis* phenotype in response to oxidative stress. A) Method for a transfer-based treatment. Plants are first grown on an agar plate containing no stressor, then transferred to a new plate containing 100 mM H_2O_2. Bleaching in response to treatment is observed after 3 days. **B)** *Col-0* plants treated with 0 mM, 50 mM, and 100 mM H_2O_2 for 3 days. On right, scale of measured hue values from minimum (fully bleached) to maximum (*Col-0* untreated). Average hue for each plate is plotted along this scale to obtain "relative greenness" (RG) metric. Relative greenness of each plate noted below image. Plants photographed on pink background for programmatic data processing.

Screening the partial Arabidopsis TF OX collection to identify TFs regulating plant stress responses

Once we had determined a suitable screening pipeline for measuring stress response, we performed two identical screens incorporating all TF-OX lines in the collection that had at least 3 finalized lines (38 TFs total) (Figure 3A, B). A *35S::TCP19* OX line from outside the collection was also included in screens as we hypothesized it would be more susceptible to stress. OX constructs can have different levels of expression depending on the location of insertion into the genome (Pérez-González & Caro 2016). As Agrobacterium-mediated transformation leads to random insertions of the OX construct, we were not expecting all lines belonging to any one TF to have the same expression level and therefore display the same phenotype upon treatment. Hence, our goal was to identify TFs that had at least one overexpression line showing a significant and consistent phenotypic change (compared to *Col-0*) in response to the H_2O_2 treatment.

In the primary screens, we found that though there were visible differences between treatment groups, none of the OX lines displayed a statistically significant phenotype difference from *Col-0* (Student's *t*-test, all p-values > 0.05). Since each line had only a single replicate in each screen, we believed that the variation in the treatment groups could be hindering our ability to identify TFs that resulted in higher resistance or susceptibility to H_2O_2 when overexpressed. Further analysis of our results determined that the standard deviation in the *Col-0* treatment group was several times larger than in the *Col-0* untreated group, pointing to large variation in treatment group results (Figure 3C). Thus, we decided to perform a secondary screen including a larger number of replicates per line. Candidates for the secondary screen were chosen from the results of the initial primary screens. As no results from the primary screens were statistically

significant, we decided to first look for lines that displayed consistent differences of at least 2 standard deviations above or below the *Col-0* group in both primary screens (Figure 3A). However, only two lines fit this criterion. Since we would be confirming any phenotypes in the secondary screen, we expanded our pool of candidates by lowering the threshold to 1 standard deviation above or below *Col-0* (Figure 3B). If a certain line was selected using this method, all lines for that specific TF were tested in the secondary screen.

We ran the secondary screen with 4 treated and 2 untreated replicates per line. By analyzing hue values in untreated plants, we first excluded lines that were significantly different from the control (TF-OX vs Col-0), as this would indicate that such plants have an underlying condition that leads to chlorosis, which may not be related to a plant's ability to deal with stress. Next, we looked for lines that, being similar to Col-0 in basal conditions, demonstrated significantly higher or lower resistance (Student's *t*-test, $p < 0.05$), to the H_2O_2 treatment compared to *Col-0* (Figure 3D). We found one line (1 TF) that was more resistant and nine lines (7 TFs) that were more sensitive to the H_2O_2 treatment. Finally, these lines were genotyped to confirm the identity of the overexpressed gene (Table 3).

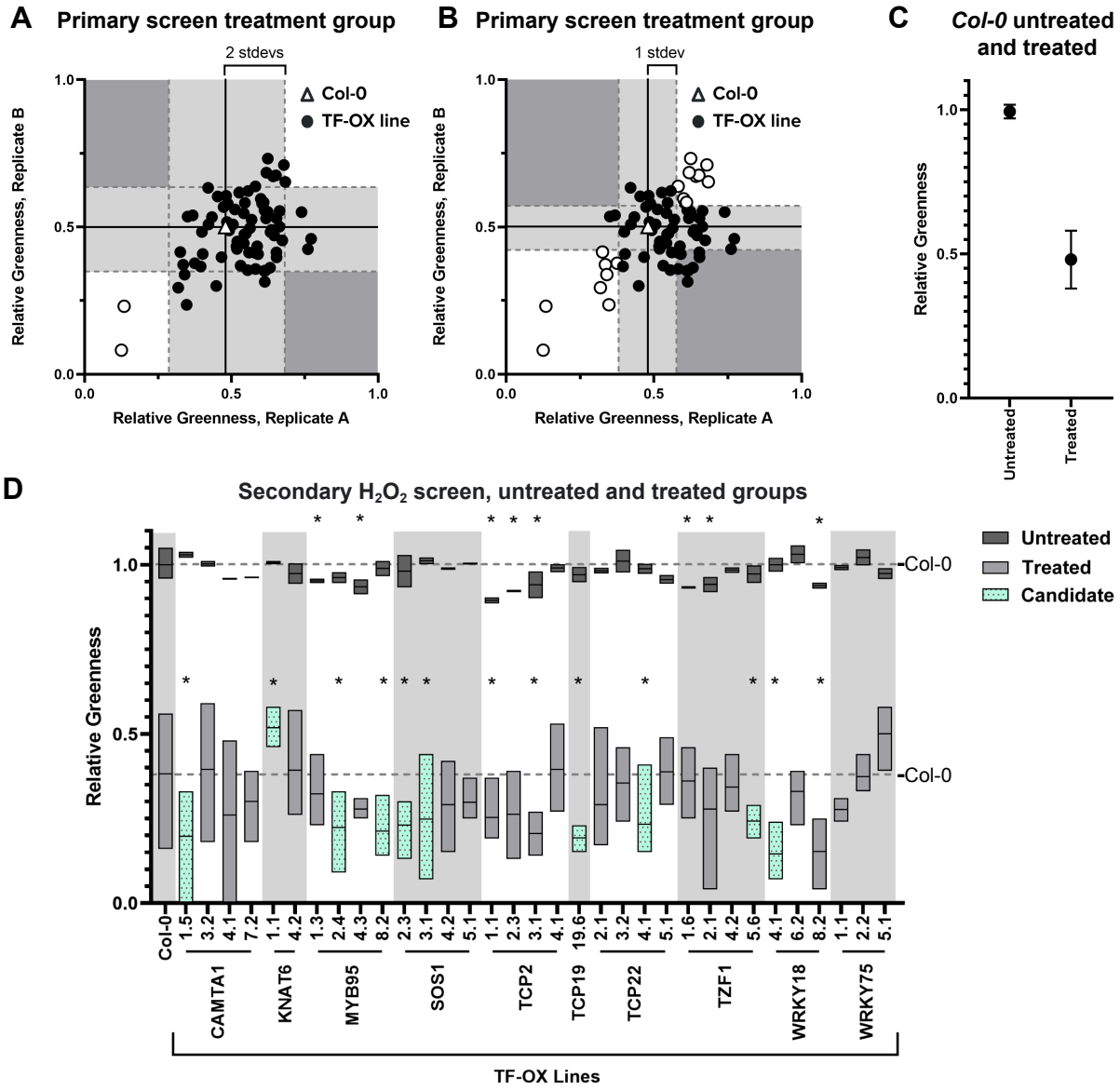


Figure 3. Results of primary and secondary oxidative stress screens. A) Treated results from both primary screens plotted against each other. Each dot corresponds to a single TF-OX line. *Col-0* means noted by lines for reference. 2 standard deviations (stdevs) are shaded in either direction. Lines falling outside of 2 stdevs in both screens are noted by a white dot. **B)** Area shaded has been shrunk to 1 stdev in either direction. Lines falling 1 stdev away in both screens are noted by a white dot. **C)** *Col-0* treated and untreated values from primary screen. Error bars display 1 stdev in either direction. n = 12 in both groups. Untreated: 0.48 ± 0.10; treated: 0.99 ± 0.02. **D)** Results from secondary screen. Each lane represents one TF-OX line. Bars portray range and mean. Dark grey bars represent measurements taken from untreated plants. n = 6 for *Col-0*, n = 2 for all other lines. Light grey and teal bars represent measurements taken from treated plants. n = 12 for *Col-0*, n = 4 for all other lines. *Col-0* mean for untreated and treated groups are denoted by dashed lines at 1.00 and 0.38, respectively. Bars significantly different from *Col-0* ($p < 0.05$) in either group are marked by an asterisk. Lines that show a significant difference after treatment but not before treatment are colored with a patterned teal.

TABLE 3 - TF-OX lines sensitive or resistant to hydrogen peroxide treatment.

Name	TF Family	AGI Code	Phenotype	Collection Code	Line #
CAMTA1	CAMTA	AT5G09410.2	Sensitive	U21-C07	1.5
KNAT6*	HB	AT1G23380.1	Resistant	U03-E11	1.1
MYB95*	MYB	AT1G74430.1	Sensitive	U05-G05	2.4, 8.2
SOS1	PHD	AT2G37520.1	Sensitive	U21-C02	2.3, 3.1
TCP19*	TCP	AT5G51910.1	Sensitive	U18-C02	19.6
TCP22*	TCP	AT1G72010.1	Sensitive	U03-H09	4.1
TZF1*	C3H	AT2G25900.1	Sensitive	U06-H04	5.6
WRKY18*	WRKY	AT4G31800.1	Sensitive	U04-H07	4.1

*TF coding sequence confirmed through sequencing of plant lines

Screening Arabidopsis TF OX lines for abnormal developmental phenotypes

In addition to the stress-tolerance screens, we performed a developmental phenotype screen to identify TF-OX lines with unusual phenotypes under non-stress conditions. We hypothesized that differing sensitivity to ROS in our oxidative stress screen is due to differing levels of ROS under basal conditions. For example, we would expect to see that a line demonstrating increased sensitivity to ROS, or poor ROS reduction mechanisms, would tend to have higher levels of ROS under all conditions. Plants with higher basal levels of ROS tend to grow smaller and flower later (Sewelam 2013). Thus, we expected to see oxidative stress resistance positively correlated with plant size, and negatively correlated with plant flowering time.

To perform this developmental screen, TF lines showing significantly increased or decreased resistance to stress treatment (Table 3) and *Col-0* controls were grown in soil until maturity. Plants were photographed periodically to measure plant size over time (Figure 4A) and the number of days to flower was recorded (Figure 4B). We next analyzed the correlation between the developmental screen and stress-tolerance results, and found that resistance to H_2O_2 treatment and plant area were positively correlated ($R^2 = 0.61$, $p = 0.022$), confirming a relationship between the two (Figure 4C). Likewise, we found that resistance to H_2O_2 treatment and days to flower were negatively correlated ($R^2 = 0.62$, $p = 0.020$) (Figure 4D). These results are consistent with our hypothesis that lines showing a sensitivity or resistance to the H_2O_2 screen have elevated or reduced ROS levels, respectively, in basal conditions. This could be further confirmed in a future experiment that directly quantifies the amount of ROS found in leaf tissue.

Additionally, while generating the TF-OX line collection, we noted that lines that overexpress certain TFs consistently displayed abnormal developmental phenotypes. To confirm these observations, these lines were also evaluated in this screen and phenotypes recorded (results are summarized in Table 4).

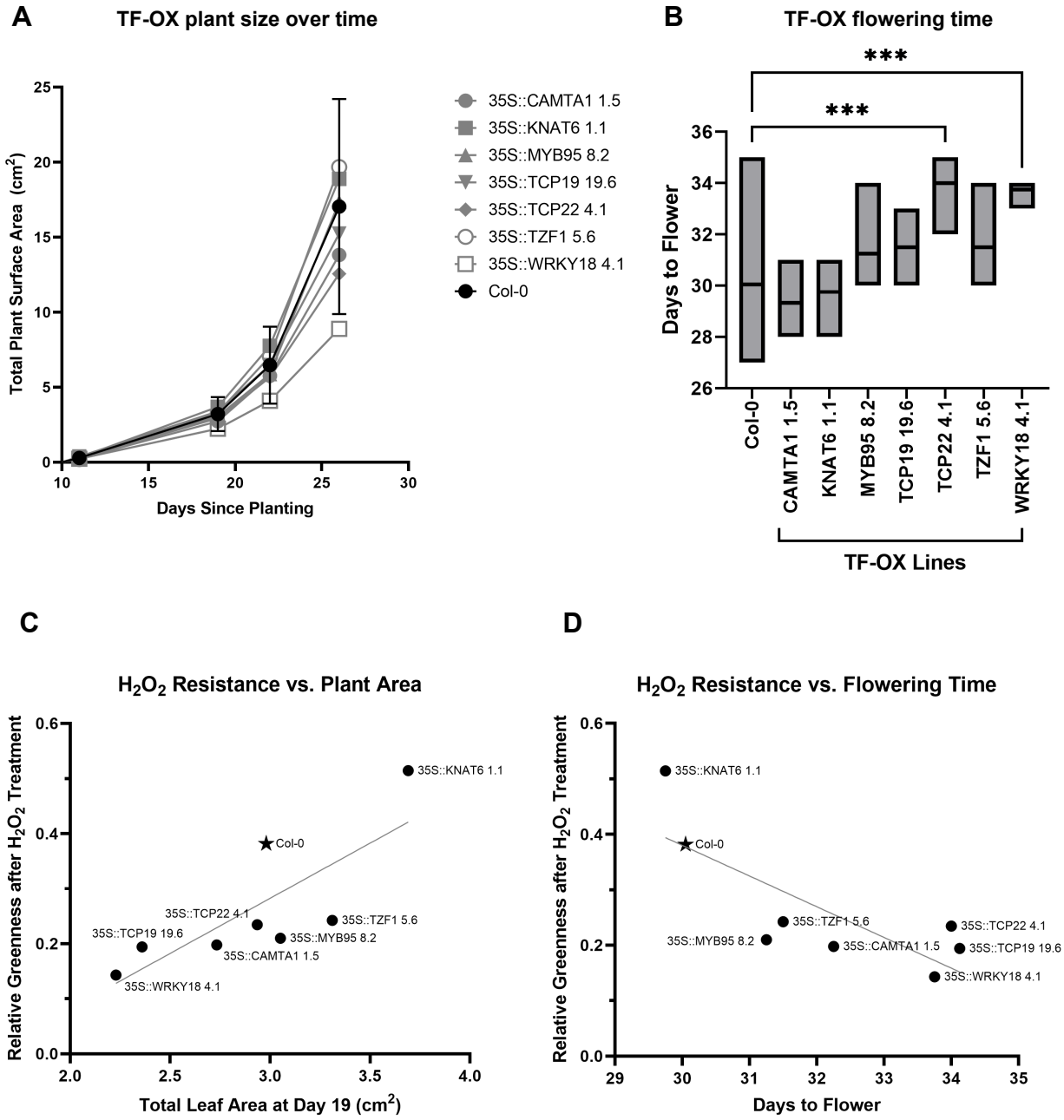


Figure 4. Results of developmental screen. A) Plant surface area in cm^2 over time. n = 16 for *Col-0*, n = 4 for all TF-OX lines. No lines were significantly different ($p < 0.05$). **B)** Days to flower for each TF-OX line. Flowering time was measured as the number of days since germination it took to produce an inflorescence >1 cm in height. Bars show min-max range and mean. n = 16 for *Col-0*, n = 4 for all other lines. $p < 0.001$ for significant lines. **C)** Mean relative greenness from secondary oxidative stress screen plotted against mean plant area at day 19. Data points fit with linear regression. $R^2 = 0.61$, $p = 0.022$. **D)** Mean relative greenness from secondary oxidative stress screen plotted against mean flowering time. Data points fit with linear regression. $R^2 = 0.62$, $p = 0.020$.

TABLE 4 - Qualitative phenotypes observed in developmental screen.

Name	AGI Code	Phenotype	Collection Code	Line #
ERF036	AT3G16280.1	Yellowing leaves	U01-E12	3.3, 6.1, 7.3
HB23	AT5G39760.1	Short, round leaves	U03-C01	2.1, 9.1
HRS1	AT1G13300.1	Narrower leaves	U03-H09	2.1, 4.1
KAPP	AT5G19280.1	Stunted growth, yellowing leaves	U04-H07	4.1, 6.2, 8.2

Characterization of TCP19 as a novel regulator of plant responses to stress

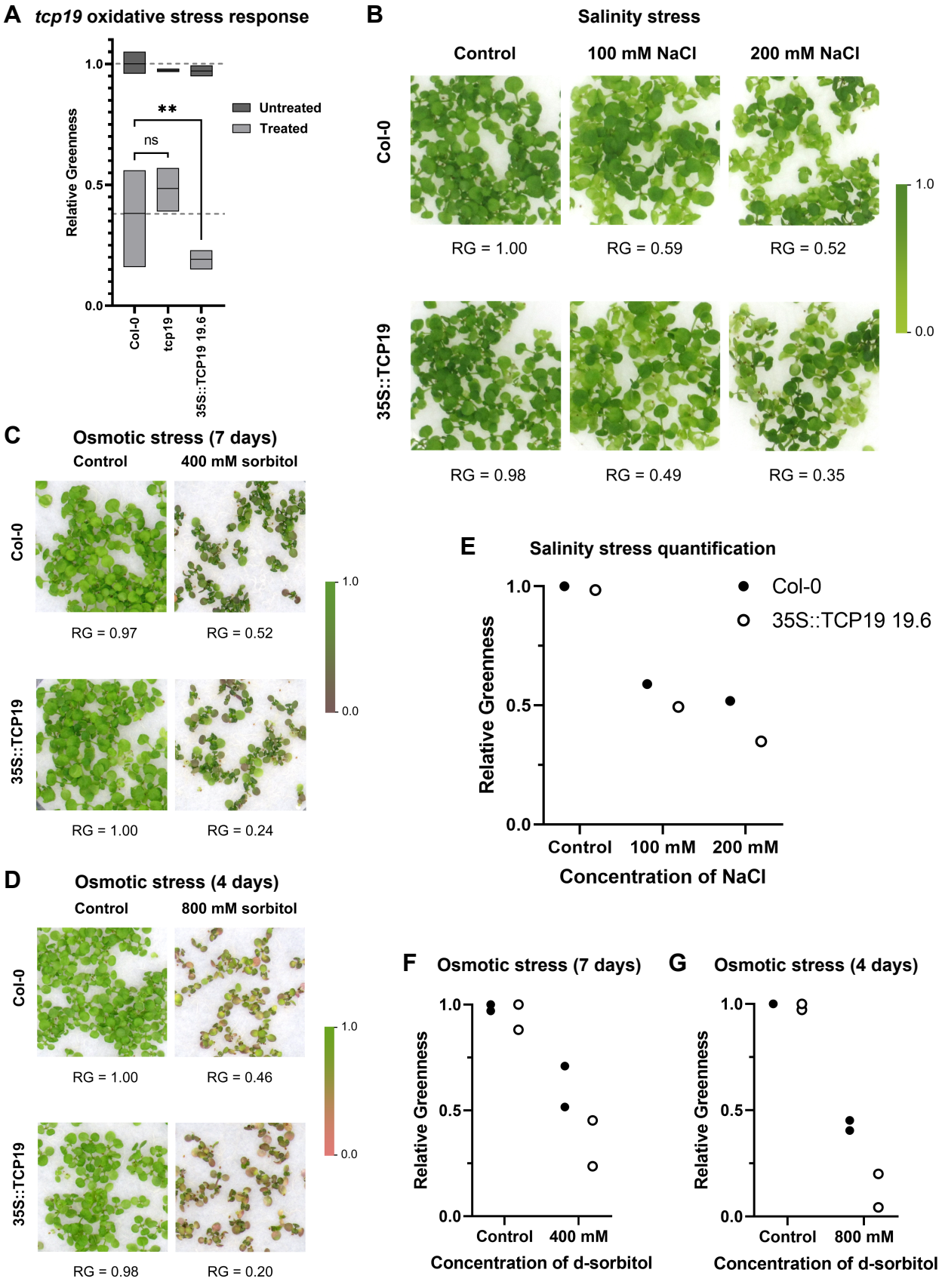
According to our screen results, *TCP19* emerged as a candidate for regulating plant stress responses as its OX line (*35S::TCP19*) resulted in a highly significant sensitivity to H_2O_2 treatment among the OX lines that we tested. We reasoned that a *tcp19* knock-out mutant could then have an increased resistance to H_2O_2 and therefore may be more resistant to stress. To test this hypothesis, we analyzed the sensitivity of *tcp19* mutant plants to the H_2O_2 treatment (following the same treatment protocol that we used in our screens). We found that *tcp19* mutant plants were on average more resistant to the H_2O_2 treatment compared to *Col-0* (+0.11 RG), however, this difference was not statistically significant (p=0.113) (Figure 5A). This could suggest that although *TCP19* is involved in stress response, other TFs in the TCP gene family may be redundant to *TCP19* in regulating stress, reducing the difference in phenotype in *tcp19*.

We also investigated if *35S::TCP19* had increased sensitivity to other stress treatments known to result in increased ROS. To test this, we designed two additional experiments to test for tolerance to high salinity stress (using NaCl treatment) (Figure 5B) and to osmotic stress (using d-sorbitol treatment) (Figure 5D). We tested various concentrations of each stressor using *Col-0* and *35S::TCP19* plants in order to find an appropriate concentration that would allow us to visualize both increased resistance or sensitivity to the treatment when comparing the response of *35S::TCP19* and wild-type plants (Figure 5B, D, F). As we were optimizing the experimental conditions, we found that NaCl treatments resulted in a less severe chlorosis (compared to H_2O_2 treatments), and that d-sorbitol treatments triggered the accumulation of anthocyanins turning green tissues purple. Due to these color variations, we also adapted the hue-analysis method in order to be able to effectively quantify the effects of each treatment (see materials and methods). After setting the experimental conditions and data analysis strategy, we performed experiments

using *35S::TCP19* and *Col-0* plants. We found that *35S::TCP19* was less resistant than *Col-0* in both stress screens at different concentrations of NaCl and d-sorbitol (Figure 5C, E, H). These results indicated that *TCP19* is a negative regulator of stress response, which is consistent with the results of our initial screen for oxidative stress.

As the increased sensitivity to salinity and osmotic stress is consistent with increased sensitivity to osmotic stress, this also affirms that our H_2O_2 screen method would likely be a useful indicator of abnormal stress-response for a variety of stress conditions. We would like to further test other ROS-regulating candidates under the salinity and osmotic stress screens to see if they demonstrate the same correlation. The hue-analysis method was effective for quantifying results in both NaCl and d-sorbitol treatments, though we observed that plant size was also a sensitive indicator of stress response in the d-sorbitol screen. These screening conditions are optimized to be used to test OX lines in the future and measure stress response phenotypes in relation to varying types of stress.

Figure 5. *35S::TCP19 Arabidopsis* line exhibits increased sensitivity to various stress conditions. A) *Col-0*, *35S::TCP19*, and *tcp19* treated with 100 mM H_2O_2. Relative greenness calculated using the same scale as previous oxidative stress screens. Bars represent min-max range and mean. n = 16 for *Col-0*, n = 4 for *35s::TCP19* and *tcp19*. $^{ns}p = 0.113$, $^{**}p < 0.001$ **B)** Images of plates containing *Col-0* or *35S::TCP19* treated at 0 mM, 100 mM, and 200 mM NaCl for 10 days. On the right, scale of Relative greenness values from minimum (arbitrary) to maximum (*Col-0* untreated). RG for each image is noted below the panel **C)** Images of plates containing *Col-0* or *35S::TCP19* treated at 400 mM d-sorbitol for 7 days. **D)** Images of plates containing *Col-0* or *35S::TCP19* treated at 800 mM d-sorbitol for 4 days. **E)** RG values from NaCl treatment. n = 1 for each data point. **F)** RG values from 400 mM d-sorbitol treatment. Replicates plotted separately. **G)** RG values from 800 mM d-sorbitol treatment. Replicates plotted separately.


A
tcp19 oxidative stress response
Relative Greenness
1.0
0.5
0.0
**
ns
Untreated
Treated
Col-0
tcp19
35S::TCP19 19.6
B
Salinity stress
Control
100 mM NaCl
200 mM NaCl
Col-0
35S::TCP19
RG = 1.00
RG = 0.59
RG = 0.52
RG = 0.98
RG = 0.49
RG = 0.35
1.0
0.0
C
Osmotic stress (7 days)
Control
400 mM sorbitol
Col-0
35S::TCP19
RG = 0.97
RG = 0.52
RG = 1.00
RG = 0.24
1.0
0.0
D
Osmotic stress (4 days)
Control
800 mM sorbitol
Col-0
35S::TCP19
RG = 1.00
RG = 0.46
RG = 0.98
RG = 0.20
1.0
0.0
E
Salinity stress quantification
Relative Greenness
1.0
0.5
0.0
Col-0
35S::TCP19 19.6
Control
100 mM
200 mM
Concentration of NaCl
F
Osmotic stress (7 days)
Relative Greenness
1.0
0.5
0.0
Control
400 mM
Concentration of d-sorbitol
G
Osmotic stress (4 days)
Relative Greenness
1.0
0.5
0.0
Control
800 mM
Concentration of d-sorbitol

Discussion

Studying the biochemical function of transcription factors in plants requires unique approaches that are both effective and scalable to encompass the large number of TFs in existence. Here, we have devised a pipeline which utilizes existing community resources to create a collection of *A. thaliana* transcription factor overexpression lines. We have also designed a straight-forward screening protocol to identify TFs involved in the regulation of plant oxidative stress that can be scaled to hundreds of TFs, identified 8 TFs as potential novel regulators of ROS homeostasis in planta, and found 4 additional TFs that regulate plant development. Our pilot experiments, using a small subset of TF overexpression lines, indicated that this would be a successful approach to identify TF function in vivo, which would require the expansion of the OX collection to include OX lines for all TFs in the *Arabidopsis* genome. We propose this approach as an alternative to the use of T-DNA insertion libraries or other genome-wide mutagenesis methods for TF screening, as TF overexpression would help avoid the confounding effects of redundant TFs on plant phenotypes.

While generating this initial collection of lines, we did find a few TFs (4 out of 131) that, when overexpressed, had prohibitively low germination rates or senesced before plants reached the reproductive stage, making it difficult to create TF-OX lines for them. We also noted that, when genotyped, the *35S::TCP2* lines held several large deletions in their coding sequences, suggesting that we were only able to obtain "OX" lines for *TCP2* if protein function was disrupted, and that *TCP2* is toxic to plants when overexpressed. It is likely that another approach will need to be adopted for these TFs on a case-by-case basis, such as an inducible-expression system or a weaker constitutive promoter. However, our constitutive OX approach worked for

the vast majority of TFs we used while building this collection, indicating strong potential for usage across almost all *Arabidopsis* TFs.

We also developed a phenotype-based screen to measure relative sensitivity/resistance to oxidative stress of the generated TF-OX lines. This screen allowed us to see lines which were strongly different from wild-type under high-stress conditions. However, the high variability in results makes this screen difficult to use for more than a dozen sets of TF-OX lines. Additionally, the measurement used in the screen, severe chlorosis, is an extreme phenotype and may not necessarily demonstrate plant tolerance under lower levels of stress (Claeys et al. 2014). Measurements of stress response obtained via severe chlorosis may not directly translate to stress response in real-world conditions, and thus would have fewer useful applications. While developing this screen, we had initially used the metric of "bleached" vs. "unbleached" tissue, so the H_2O_2 concentration was adjusted to give the *Col-0* control group a ~50% bleaching rate. However, we later switched to using the more sensitive and accurate metric of hue (color) value. Due to this improvement in data quantification, the concentration of H_2O_2 could be lowered in future screens to induce a smaller but significant chlorosis in treated plants, rather than the bleached phenotype observed in this screen. Alternatively, the screen could be adapted to measure a different, more sensitive phenotype, such as plant size (Claeys et al. 2014). Both approaches have trade-offs, but would likely improve both the replicability and scalability of oxidative stress-response screens overall. Similarly, we developed stress-response screens using NaCl to induce salinity stress and d-sorbitol to induce osmotic stress. These screens were designed with the intention of being secondary (or tertiary) screens to test candidates already identified from initial oxidative stress screens. Salt stress induces chlorosis in treated plants, so this screen benefits from the use of the hue value metric to quantify results. With an adjustment

to the range of hues used, hue value analysis can also be used to quantify osmotic stress response, due to anthocyanin accumulation. We noted that plant size may also be a sensitive indicator of osmotic stress response, so further experiments should be done to determine which quantification would be best for a large-scale screen.

Ultimately, with the use of the oxidative stress screen, we identified lines in our collection that were significantly more or less resistant to oxidative stress than *Col-0* (Refer to Table 3 for a list). We believe that the TFs expressed in these OX lines are most likely part of pathways involving ROS production (thus affecting the rate that the plant creates ROS), or pathways regarding ROS scavenging (thus affecting the rate that ROS is removed from plant tissues). Results from the developmental screen show further evidence that these TFs also have an effect on basal ROS levels, not just under induced oxidative stress. This makes them interesting candidates for functional characterization in the future, and suggests that these TFs may be involved in the regulation of plant responses to various stress conditions. Indeed, we found that *TCP19*, one candidate that negatively related plant response to oxidative stress, also negatively regulated responses to salinity stress and osmotic stress. Future work on this project should test stress-response phenotypes of plants mutant for the candidate genes. It would be of particular interest to evaluate if TFs that resulted in high sensitivity to the stress when overexpressed demonstrate the opposite phenotype when mutated. If mutant lines do not demonstrate any significant phenotypes as expected, higher order mutants with increased resistance could be designed by knocking out the function of multiple TFs identified using our screening methods.

Materials and Methods

Generation of *35S::TF* constructs

Arabidopsis thaliana TF coding sequences were transferred from pENTR/D plasmids containing each coding sequence (Pruneda-Paz et al. 2014) to pB7WG2 via Gateway recombination-based cloning (Life Technologies).

Creation of transgenic plants

Transgenic *Arabidopsis thaliana* lines were created via *Agrobacterium*-mediated transformation. Desired constructs were transformed into AGL0 *Agrobacterium* through electroporation. Transformed bacteria were then incubated, selected for the construct, and further incubated in LB broth. Columbia ecotype (*Col-0*) *A. thaliana* plants were grown until flowering, then flowers were dipped into the LB broth to infect the plants with *Agrobacterium*. Plants were temporarily placed under plastic bags to promote bacterial growth, then allowed to grow until T_1 seeds could be harvested.

Selection of single insertion homozygous lines

For selection against glufosinate-ammonium, sterile seeds were cold-stratified for 2-3 days at 4°C and grown in plates containing Murashige-Skoog medium, 1.5% agar, 1% sucrose, and 0.016% glufosinate ammonium under 12 hour light/dark cycles (100 uE) at 22°C for 2-3 weeks. Any selected plants were then transferred to soil and grown under 12 hour light/dark cycles until maturity and seed collection. Eight T_1 progeny (from eight independent insertion events) showing glufosinate resistance were chosen for each TF, then harvested to obtain the T_2 generation. T_2 progeny was grown on selection medium, and four lines displaying a 3:1 segregation ratio for the glufosinate resistance phenotype were chosen. From each line, six individuals were transferred to soil and grown until maturity and seed collection. T_3 progeny

from chosen individuals were again grown on selection medium, and one line displaying glufosinate resistance in 100% of progeny (homozygous for the insertion) was chosen.

Transfer-method stress screens

Sterile seeds were cold-stratified for 2-3 days at 4°C and grown in plates containing Murashige-Skoog medium, 1.5% agar, and 0% sucrose under 12 hour light/dark cycles (100 uE) at 22°C. A circular Whatman cellulose filter paper was placed on top of the growth medium before adding seeds on top, to later facilitate the transfer of plants to a new plate. After growing for 10 full days (for H_2O_2 treatment), 7 full days (for NaCl treatments) or 10 full days (for d-sorbitol treatments), filter papers with plants on top were moved to new plates, containing 100 mM H_2O_2, 100/200 mM NaCl, or 400/800 mM d-sorbitol in the growth medium. Plants were placed back in the incubator to grow on treatment medium for 3 days (for H_2O_2 treatment), 10 days (for NaCl treatments), 4 days (for 800 mM d-sorbitol treatments), or 7 days (for 400 mM d-sorbitol treatments), then photographed.

Hue value quantification

Photographs from each screen were obtained by photographing plants directly from above under bright light. Images were automatically analyzed using ImageJ. First, plant material in the image was selected by excluding the background color(s). Then the image was converted into an HSB stack and the average Hue value was taken for the selection. Hue values were normalized to a minimum value (hue of a fully-bleached section of leaf for H_2O_2 screen, arbitrary boundary for salinity and osmotic screens) and a maximum value (hue of untreated *Col-0* control group), then converted into a percentage along that scale.

Developmental phenotype screen

To monitor for leaf surface area, flowering time, and qualitative phenotypes such as leaf shape, TF-OX lines were grown in individual pots (to prevent crowding effects) under long day conditions (16 light/8 dark) at 22°C in sterile soil. Plants were watered weekly with a solution containing 1.25g/L fertilizer (excess fertilizer solution was removed 24 hours after watering). Photographs were taken from above periodically. Leaf surface area was automatically calculated in ImageJ by counting yellow-green pixels. Flowering date was recorded when the first inflorescence of each plant reached 1 cm in height.

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