WRKY transcription factors and ethylene signaling modify root growth during the shade avoidance response

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Low red:far-red light in shade induces genes in roots regulated by WRKY transcription factors and ethylene, restricting primary and lateral root growth.

List of author contributions:
U.V.P. conceived the study. D.R., M.R., and U.V.P. designed experiments. D.R. performed most of the experiments and analyzed the data; A.A. and O.S. performed genotyping, generation of transgenic plants, and immunoblots, D.R. and U.V.P. wrote the paper and collected contributions of all authors.

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Abstract

Shade-intolerant plants rapidly elongate their stems, branches, and leaf stalks to compete with neighboring vegetation, maximizing sunlight capture for photosynthesis. This rapid growth adaptation, known as the shade avoidance response (SAR), comes at a cost: reduced biomass,

crop yield, and root growth. Significant progress has been made on the mechanistic understanding of hypocotyl elongation during SAR; however, the molecular interpretation of root growth repression is not well understood. Here, we explore the mechanisms by which SAR induced by low red:far-red light restricts primary and lateral root (LR) growth. By analyzing the whole-genome transcriptome, we identified a core set of shade-induced genes in roots of Arabidopsis (*Arabidopsis thaliana*) and tomato (*Solanum lycopersicum*) seedlings grown in the shade. Abiotic and biotic stressors also induce many of these shade-induced genes and are predominantly regulated by WRKY transcription factors. Correspondingly, a majority of *WRKY* genes were among the shade-induced genes. Functional analysis using transgenics of these shade-induced *WRKYs* revealed that their role is essentially to restrict primary root and LR growth in the shade; captivatingly, they did not affect hypocotyl elongation. Similarly, we also found that ethylene hormone signaling is necessary for limiting root growth in the shade. We propose that during SAR, shade-induced WRKY26, 45, and 75, and ethylene reprogram gene expression in the root to restrict its growth and development.

Introduction

Plants are exposed to various environmental challenges throughout their life cycles, such as suboptimal access to sunlight, low water, nutrient availability, extreme temperatures, presence of competitors, herbivores, and pathogens (Casal, 2012). Plants exhibit incredible plasticity to withstand these adverse conditions and respond by locally modifying growth rhythms, metabolism, and reproduction to best adapt to their environment (Chory, 2010; Kohnen et al., 2016). An excellent example of adaptive phenotypic plasticity is the shade-avoidance response (SAR). In shade-intolerant plants, SAR is triggered when they are in close proximity to other plant competitors or under a canopy by activating a series of morphological changes to maximize sunlight capture and ensure reproductive fitness (Smith, 1982). The characteristic phenotypes of SAR include rapid stem and petiole elongation, leaf hyponasty, accelerated reproduction, apical dominance, and reduced root growth and development (Salisbury et al., 2007; Casal, 2012). The molecular mechanisms controlling gene expression changes leading to the phenotypic alterations in the shoot organs during SAR are well understood in the model plant Arabidopsis (*Arabidopsis thaliana*) (Casal, 2012; Li et al., 2012; Galvão and Fankhauser, 2015; Pedmale et al., 2016). But the impact of shade on the growth of underground root systems and the molecular account leading to this phenomenon are poorly understood.

Under a dense canopy, plants sense vegetational shading by detecting either a reduction in the ratio of red to far-red (R:FR) light, blue light, or photosynthetically active radiation (PAR) (Keller et al., 2011; Keuskamp et al., 2011; Hornitschek et al., 2012). Any changes in the red/far-red light (R: FR) and blue light in the environment are largely perceived by the R/FR light-sensing phytochrome B (PHYB) and UV-A/blue light-sensing cryptochrome (CRY) 1 and 2 photoreceptors, respectively. In seedlings, CRY- and PHY-mediated shade perception induces the expression of growth-promoting genes in the hypocotyl, such as those involved in hormone biosynthesis and cell-wall remodeling proteins and enzymes, which are both required for the rapid stem elongation (Kohnen et al., 2016; Pedmale et al., 2016; Wit et al., 2016; Paik et al., 2017).

A handful of studies have linked root growth and development with the SAR, and those have mainly focused on the lateral root (LR) emergence and development (Salisbury et al., 2007; Chen et al., 2016; Gelderen et al., 2018; Gelderen et al., 2021). Salisbury et al. (2007) showed that mainly PHYB induces LR formation via auxin signaling and suggested that the inhibition of LR number under low R:FR might be caused by decreased auxin transport or responsiveness in the roots. Findings from Chen et al. (2016) demonstrated that LR development is induced by shoot illumination regardless of the light conditions in which the roots

are cultivated, suggesting that a long-distance signal produced in the shoots causes LR formation. It was suggested that ELONGATED HYPOCOTYL 5 (HY5) transcription factor (TF), which is stabilized in the shoots under shade (Pacín et al., 2016), is transported to the roots, where it induces its own expression and regulates LR formation. Based on this observation, van Gelderen et al. (2018) demonstrated that HY5 locally represses LR development in the shade by controlling auxin-dependent pathways at the LR primordia. In a recent study, it was reported that the expression of hypocotyl-localized HY5 was insufficient to complement the LR growth defects seen in *hy5* mutant Arabidopsis plants (Burko et al., 2020).

SAR imparts an important adaptative function to a plant under suboptimal conditions by allowing plants to compete for light. However, such adaptation comes at a cost. For instance, plants prioritize rapid stem and petiole elongation over immunity defense response to herbivores in the shoot, and thus shaded plants are more susceptible to microbial diseases and herbivory (Ballaré, 2014). This prioritization of growth responses over defense is likely to make use of the limited resources efficiently. The presence of pathogens or herbivores activate pattern-recognition receptors present on the cell surface to activate pattern-trigger immunity (PTI), which leads to the induction of salicylic acid (SA) and jasmonic acid (JA)-mediated pathways as a defense response (Ballaré et al., 2012). It has been demonstrated that defense responses including JA signaling are lowered in *phyB* mutant and WT plants exposed to low R:FR shade (Leone et al., 2014; Ortigosa et al., 2020).

Plant disease resistance or biotic stress and abiotic stress responses are primarily mediated by WRKY TFs (Pandey and Somssich, 2009). They constitute the largest family of plant-specific transcriptional regulators, acting as either repressors or activators (Bakshi and Oelmüller, 2014). Accumulating evidence shows that a large number of *WRKY* genes take center stage to regulate various aspects of plant innate immunity by responding to herbivores, PTI elicitors, regulation of defense-related SA and JA hormones, synthesis of defense-related compounds, and phytoalexins (Chi et al., 2013). Apart from their role in stress responses, WRKYs also have diverse biological functions in many plant processes not limited to nutrient homeostasis, seed and trichome development, embryogenesis, seed dormancy, senescence, etc. (Eulgem et al., 2000; Skibbe et al., 2008; Mao et al., 2011; Birkenbihl et al., 2018; Karkute et al., 2018; Viana et al., 2018; Chen et al., 2019). WRKY proteins are largely defined by the presence of a conserved WRKY DNA-binding domain defined by the WRKYGQK amino acid sequence. Apart from the WRKY domain, these transcription factors contain an atypical zinc-finger domain in their carboxyl-terminal (Rushton et al., 2010; Chen et al., 2019). WRKY TFs primarily bind to the W-box cis-elements in the promoter of their target genes (Ciolkowski et al.,

2008; Rushton et al., 2010) Thus, WRKYs are essential regulators in responding to internal and external developmental signals as well as stresses.

To understand the molecular account of how low R:FR shade leads to the inhibition of primary root growth, we analyzed the whole genome transcriptome of the roots of Arabidopsis and tomato (*Solanum lycopersicum*) seedlings grown in the shade. We identified a core set of shade-induced genes in the roots of shaded plants, and most of them were also induced by abiotic and biotic stressors. The majority of the shade-induced genes contain W-box promoter elements and are considered the targets of WRKYs. Many *WRKY* family members were also significantly upregulated in the roots of shaded plants. To decipher the contribution of individual WRKYs in controlling root growth during the SAR, we overexpressed in Arabidopsis a large number of shade-induced WRKYs. We identified that *WRKY26* and *WRKY45* overexpression led to a constitutive-shaded, short primary root phenotype even in the absence of shade. In contrast, overexpression of *WRKY75* lead to a decrease in the LR number in the shade but did not affect the primary root growth. Interestingly, the overexpression of these WRKYs affected only the roots, and it did not lead to any hypocotyl elongation defects seen during the SAR. Similarly, like WRKYs, our study implicates ethylene hormone to be necessary to limit root growth but was insignificant for hypocotyl growth in the shade. In summary, we found that low R:FR shade induces a large number of *WRKY*'s, particularly to restrict root growth and development. We hypothesize that the reduced growth of root organs helps the plant divert its critical resources to the elongating organs in the shoot to ensure competitiveness under limiting photosynthetic radiation.

RESULTS

Shade-induced genes in the roots resemble biotic and abiotic stress-induced transcriptome

To determine how the low R:FR of vegetational shade affects root growth and development, we had performed a whole-genome transcriptomic analysis using RNA-seq as a time course on the roots excised from Arabidopsis seedlings grown in white light (unshaded; high R:FR) and white light supplemented with FR (shade; low R:FR) conditions (Fig. 1A and Supplemental Fig. S1A). 5-day-old WL grown Arabidopsis seedlings were transferred to shade or mock-treated, then their roots were harvested after 30 min, 3h, 7h, 1d, 3d, and 5d of treatment duration. Similarly, we performed a comparable experiment in 7d tomato seedlings,

and the root tissue was harvested from them after 3h, 6h, 12h and 24h. Total RNA was isolated from these root tissues and the whole genome transcriptome analysis (RNA-seq) was performed using short-read sequencing. Gene expression matrices and statistically significant (false discovery rate; FDR <0.05) differentially expressed genes (DEG) were determined by comparing the shade and unshaded samples to its own developmental time point (Supplemental Table S1). Pearson's correlation coefficient calculated from the FPKM (fragments per kilobase of exon per million reads) values for each biological replicate indicated a very high correlation (R>0.9) between them (Supplemental Fig. S1B). We identified a total of 4,835 DEGs that were induced along the time-course in Arabidopsis, and 2,523 in tomato in all the time points combined (Supplemental Fig. S1C; Supplemental Table S1). Henceforth, we will refer these upregulated DEG as shade-induced genes. Next, we subjected the shade-induced genes up to 24h time point for Gene Ontology (GO) analysis to assign them a biological function. Our GO analysis on the shade-induced genes in the roots was largely enriched and overrepresented with GO terms related to stress responses, defense against pathogens, and innate immune responses in both Arabidopsis and tomato (Fig. 1C; Supplemental Table S2).

As our GO analysis revealed that the shade-induced genes were also induced during biotic and abiotic stress, and plant's defense against pathogens, therefore, we compared our dataset with the publicly available published RNA-seq datasets, especially related to immunity and defense responses. In one of the comparisons, we chose a study in Arabidopsis that identified 776 common genes that are induced when treated with seven separate elicitors (3-OH-FA, flg22, elf18, nlp20, CO8, OGs and Pep1) of pattern triggered immunity (PTI) (Bjornson et al., 2021). More than half (51%) or 396 of the 776 elicitor-induced genes overlapped with our shade-induced genes (Fig. 1C), representing an enrichment of 3.5-fold over the number of genes that would be expected by random chance (p-value < 10^{-129}). These 396 genes, commonly induced by shade and elicitors of PTI displayed an increased temporal expression in roots of Arabidopsis seedlings exposed to shade (Fig. 1D), suggesting that prolonged exposure to shade activates defense-like responses in the roots in absence of pathogens.

Promoters of the shade-induced genes contain W-box elements

To obtain further insights on the nature of the genes that are universally responding to shade stimuli in the roots, we sought to identify the conserved cis-elements in the promoters of shade-induced genes. We performed *de novo cis*-motif analysis on the promoter sequences (500 bp upstream and 50 bp downstream) of the transcription start site of the shade-induced genes in Arabidopsis (Supplemental Fig. S1C; Supplemental Table S1) as well as those

overlapping with the PTI elicitor-induced genes (Fig. 1C). We identified W-box motif [TTGACC/T] as one of the top enriched *cis*-element among the promoters of the shade-induced genes (*p*-value 10^{-17}; Supplemental Fig. S2A), as well among the shade and PTI elicitor-induced genes (*p*-value 10^{-27}; Fig. 1E). Approximately 33% of the promoters of the shade-induced genes in Arabidopsis roots contained W-box motifs. Interestingly, we did not identify the W-box motif in the promoters of the downregulated genes (Supplemental Fig. S2B). Similarly, W-box is among the top enriched motifs in the promoters of tomato shade-induced genes (Supplemental Fig. S2C). Therefore, considering that WRKYs are central to both biotic and abiotic stresses, we hypothesized that they are likely responsible for the induction of stress and defense-related gene expression program that we observed in the roots of shaded plants (Fig. 1B). Consistently, 48% of the genes known to be directly regulated by WRKY18, WRKY33, and WRKY40 TFs, were also induced by shade (Fig. 1F-G) (Birkenbihl et al., 2017). Among them, well characterized defense marker genes, *CYP71A12, MYB51*, and *PIP1* (Lakshmanan et al., 2012; Hou et al., 2014; Birkenbihl et al., 2017), were found to be substantially induced in the roots of Arabidopsis under shade (Supplemental Fig. S2D). Combined, these results suggest that the shade induces genes in the roots that are also upregulated when a plant encounters abiotic and biotic stress, and a large proportion of these genes contain W-box promoter elements, which are binding sites for WRKY TFs.

Large number of WRKY transcription factors are induced in response to shade

The RNA-seq and GO analysis on the shade-induced genes, and the discovery of W-box promoter elements in them, indicated the involvement of WRKY TFs in mediating root responses to shade. To test this hypothesis, first we surveyed the expression of all the *WRKY* genes in Arabidopsis and tomato in our transcriptomic data. We found a large number of *WRKY*s were induced along the time-course in response to shade. 33 out of 74 *WRKY*s in Arabidopsis were significantly expressed (FDR <0.05) in one or more time points, similarly, 21 out of 83 *WRKY*s were upregulated in tomato (Fig. 2A-B; Supplemental Fig. S3A).

Next, to perform in-depth functional analysis of the shade-induced *WRKY*s in Arabidopsis, we sought to narrow down the candidates as large number of them were induced (Fig. 2B). In order to do this, we selected *WRKYs* with a minimum threshold of 0.5 $\log_2$ fold-change induction relative to the unshaded control along the time-course. Using this parameter, we identified 12 *WRKYs* that were consistently up-regulated in the shade (Fig. 2C). We further classified these 12 genes in to three groups, namely, “early”, “middle”, and “late”, based on the time they were upregulated post shade treatment. *WRKY8*, *WRKY70,* and *WRKY75* were

upregulated within the first 30 min of the shade treatment, there we classified them as immediate early-induced genes. Next, *WRKY25*, *WRKY26*, *WRKY33*, *WRKY45,* and *WRKY51* were classified as intermediate middle, as their expression was seen between 3-7 hours of shading. Lastly, *WRKY13*, *WRKY29*, *WRKY31,* and *WRKY58* were classified as late-induced genes as they were expressed only after 24h of shading (Fig. 2C). We also compared the Arabidopsis *WRKY* expression profile to its putative orthologs in tomato (Supplemental Fig. S3B-C). There were some similarities between tomato and Arabidopsis *WRKY* temporal expression patterns; however, several *WRKY*s showed statistically significant induction at 24h time point in the shade. It is possible that temporal expression of *WRKY* genes could be delayed in tomato when compared to Arabidopsis and, thus, a longer time-course experiment in tomato could be more suitable to address ortholog-specific similarities. Nevertheless, it is clear that shade activates similar pathways in these two distantly-related species.

Focusing on Arabidopsis, we investigated whether the early, middle, and late shade-induced *WRKY* genes were closely related phylogenetically. For this, we constructed a Maximum Likelihood phylogenetic tree based on their polypeptide sequences of the 12 WRKY that was classified above and induced by shade. We observed that the pattern of *WRKY* expression did not reflect the phylogenetic relationship between them. For instance, WRKY75 and WRKY45 that are closely related to each other (Fig. 2D), were induced in the middle and at earlier time points (Fig. 2C). An exception to this was the branch comprising WRKY25, WRKY26 and WRKY33, which are phylogenetically close and were induced in the middle of the time course (Fig. 2C). This was consistent with previous studies, which indicated that these WRKYs (25, 26, and 33) act redundantly in Arabidopsis' response to high temperature, gibberellin (GA), and abscisic acid (Li et al., 2011; Zhang et al., 2015). It is not surprising, however, that closely-related genes, such as *WRKY45* and *WRKY75*, have different expression profiles. It is very common that paralogous genes initially diverge by changes in the promoter region, which leads to sub- or neo-functionalization of duplicated genes (Rosado et al., 2016; Teufel et al., 2016). Collectively, our results suggest that a large number of WRKY TFs are specifically induced in both Arabidopsis and tomato roots in response to the shade stimuli.

Shade-induced WRKY proteins accumulate in the roots and largely absent in the shoot

As known with the large gene families, various members of the *WRKY* genes are paralogous, and are documented for functional redundancy due to gene duplications, which complicates genetic analysis to determine the role of individual WRKY TF (Eulgem et al., 2000; Zhang et al., 2015). In this scenario, we decided that the best strategy for studying the role of

the shade-induced *WRKY*s (Fig. 2C) in root growth during the SAR is by overexpressing them. It is documented that overexpression of WT genes can also cause mutant phenotypes; be used to assess the impact of genetic alterations and gene activity in generating phenotypes and is comparable to traditional loss-of-function methods (Palatnik et al., 2003; Chua et al., 2006; Prelich, 2012; Kaiserli et al., 2015). Therefore, we generated transgenic Arabidopsis lines overexpressing the selected shade-induced 12 WRKYs as a mCitrine fluorescent protein fusion (*WRKYox*) under the control of the constitutive Arabidopsis *UBIQUITIN 10* (*UBQ10*) promoter. We identified multiple independent transgenic lines with a single insertion of the transgene and we selected a minimum of three lines for further analysis, except for *WRKY8ox* and *WRKY33ox*, as we could not recover stable transgenic lines for them.

First, we performed immunoblot analysis to ensure that the transgenic lines for rest of the 10 shade-induced WRKYs were expressing full-length mCitrine fusion proteins, and not partial fusions or free mCitrine alone. Using total protein lysates obtained from the whole 5-day old transgenic seedling grown in unshaded or exposed to the shade for 3-24 hours, we performed immunoblot analysis using an anti-GFP antibody. Immunoblot analysis could detect the presence of full-length mCitrine fusion with WRKY 25, 26, 31, 45, 51, and 75 among the independent transgenic lines (Supplemental Fig. S4). Differential protein accumulation was not observed in the shade and unshaded growth conditions in these transgenes. However, the specific protein for WRKY 13, 29, 58, and 70 and some indicated independent lines could not be detected in the immunoblot. This could be due to one or more reasons; transgenic protein expression was below the detection limit of the antibody used in the immunoblot assay, low expression of the transgene, dilution of the specific signal due to the use of the whole seedling lysates, or instability of the protein.

Nevertheless, we performed confocal microscopy to visualize WRKY-mCitrine fusion proteins in three independent transgenic lines for each of the ten *WRKYox* that were exposed to a minimum of 24 h of shade. For all of the ten WRKYs, we observed them to be present in the nucleus of the root epidermal cells within the maturation zone in shade (Fig. 3A), consistent with their role as a nuclear-localized TF (Eulgem et al., 2000). As we had used a constitutive *UBQ10* promoter to express these *WRKY*s, however, their protein expression and distribution varied considerably within the cell-types of the roots (Fig. 3A; upper panel). For example, in the elongation and meristematic zones of the root (Fig. 3A; lower panel), WRKY25, WRKY45, and WRKY51 were detected in both the epidermis and cortex. In the meristematic zone, WRKY26 was detected only in the columella cells and WRKY58 was observed in the LR cap. In the shoots (Fig. 3B), we detected fluorescence signal for WRKY26 and WRKY45, whereas,

WRKY31 signal was observed only in the trichomes. Remarkably, we did not detect any signals for rest of the WRKY proteins in the shoot. This varied expression profile might be due to regulation of these WRKYs post-transcriptionally or post-translationally. However, it is also likely that technical limitations of confocal microscopy or poor expression levels impede us from detecting WRKY-mCitrine fusions in all cell types.

Shade-induced WRKYs affect primary root and lateral root growth in shaded and unshaded conditions

Since many *WRKY*s are upregulated in the roots of Arabidopsis and tomato seedlings in the shade, we sought to assess their functional contribution in regulating root growth. We chose to determine the effect of their overexpression in the low R:FR mediated SAR, specifically on hypocotyl elongation and root growth inhibition. For each of the shade-induced selected WRKY overexpressors, we used three independent Arabidopsis transgenic lines (described in Fig. 3) for phenotyping, except for *WRKY51*, for which we recovered only two separate lines (Supplemental Fig. S5). We analyzed four phenotypic traits under unshaded and shade light conditions: length of the primary root, LR number, LR density, and hypocotyl length. We report the average phenotypic values in Fig. 4, by combining the measurements from all the independent transgenic lines employed for each of the WRKYs.

5-day old seedlings grown in unshaded condition were transferred to the shade or mock-treated for 4-days and then their primary root length, LR number, and LR density was measured. Most of the *WRKY* overexpressing seedlings produced primary roots whose length was comparable to the WT (Fig. 4A-B). However, *WRKY26* and *WRKY45* overexpressors displayed a constitutive primary root growth and LR branching inhibition even in the absence of the shade stimuli (Fig. 4A-B). The primary root length of *WRKY26* and *WRKY45* did undergo a modest decrease in size in the shade compared to other *WRKY* overexpressors and the WT (Fig. 4B, Supplemental Fig. S5A). However, the root and hypocotyl growth of mutants that harbor T-DNA insertion in the protein coding region of *WRKY26* and *WRKY45* were indistinguishable from WT in the shade and unshaded light (Supplemental Fig. S6A-C). Consistently, transgene expression levels in the *WRKY26ox* and *WRKY45ox* did not vary with the light conditions as determined by reverse-transcription quantitative PCR (RT-qPCR) analysis using transgene specific oligos that hybridizes to the mCitrine tag. We observed an overall increase in *WRKY26* and *WRKY45* expression levels in the root as opposed to the whole seedling in response to shade by RT-qPCR analysis using oligos that recognizes both the transgene and the WT gene (Supplemental Fig. S6D).

Surprisingly, all the *WRKY* overexpressing transgenic seedlings did not have any measurable defects in their hypocotyl length and were comparable to the WT (Fig. 4C; Supplemental Fig. S5B) in the shade and non-shading control conditions. *WRKY26ox* and *WRKY45ox*, which displayed hypersensitive shorter roots in non-shading conditions, did not have any obvious hypocotyl growth defects in the shade. However, the expression of WRKY26 and WRKY45 protein was detected in the hypocotyl (Fig. 3B; Supplemental Fig. S6E). So, the lack of hypocotyl defects in *WRKY26ox* and *WRKY45ox* likely reflects their specialized role in the avoidance response, which is restricted in limiting root growth.

In all the ten *WRKY* overexpressing seedlings, we observed reduced LR number in the shaded seedling compared to their unshaded counterparts, similar to WT (Fig. 4D; Supplemental Fig. S5C). In *WRKY26ox* and *WRKY45ox* seedlings, significantly reduced to nearly absent LRs were noted (Fig. 4A, D), in the shade and non-shading conditions, similar to its constitutive primary root growth inhibition in these conditions (Fig. 4A, B). During the duration of our assay (9 days) in the shade, the LRs were not detected for *WRKY26ox* and *WRKY45*ox seedlings; in contrast, up to 6 LR could be seen in each WT seedlings (Fig. 4D, Supplemental Fig. S5C). Although *WRKY51ox* and *WRKY75ox* did not influence the inhibition of the primary root growth, we observed a marked decrease in their number of LR in the shade (Fig. 4D, Supplemental Fig. S5C).

Of note, we could only detect a few statistically significant differences in the number of LRs in our transgenic lines. This is likely due to variation in LR produced by individual seedlings and accounted for by WRKY's expression levels in the cells. Significant differences were not observed in the LR density, measured as a number of LR per 1 centimeter (cm) of the primary root (Fig. 4E; Supplemental Fig. S5D). This result was not particularly unexpected, considering that most *WRKYox* seedlings did not have much of an impact on the primary root growth and LR number, except for *WRKY26*, *WRKY45*, and *WRKY75*. *WRKY51ox* seedlings also displayed a slight shortening of the primary root and the hypocotyl, but these transgenic lines also showed variable and delayed seed germination, and other pleiotropic defects. Therefore, other confounding factors could be influencing the resulting phenotype of *WRKY51ox* in the shade (Fig. 4A-C).

Overexpression of *WRKY13*, *WRKY29,* and *WRKY58* showed a slight increase in their root length, LR production, and LR density compared to the WT in the shade and non-shading conditions (Supplemental Fig. S7A-C). However, these observations were reinforced only in the *WRKY13ox* independent lines, but not on *WRKY29ox* and *WRKY58ox* individual seedlings (Supplemental Fig. S7D-F). Considering that these three *WRKY*s are shade-induced in the

368 “late” stages of our time-course analysis (Fig. 2C), it is plausible that they could have a
369 secondary role or oppose the shade-mediated repression of root growth.
370 Together, our results here indicate the significance of WRKYs, especially the
371 upregulation of *WRKY26* and *WRKY45* in the roots of shaded plants to limit their growth and not
372 that of the hypocotyl. Overexpression of *WRKY26* and *WRKY45* resulted in reduced primary
373 root growth and LR number under the control unshaded condition, as to resemble the
374 phenotype of WT roots in the shade. Overexpression *of WRKY75* had no substantial role in
375 repressing the primary root growth, but had an effect by repressing LR emergence in the shade
376 and not in unshaded growth.
377
378 **Ethylene is required for root growth inhibition in the shade**
379 ‘Response to ethylene’ was one of the top enriched GO terms among the shade-induced
380 genes in both Arabidopsis and tomato (Fig. 1B). WRKY TFs integrate ethylene hormone
381 responses along with environmental and developmental signals (Koyama, 2014). Furthermore,
382 ethylene and components of the ethylene signaling pathway are required for efficient resistance
383 towards certain plant pathogens. For example, ethylene-insensitive Arabidopsis mutant *ein2*
384 was more susceptible than WT plants to infection by *Botrytis cinerea, a* fungal pathogen
385 (Thomma et al., 1999). Involvement of ethylene signaling and multiple WRKYs in response to
386 senescence and high temperature have been documented (Li et al., 2011; Koyama et al., 2013;
387 Koyama, 2014). Ethylene also regulates root growth by mostly restricting cell elongation
388 (Růžička et al., 2007). Previous studies have determined the importance of ethylene in petiole
389 elongation but not for hypocotyl elongation during SAR (Pierik et al., 2009; Das et al., 2016),
390 although ethylene biosynthetic pathway was particularly upregulated in the hypocotyl in
391 response to shade (Kohnen et al., 2016). Therefore, in light of this knowledge and observation,
392 we tested the effect of ethylene and the components of ethylene signaling on root growth during
393 shade avoidance.
394 We grew WT seedlings in unshaded and shaded conditions on a growth media
395 supplemented with different concentrations of 1-aminocyclopropane-1-carboxylic acid (ACC), a
396 routinely used biosynthetic precursor of ethylene (Růžička et al., 2007). In the shade, a lower
397 dose of 0.2 μM ACC had a stimulatory effect on the hypocotyl elongation. In contrast, higher
398 doses of 2 and 10 μM had a moderate impact on the hypocotyl elongation (Fig. 5A), confirming
399 previous results (Das et al., 2016). But ACC treatment profoundly affected the primary root
400 growth in the seedlings grown in both shade and unshaded conditions (Fig. 5B). At 0.2 μM ACC,
401 the primary root growth of unshaded seedlings was indistinguishable from that of shaded

seedlings, having a shorter root length which was comparable to untreated roots in the shade. Increasing concentrations of ACC led to further attenuation of the root growth in both unshaded and shade conditions with similar root length, especially at 10 µM of ACC. ACC treatment, notably at 10 µM, led to a modest reduction in LR number in unshaded seedlings (Fig. 5C). However, ACC had the opposite effect on shaded seedlings, as we observed increased LR density at 10 µM ACC (Fig. 5D). At 2 and 10 µM ACC, the LR density in shaded and unshaded seedlings was similar (Fig. 5D). Therefore, these results indicate that ethylene is required for root growth inhibition in the shade.

Next, to further explore the importance of ethylene in regulating root growth in the shade, we analyzed mutants defective in ethylene signaling, namely, *ein2* and *ein3eil1* double mutant, respectively. EIN2 (ETHYLENE INSENSITIVE 2) is a crucial signaling transducer, and EIN3 (ETHYLENE INSENSITIVE 3) and EIL1 (EIN3-LIKE 1) are critical downstream TFs in the ethylene response (Dolgikh et al., 2019). Hypocotyl growth defect was not observed in *ein2* and *ein3eil1* seedlings in the shade (Fig. 5E), agreeing with a previous study (Das et al., 2016). Interestingly, the primary roots of both *ein2* and *ein3eil1* mutants did not respond to shade-induced growth inhibition, regardless of the shade or unshaded growth conditions (Fig. 5F). The root phenotypes of *ein2* and *ein3eil1* further resembled *WRKY26ox* and *WRKY45ox* seedlings (Fig. 4B-E). Accordingly, *ein2* and *ein3eil1* mutants presented much fewer LRs and LR density compared to the WT, both in the shade and non-shading conditions (Fig. 5G-H). Together, the data presented in Fig. 5 indicates that ethylene and its associated signaling are required to restrict root growth in the shade. Also, the data supports the requirement of ethylene signaling along with WRKYs, analogous with the plant defense responses.

Roots of *WRKY26ox* and *WRKY45ox* are sensitive to ACC

To further dissect the nexus between WRKYs and ethylene, we tested whether ethylene can trigger the expression of *WRKY45* and *WRKY26*, whose overexpression resulted in root-specific phenotypes in the shade and unshaded light (Fig. 4B). WT Arabidopsis seedlings were treated with 2µm ACC in the shade or unshaded light and RNA was extracted from the whole seedlings or from dissected roots. RT-qPCR was performed to measure the expression levels of *WRKY26* and *WRKY45* along with the ethylene responsive *ERF1B* marker gene. *ERF1B* displayed increased expression in the root after ACC treatment when compared to the mock control and unshaded condition (Fig. 6A). Likewise, we observed an increased expression of *WRKY45* in the unshaded and shaded WT seedlings treated with ACC (Fig. 6A). However, the increased expression of *WRKY26* was not statistically supported in our RT-qPCR assay on the

RNA from the roots. In whole seedlings, ACC triggered the expression of *ERF1B* in WT seedlings, but not in *WRKY26ox* and *WRKY45ox*. But we did not observe increased expression of *WRKY26* and *WRKY45* in whole seedlings (Fig 6B). Next, we measured the primary root length of *WRKY26ox* and *WRKY45ox* supplemented with different amounts of ACC. The roots of *WRKY26ox* and *WRKY45ox* showed increased sensitivity towards ACC in both shaded and unshaded light when compared to the WT (Fig. 6C). ACC treatment also affected lateral root number and density in *WRKY26ox* and *WRKY45ox* (Supplemental Fig. S8A-B). These results indicate that ACC, precursor of ethylene has the ability to induce *WRKY45* in the root in the shade as well as in unshaded light. In addition, the ACC further reduced the root length of *WRKY26ox* and *WRKY45*ox transgenic lines indicating that ethylene and WRKYs likely work together or in parallel to inhibit root growth.

DISCUSSION

Mechanisms underlying stem and petiole elongation under shade have been widely studied for several decades (Hornitschek et al., 2009; Pierik et al., 2009; Li et al., 2012; Pedmale et al., 2016). However, our understanding of how shade perceived by the above-ground shoots leads to the reduced growth of the belowground primary root and LR has been limited. This study presents evidence that many *WRKY* genes are transcriptionally upregulated in the roots of shaded Arabidopsis and tomato plants. We further demonstrate that several WRKYs (26, 45, 75) and ethylene function in restricting root and LR growth but did not affect hypocotyl elongation in the shade.

We discovered genes induced by biotic and abiotic stressors overlapped with a large proportion of the shade-induced genes in the roots of Arabidopsis and tomato seedlings grown in the shade (Fig. 1B). PTI-induced genes also coincided with a significant portion of the shade-induced genes in the roots, which are known to be regulated by WRKY TFs (Fig. 1C-G). Importantly we found W-box promoter elements in a large number of shade-induced genes in the roots (Fig. 1E, Supplemental Fig. S2A), suggesting the involvement of WRKYs in the reprogramming of the gene expression to restrict root growth, typically observed during SAR. Furthermore, a large number of *WRKY* genes were significantly (FDR <0.05) upregulated in the roots of both shaded Arabidopsis and tomato, progressively increasing through the time course in the shade (Fig. 2; Supplemental Fig. S2). To identify the contribution of shade-induced WRKYs in regulating root growth in the shade, we performed functional analysis on a select ten WRKY members by overexpressing them. We chose overexpression as an alternate yet powerful tool to generate mutant phenotypes (Chua et al., 2006; Prelich, 2012) and also to

overcome known functional and genetic redundancy among the WRKY gene family members and potential gene-compensation (Rushton et al., 2010; Zhang et al., 2015). Overexpression of *WRKY26* and *WRKY45* led to a retarded root growth and LR emergence, irrespective of shade or unshaded light. *WRKY26ox* and *WRKY45ox* seedlings had a constitutive shade avoiding shorter primary root and reduced LR in unshaded light, mimicking a WT seedling in the shade. Importantly, in *WRKY75ox* seedlings, there was no effect on the primary root length, but a marked reduction in LR number was seen, similar to *WRKY26* and *WRKY45* overexpressors. But, none of the ten shade-induced WRKYs that we characterized affected hypocotyl elongation, indicating that the roles of these WRKYs are primarily limited to regulate root growth during the SAR.

Our results here demonstrate that phenotypic activation of WRKY TFs is feasible as a general approach to identify their functional roles in plant growth and development (Fig. 4). Previous studies have shown enhanced tolerance towards pathogens, salt, and drought by overexpressing *WRKY*s that was under investigation in *Oryza sativa* (rice), *Glycine max* (soybean), and Arabidopsis (Rushton et al., 2010). However, apart from this study, only a few prior reports have associated WRKYs with light signaling and adaptation. For instance, Arabidopsis WRKY18 and WRKY40 have been shown to co-localize with PHYB and PHYTOCHROME-INTERACTING FACTORS (PIFs) in the nuclear speckles or photobodies, but their role in red/far-red light signaling is unknown (Geilen and Böhmer, 2015). WRKY40 is required for adaptation towards high light stress in Arabidopsis (Aken et al., 2013), and WRKY22 is involved in dark-induced senescence (Zhou et al., 2011).

We characterized root-specific and shade-induced *WKRY*s by overexpressing them in Arabidopsis. We reasoned that overexpression of a WT protein can also cause mutant phenotypes, which is an alternative yet powerful tool to traditional loss-of-function analysis to infer gene function, especially when loss-of-function opportunities are not available (Palatnik et al., 2003; Prelich, 2012; Kaiserli et al., 2015). However, overexpression of several shade-induced *WRKY*s produced no observable mutant primary root and LR growth phenotypes. Out of the ten *WRKYox* lines that we characterized, we observed primary root-specific phenotypes only in two of them, for *WRKY26* and *WRKY45* respectively. Importantly, hypocotyl growth was not affected in *WRKY26ox* and *WRKY45ox* in the shade indicating their specificity in regulating root growth in spite of overexpression. Among the independent transgenic plants for a given *WRKY*, we observed variation in their protein expression. For example, among the three independent lines we characterized for *WRKY26ox*, line #2 had the strongest root phenotype (Supplemental Fig. S5A) and protein levels in whole seedlings (Supplemental Fig. S4). The

variation in protein expression in these transgenic lines could be due to the positional effect of the transgene in the genome. *WRKY51ox* seedlings had detectable protein levels by immunoblot analysis, but they exhibited poor seed germination and we were unable to phenotype them adequately due eliminate confounding interpretation. Our microscopic analysis revealed that WRKY26 and WRKY45 were expressed in most of the cell-types in the shoot. However, even with the use of constitutive expression, transgenic protein in several *WRKYox* lines were not detected or they were below the detection limit in our immunoblot assay and confocal microscopy. This lack of protein detection could be attributed due to post-translational degradation or post-transcriptional regulation in some of the *WRKYox* lines. Therefore, we cannot completely rule out the lack of phenotypes in many *WRKY* overexpressing lines (for example, in *WRKY13ox*, *WRKY29 ox*, *WRKY58 ox*, and *WRKY70 ox*) could be attributed to lack of optimal protein expression or accumulation. The lack of phenotypes in some WRKYs (for example, WRKY31 and WRKY75), could be due to the absence of required activating factor, which had to be overexpressed along with the WRKYs. Several WRKYs are also known to be regulated by Ca^{2+} and bind to 14-3-3 proteins (Rushton et al., 2010). Another aspect could be the feedback loops, which could interfere with WRKYs as several reports point that WRKYs are capable binding to their own promoters or of other *WRKY* genes in response to stress (Skibbe et al., 2008; Rushton et al., 2010; Li et al., 2011; Li et al., 2015; Birkenbihl et al., 2018). Expression and post-translational modification could further limit the function of the shade-induced *WRKY*s when overexpressed. Epitope tags are routinely fused with TFs, and they rarely interfere with the function of the TFs as determined by various *in vitro* and *in vivo* experiments such as ChIP-seq (chromatin immunoprecipitation-sequencing), etc. However, we cannot rule out completely whether the fusion of the WRKYs with mCitrine could be interfering with their activity. However, in future studies, tissue or organ-specific expression of *WRKY*s, analysis of multi-genic or higher order mutants of *WRKY*s will help decipher the exact contribution of shade-induced *WRKY*s in the shade avoidance response.

Previously, WRKY26 was identified as a positive regulator of thermotolerance in Arabidopsis plants, working synergistically with *WRKY25*, *WRKY33,* and ethylene signaling (Li et al., 2011). In this literature, overexpression of *WRKY26* led to the reduction of fresh weight in adult Arabidopsis plants, mirroring our results, where its overexpression led to shorter roots in the shade and unshaded light (Fig. 4). Arabidopsis WRKY45 is a positive regulator of GA-mediated age-induced leaf senescence (Chen et al., 2017), and has been implicated in the activation of *PHOSPHATE TRANSPORTER1;1 (PHT1;1)* in the roots of plants undergoing phosphate deficiency (Wang et al., 2014). Wang et al. (2014) further showed that *WRKY45ox*

lines had shorter primary roots in the presence of arsenate. This result is similar to our study, where *WRKY45ox* led to constitutive primary root length shortening in the shade and unshaded light. Furthermore, we report that *WRKY75* overexpression had no effect on the primary root length but caused a reduction in lateral root frequency in seedlings grown only in the shade but not in unshaded conditions (Fig. 4E;Supplemental Fig. S5D). Silencing of *WRKY75* by RNAi resulted in more LR than WT Arabidopsis but no differences in the primary root length (Devaiah et al., 2007). Also, WRKY75, like WRKY45, can promote leaf senescence, participating in a positive feedback loop with hydrogen peroxide and SA to accelerate leaf senescence (Guo et al., 2017). WRKY75 is also a positive regulator of GA-mediated control of flowering time (Zhang et al., 2018). Surprisingly, although WRKY45 and WRKY75 have overlapping roles in phosphate acquisition, leaf senescence, and GA-mediated signaling, yet, only *WRKY45ox* seedlings displayed inhibitor of root length, indicating that many WRKYs share many overlying as well as divergent functional roles.

Ethylene is necessary for petiole elongation during the SAR but had a limited effect on hypocotyl elongation (Pierik et al., 2009; Das et al., 2016). Our results (Fig. 5) show that ethylene and its associated signaling are required to restrict root growth in the shade, suggesting an organ-specific role of ethylene signaling in the shade. Ethylene signaling is critical for a plants' defense responses and molecular links between this hormone and WRKY activity have been proposed (Bakshi and Oelmüller, 2014). Ethylene induces the expression of several *WRKY*s, and WRKY25, 26 and 33, induce the expression of *EIN2*, forming a positive feedback loop (Li et al., 2011; Li et al., 2013). Here, ACC, precursor of ethylene was capable to induce the expression of *WRKY45* in absence of the shade in the root. Expression of *EIN3* is induced by ethylene via EIN2, and also by the defense-hormone JA, and by light via PIF4 and PIF5 bHLH transcription factors (Li et al., 2013; Sakuraba et al., 2014). Our data showed that ethylene-treated WT seedlings and *ein2* and *ein3eil1* had similar root phenotypes to *WRKY26ox* and *WRKY45ox* lines in the shade (Fig. 4; Supplemental Fig. S5; Fig. 5). In addition, *WRKY26ox* and *WRKY45ox* were more sensitive to ACC compared to the WT. WRKYs can function up, and downstream of the many phytohormone pathways (Antoni et al., 2011). Therefore, it is conceivable that ethylene and the shade-induced WRKYs act in concert to antagonize root growth and development, and signaling dependent upon auxin, brassinosteroid, and cytokinin hormones in the shade.

WRKYs are plant-specific TFs and are central components of the plant's resistance to pathogens and responses to abiotic and biotic stresses (Bakshi and Oelmüller, 2014). Arabidopsis and tomato genomes have 74 and 83 WRKY genes, respectively, and a great

diversity of WRKYs allows plants to cope with various adverse conditions (Bakshi and Oelmüller, 2014). WRKYs have been well studied in their involvement with biotic stress compared to abiotic responses (Bakshi and Oelmüller, 2014). SAR is also stressful for the plant, as it leads to photosynthetic impairment and reduced carbon acquisition, reducing the overall fitness of the plant (Smith, 1982; Smith, 2000). In addition, low R:FR SAR downgrades plant defense against pathogens and herbivorous insects in the shoots (Moreno et al., 2009; Ballaré et al., 2012; Courbier and Pierik, 2019). Likewise, other studies have shown that many *WRKY* genes are rapidly induced in response to wounding, drought, salinity, osmotic, cold, carbon starvation, and heat stress (Pandey and Somssich, 2009; Bakshi and Oelmüller, 2014; Rinerson et al., 2015; Viana et al., 2018). Aptly, our finding on the speedy upregulation of *WRKY* gene expression in the roots of shaded plants is conceivable. During SAR, stress-like responses likely help the plant to relocate critical resources from the root to the growing shoot organs in order to maintain competitiveness. Future studies will be required to determine the nature of the signal downstream of the photoreceptors that leads to the induction of many *WRKY* genes in the shade. Also, which are the specific gene targets of the shade-induced *WRKY*s required to repress root growth and development in the shade?

Conclusions

Low R:FR shade represses root growth and development by triggering gene expression changes in tomato and Arabidopsis, mainly resembling the transcriptional changes caused by biotic and abiotic stressors. These shade-induced genes in the roots are known to be regulated by WRKY transcription factors, and many *WRKY*s were significantly upregulated in the shade. Here, we report the involvement of crucial WRKY TFs, namely WRKY26, WRKY45, and WRKY75, along with ethylene in the SAR to inhibit primary root and LR growth. These factors had no involvement in regulating hypocotyl elongation.

MATERIALS AND METHODS

Plant Material, growth conditions and light treatments

Arabidopsis (*Arabidopsis thaliana*) genotypes used in this work are in Columbia WT (Col-0) ecotype background. Seeds of *ein2-5* and *ein3-1 eil1-1* double mutant were obtained from Dr. Hong Qiao (University of Texas, Austin). T-DNA insertional mutant for *wrky26* (SAIL_121_C01C1) and *wrky45* (CS333963/GK-684G12) were obtained from Arabidopsis Biological Resource Center (ABRC; Ohio). For phenotyping of Arabidopsis seedlings, seeds were surface sterilized with 70% (v/v) ethanol and 0.1% triton X-100 (v/v) and rinsed several times with sterile water. Seeds were plated on 0.5× Linsmaier and Skoog (LS) medium (HiMedia Laboratories) pH 5.8 containing 0.8% phyto agar (w/v). Shaded and unshaded light conditions were used as described previously (Tao et al., 2008). Briefly, Petri plates containing growth medium with the seeds were then stratified at 4°C for 4-5 d before being placing them vertically in the growth chamber (Percival) with constant white growth light from a LED source (unshaded; PAR ~120 µmol m^{-2} s^{-1}) at 22°C. After 4 d, seedlings were either kept under unshaded control light or transferred to constant stimulated low R:FR shade where growth light was supplemented with FR (R:FR ratio of 0.35) for 1d (microscopy and immunoblot analysis) or 3-5 d (phenotypic measurements). Light measurements were obtained using a full quantum sensor (Apogee Instruments). For phenotypic measurements, Petri plates with 7-9 d-old seedlings were scanned using a flatbed scanner (Epson V600), primary root length and later root (LR) numbers (counted as emerged LR) were obtained with SmartRoot (Lobet et al., 2011) and hypocotyl length was measured using NIH ImageJ software.

Cloning and generation of transgenic lines in Arabidopsis

Arabidopsis root tissue cDNA library was used as a template to amplify and clone the coding sequence of *WRKY13, WRKY25, WRKY26, WRKY29, WRKY31, WRKY45, WRKY51, WRKY70* and *WRKY75*; while Arabidopsis genomic DNA was used as a template to amplify *WRKY58*. PCR was performed using KOD polymerase (Toyobo) with the primers listed in Supplemental Table S3 and introduced in to the Gateway donor vectors, either pDONR207 or pDONR221 (Thermo Fisher Scientific) using BP clonase II enzyme (Thermo Fisher Scientific). Multisite Gateway reaction using LR clonase II mix (Thermo Fisher Scientific) was performed to combine the donor constructs with either pB7m34GW or pK7m34GW binary vector (Karimi et al., 2007) along with the *UBQ10* promoter in pDONR P4-P1R donor vector and Citrine in pDONR P3-P1R vector (destination vectors used for each *WRKY* gene are listed in Supplemental Table 2).

Destination constructs were introduced in *Agrobacterium tumefaciens* to transform Arabidopsis using the floral dip method (Clough and Bent, 1998). T1 transgenic Arabidopsis plants were selected on 0.5× LS medium supplemented with either kanamycin or basta according to the vector used for transformation (Supplemental Table S3). Segregation analysis was performed on T2 plants grown on the selective agar media and lines carrying a single copy of the transgene were propagated further and the T3 seeds were used for experiments and other analysis.

Immunoblot analysis of transgenic lines

Arabidopsis seedlings were grown under constant white light for 4d and either kept in unshaded conditions or shade for additional 24h. 20 excised roots or whole-seedlings were harvested from 5-day old plants and immediately frozen in liquid nitrogen. Samples were ground to a fine powder, resuspended in 2× LDS sample buffer (53mM Tris-HCl, 70.5 mM Tris base, 1% (v/v) LDS, 5% (v/v), glycerol, 0.255mM EDTA, 0.11mM Serva Blue G250, 0.0875 mM phenol red, pH 8.5) with 50 mM TCEP and heated to 90°C for 10 min, cooled to room temperature and then centrifuged for 5 minutes to obtain the total protein lysate. For electrophoretic separation of proteins, equal amount of total protein was loaded on 10% (v/v) Bis-Tris polyacrylamide gel and electrophoretically separated using MOPS-SDS buffer (2.5mM MOPS, 2.5mM Tris base, 0.005% (w/v) SDS, 0.05mM EDTA). Separated proteins were then transferred electrophoretically to a nitrocellulose membrane (GE Lifesciences) using transfer buffer (10% (v/v) methanol, 1.25mM bicine, 1.25mM Bis-Tris base, 0.05 mM EDTA). After transfer, the membrane was stained with ponceau red, imaged, and blocked with 5% (w/v) non-fat dry milk prepared in Tris-buffered saline with 0.05% (v/v) Tween-20 (TBST) for 30 min. Next, membranes were incubated for 1h in 1% (w/v) milk prepared in TBST with anti-GFP antibody (Roche). The blot was washed 3 times with TBST and incubated for 30 min in 1% (w/v) milk prepared in TBST with anti-mouse HRP conjugate (Bio-Rad). Chemiluminescent detection was performed using SuperSignal West Dura Extended Duration (Thermo) HRP substrate to detect the WRKY-mCitrine fusion protein.

Confocal microscopy

Stable transgenic Arabidopsis seedlings expressing *UBQ10::WRKY-Citrine* in their T3 generation were grown in unshaded light for 4-5 days and transferred to shade for 24 h. Seedlings were stained with 5 µg/mL propidium iodide and then mCitrine and propidium iodide

fluorescence were detected in a high-resolution laser scanning confocal microscope (LSM900 with Airyscan2, Zeiss) using 488 and 561 nm lasers along with BP 620/60 emission filter.

Phylogenetic analysis

Protein sequences of the 12 candidate Arabidopsis WRKYs were retrieved from The Arabidopsis Information Resource (TAIR) database (Garcia-Hernandez et al., 2002). Sequences were aligned with Clustal-W algorithm, and the phylogenetic tree was inferred by using the Maximum Likelihood method and JTT matrix-based model in Mega X software (Kumar et al., 2018).

Short-read mRNA-sequencing (RNA-seq) and analysis

The raw short-read sequencing data and expression files are available in the NCBI Gene Expression Omnibus (GEO) database with accession number GSE175963. In Arabidopsis, as described earlier, 5-day-old seedlings grown in continuous unshaded light was mock-treated or exposed to simulated shade (R:FR ~0.6) at the indicated time-points and the roots were harvested using a razor blade and flash frozen in liquid nitrogen. For tomato (*Solanum lycopersicum*, M82 cultivar), seedlings were grown in the dark for 3 days followed by 4 days in constant unshaded light at 25°C before mock-treatment or shade treatment (R:FR ~0.3) and then roots were collected at the indicated time points and flash frozen in liquid nitrogen. Total RNA from Arabidopsis roots was extracted using RNeasy Micro kit (Qiagen). Total RNA from tomato roots were extracted using ReliaPrep RNA kit (Promega). We prepared short-read sequencing libraries from two biological replicates for each time-point and treatment with either Ultra II DNA Library Prep Kit for Illumina (New England Biolabs) or TruSeq RNA-seq library kit (Illumina) according to manufacturer's instruction. All sequencing libraries for each species were pooled together and sequenced using 1 x 76 bp in NextSeq 500. Single short reads were mapped against Arabidopsis (TAIR 10) or tomato (ITAG 4.0) reference genomes using STAR v2.7.2 (Dobin et al., 2013). Assembly and quantification of transcripts was done with Cufflinks v2.1.1 (Trapnell et al., 2012). Pearson's correlation (Supplemental Fig. S1B) was used with the FPKM values to evaluate reproducibility of biological replicates, which displayed very high correlation ($R < 0.98$ in most cases, with lowest $R < 0.94$) which exceeds the ENCODE standard of 0.90 (Dunham et al., 2012). Relative gene expression was calculated as $\log_2$ fold-change in shade relative to unshaded control for each time-point using Cuffdiff (part of Cufflinks v2.1.1). Genes were considered differentially expressed if FDR-adjusted p-value was below 0.05.

De novo identification of cis-motifs

De novo *cis*-motifs in the promoters of differentially expressed genes were identified with HOMER (Heinz et al., 2010) by analyzing the 500 bp upstream and 50 bp downstream of the transcriptional start site.

Computational analysis and graphics generation

Gene Ontology (GO) term enrichment was performed on Panther Classification System (Mi et al., 2020) using Fisher's Exact test and Bonferroni correction to obtain Biological Processes enriched relative to Arabidopsis reference genome. Since tomato GO annotation is comparatively poorer, we generated a list of Arabidopsis and tomato orthologs (Supplemental Table S2). We then used the Arabidopsis accessions corresponding to tomato genes up-regulated by shade for the GO analysis. We then compared the list of significantly (Bonferroni correction; $p<0.05$) enriched GO terms between the species and manually filtered based on hierarchy to remove term redundancy. All GO terms retrieved are reported in Supplemental Table S2. R environment (R Foundation) and its packages (ggplot2, pheatmap, ComplexHeatmap, dendsort, rstatix, ggpubr, dplyr, reshape2, tidyverse, RColorBrewer, circlize, ggfortify, gridExtra) were used for statistical analysis and to visualize results.

ACC treatment

For ACC treatments, WT, *WRKY26ox* or *WRKY45ox* seedlings were grown for 4 days as previously described in unshaded conditions, then transferred to media containing 0, 0.2, 2 or 10 μM of ACC (Sigma). Then, they were either kept in unshaded or shaded conditions for 3-4 days, as indicated. Seedlings on the Petri plates were then scanned for phenotypic analyses.

RT-qPCR analysis

Whole seedlings or roots of Arabidopsis WT, *WRKY26ox* or *WRKY45ox* were used for RT-qPCR analysis. RNA was extracted using Trizol (Thermo) and DNaseI (New England Biolabs) was used to eliminate genomic DNA contamination. cDNA was synthesized using iScript cDNA Synthesis Kit (BioRad) according to manufacturer instructions. qPCR was carried out in the QuantStudio 6 Pro PCR system (Thermo) using Power SYBR Green Master Mix (Thermo) in a 10-μL reaction volume. Expression values were normalized with *ACTIN7* and *PP2A* reference genes using the $2^{-\Delta\Delta Ct}$ method. All primers and accession numbers can be found in Supplemental Table S3.

Statistical Analysis

Most statistical analyses were performed in RStudio. Analysis of variance (two-way ANOVA) and pairwise *post-hoc* Tukey analysis were performed in InfoStat statistical software (InfoStat). Phenotypic data was analyzed by comparing the means between treatments or genotypes according to the test specified at the figures.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers WRKY13 (AT4G39410), WRKY25 (AT2G30250), WRKY26 (AT5G07100), WRKY29 (AT4G23550), WRKY31 (AT4G22070), WRKY45 (AT3G01970), WRKY51 (AT5G64810), WRKY58 (AT3G01080), WRKY70 (AT3G56400), WRKY75 (AT5G13080), EIN2 (AT5G03280), EIN3(AT3G20770), EIL1(AT2G27050), Actin7 (AT5G09810), PP2A (AT1G13320), ERF1B (AT3G23240), SlWRKY04 (Solyc05g012770), SlWRKY05 (Solyc03g104810), SlWRKY06 (Solyc02g080890), SlWRKY13 (Solyc04g051540), SlWRKY16 (Solyc02g032950), SlWRKY29 (Solyc08g081610), SlWRKY31 (Solyc06g066370), SlWRKY33 (Solyc09g014990), SlWRKY38 (Solyc02g094270), SlWRKY51 (Solyc04g051690), SlWRKY71 (Solyc02g071130), SlWRKY75 (Solyc05g015850), SlWRKY80 (Solyc03g095770), SlWRKY81 (Solyc09g015770).

SUPPLEMENTAL DATA

Supplemental Figure S1. Transcriptional changes induced by shade in the roots.

Supplemental Figure S2. Shade induces transcriptional changes via similar transcription factors in Arabidopsis and tomato.

Supplemental Figure S3. Shade induces the expression of a large group of *WRKYs* in tomato.

Supplemental Figure S4. WRKYox-Citrine protein levels are unaffected by shade.

Supplemental Figure S5. Phenotype of independent Arabidopsis *WRKYox* transgenic lines in the shade.

Supplemental Figure S6. Expression dynamics of *WRKY26ox* and *WRKY45ox*.

Supplemental Figure S7. Overexpression of *WRKY13*, *29* and *58* does not affect root growth.

Supplemental Figure S8. Ethylene further represses root growth in *WRKY26ox* and *WRKY45ox* lines.

Supplemental Table S1. Differential expression in Arabidopsis and tomato roots in response to shade.

Supplemental Table S2. Gene ontology (GO) terms enriched among the shade-induced genes in roots of Arabidopsis and tomato.

Supplemental Table S3. Oligonucleotide sequences and plasmids used in this work.

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FIGURE LEGENDS

Figure 1. Shade-induced genes in the roots resemble biotic and abiotic stress-induced transcriptome.

A) Phenotypic representation of a 9-day-old Arabidopsis seedling grown under constant white light (unshaded) and shade (low R:FR); and the time-points used for RNA-seq analysis. Dashed lines indicate the region where the root tissue was excised from the shoot for RNA-seq analysis. B) Gene Ontology (GO) terms of biological processes, commonly enriched among the genes upregulated in Arabidopsis and tomato roots during the first 24h (30m, 3h, 7h, 1d; and 3h 6h, 12h, 24h, respectively) under shade. C) Arabidopsis genes induced by the shade in the roots along the time-course (up to 5d) and by various PTI-elicitors from a prior study (Bjornson et al. 2021). Over-representation and *p*-value were calculated based on hypergeometric distribution and Fisher's exact test. D) Expression profile of common Arabidopsis shade-induced and elicitor-induced genes along the time-course. Values represent $\log_2$ fold change in the shade relative to unshaded control. E) *de novo* enriched cis-motif element found in the promoters of the 396 genes induced by both shade and elicitors as determined in a previous study (Bjornson et al. 2021). F) Arabidopsis genes induced by the shade in the roots along the time-course (up to 5d) and by WRKY18, WRKY33 and WRKY40 in response to immune response flg22 elicitor from a prior study (Birkenbihl et al. 2017). Over-representation and *p*-value were calculated based on hypergeometric distribution and Fisher's exact test. G) Expression profile of common Arabidopsis shade-induced and WRKY-induced genes along the time-course. Values represent $\log_2$ fold change in the shade relative to the unshaded control. D and G) Boxplot components correspond to: box limits, first and third quartile; central line, median; whiskers, upper and lower 1.5x interquartile range; pink dots, individual genes; black dots, outliers.

Figure 2. Large number of WRKY transcription factors are induced in response to shade

A) Global transcriptional profile of *WRKY* gene family members in Arabidopsis and tomato roots in the shade. Values represent $\log_2$ fold expression of *WRKY* genes in the shade compared to its unshaded developmental control. B) *WRKY* genes that are significantly up-regulated in the Arabidopsis roots relative to its unshaded control (FDR $\leq$0.05). C) Transcriptional profile of the selected *WRKYs* consistently induced by shade, *i.e*., those up-regulated above the threshold of 0.5 $\log_2$ fold relative to the unshaded control. Expression groups are color coded and characterized by the timing of their significant induction in the shade during the time course. D) Dendrogram of selected Arabidopsis WRKY proteins, classified as early, middle, and late as in

panel B. The phylogenetic tree was inferred by Maximum Likelihood and JTT model. Branch lengths are scaled according to number of substitutions per site. Numbers represent the percentage of trees in which the associated proteins clustered together considering 500 bootstrap replicates.

Figure 3. Shade-induced WRKY proteins accumulate in the roots.

A,B) Confocal microscopy images of the indicated 5-day-old Arabidopsis shaded *WRKYox* (fused with mCitrine protein) transgenic seedlings. Prior to the shade treatment, the seedlings were grown in unshaded light for 4 days. A) Arabidopsis roots, and B) Arabidopsis shoots largely encompassing the hypocotyl, petiole, and the first true leaves. Magenta color indicates the propidium iodide (PI) counterstaining of the cell wall and the green signal indicates mCitrine signal.

Figure 4. Shade-induced WRKYs primarily affect root growth in the shaded and has no effect on the hypocotyl growth during the SAR.

A) Phenotype of representative 9-day-old WT and WRKYox seedlings under unshaded light and shade (low R:FR). B) Primary root length of the indicated genotypes in cm, C) Hypocotyl length of the indicated genotypes in cm, D) Frequency of seedlings with 0-10, or more lateral roots (LR) under unshaded or shade conditions in 9-day-old seedlings, E) LR density as the number of LR per 1 cm of the primary root. B-E represent means ± SE of combined independent transgenic lines (*UBQ10::WRKYox*) for each candidate gene; dots represent individual data points; 26-72 individual seedlings were used per genotype. Asterisks represent significant difference (p<0.05) with WT for each light condition (black for unshaded and red for shade) in two-tailed *t*-test with Benjamini-Hochberg correction for multiple testing. Green shading highlights root length reduction in *WRKY26ox* and *WRKY45ox*.

Figure 5. Ethylene hormone signaling is required for the repression of root growth and development in the shade.

A-D) Phenotype of unshaded and shaded WT seedlings treated with the indicated concentrations of 1-aminocyclopropane-1-carboxylic acid (ACC). E-H) Seedlings of WT and ethylene signaling mutants grown in shaded and unshaded light. The phenotypic measurement represents 4-day-old seedlings grown in unshaded light and then transferred to the shade or mock-treated for 4 days. A, E) Hypocotyl length in cm, B, F), Primary root length in cm, C,G) Frequency of seedlings with 0 – 10, or more lateral roots, D,H) Lateral root density is the

number of lateral roots per cm of the primary root. Values represent means ± SE of 8-15 seedlings; dots represent individual data points. Different letters denote significant differences in *post-hoc* Tukey test (ANOVA $p<0.05$).

Figure 6. Ethylene further represses root growth in *WRKY26ox* and *WRKY45ox* lines. A,B) RT-qPCR analysis in 4-day-old seedlings grown in unshaded light and then treated with shade and/or 1-aminocyclopropane-1-carboxylic acid (ACC) (2 μM) as indicated; C) Primary root length in cm of unshaded and shaded seedlings treated with the indicated concentrations of ACC. The phenotypic measurement represents 4-day-old seedlings grown in unshaded light and then transferred to the shade or mock-treated for 3 days. Values represent means ± SE of 3 biological replicates (composed of 10-20 seedlings each) for A and B, and 9-15 seedlings for C. Dots represent individual data points. Different letters denote significant differences in *post-hoc* Tukey test (ANOVA $p<0.05$).

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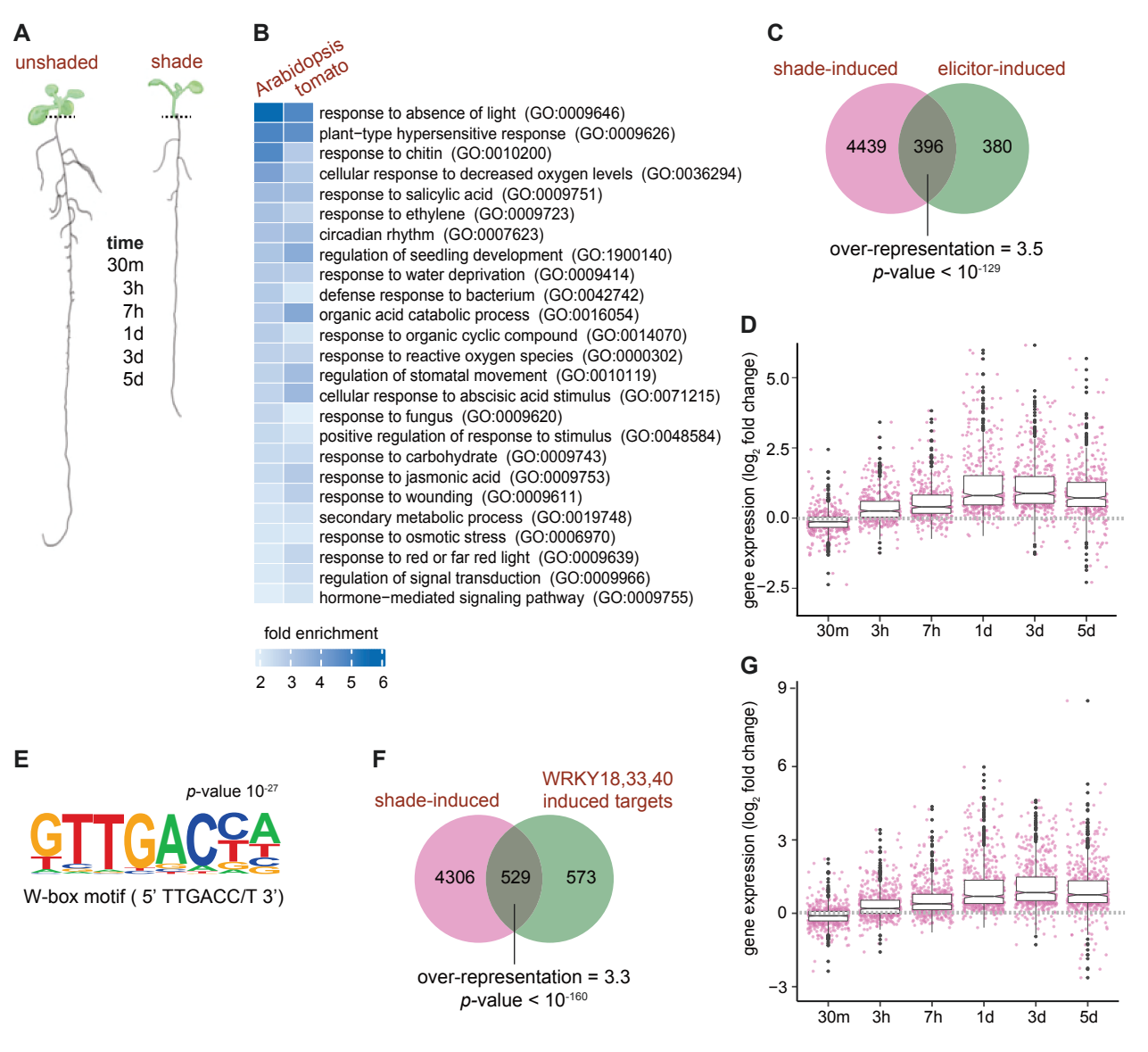
A
unshaded
shade
time
30m
3h
7h
1d
3d
5d
B
Arabidopsis
tomato
response to absence of light (GO:0009646)
plant−type hypersensitive response (GO:0009626)
response to chitin (GO:0010200)
cellular response to decreased oxygen levels (GO:0036294)
response to salicylic acid (GO:0009751)
response to ethylene (GO:0009723)
circadian rhythm (GO:0007623)
regulation of seedling development (GO:1900140)
response to water deprivation (GO:0009414)
defense response to bacterium (GO:0042742)
organic acid catabolic process (GO:0016054)
response to organic cyclic compound (GO:0014070)
response to reactive oxygen species (GO:0000302)
regulation of stomatal movement (GO:0010119)
cellular response to abscisic acid stimulus (GO:0071215)
response to fungus (GO:0009620)
positive regulation of response to stimulus (GO:0048584)
response to carbohydrate (GO:0009743)
response to jasmonic acid (GO:0009753)
response to wounding (GO:0009611)
secondary metabolic process (GO:0019748)
response to osmotic stress (GO:0006970)
response to red or far red light (GO:0009639)
regulation of signal transduction (GO:0009966)
hormone−mediated signaling pathway (GO:0009755)
fold enrichment
2 3 4 5 6
C
shade-induced
elicitor-induced
4439
396
380
over-representation = 3.5
p-value < 10-129
D
gene expression (log2 fold change)
5.0
2.5
0.0
−2.5
30m 3h 7h 1d 3d 5d
E
p-value 10-27
W-box motif (5’ TTGACC/T 3’)
F
shade-induced
WRKY18,33,40
induced targets
4306
529
573
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9
6
3
0
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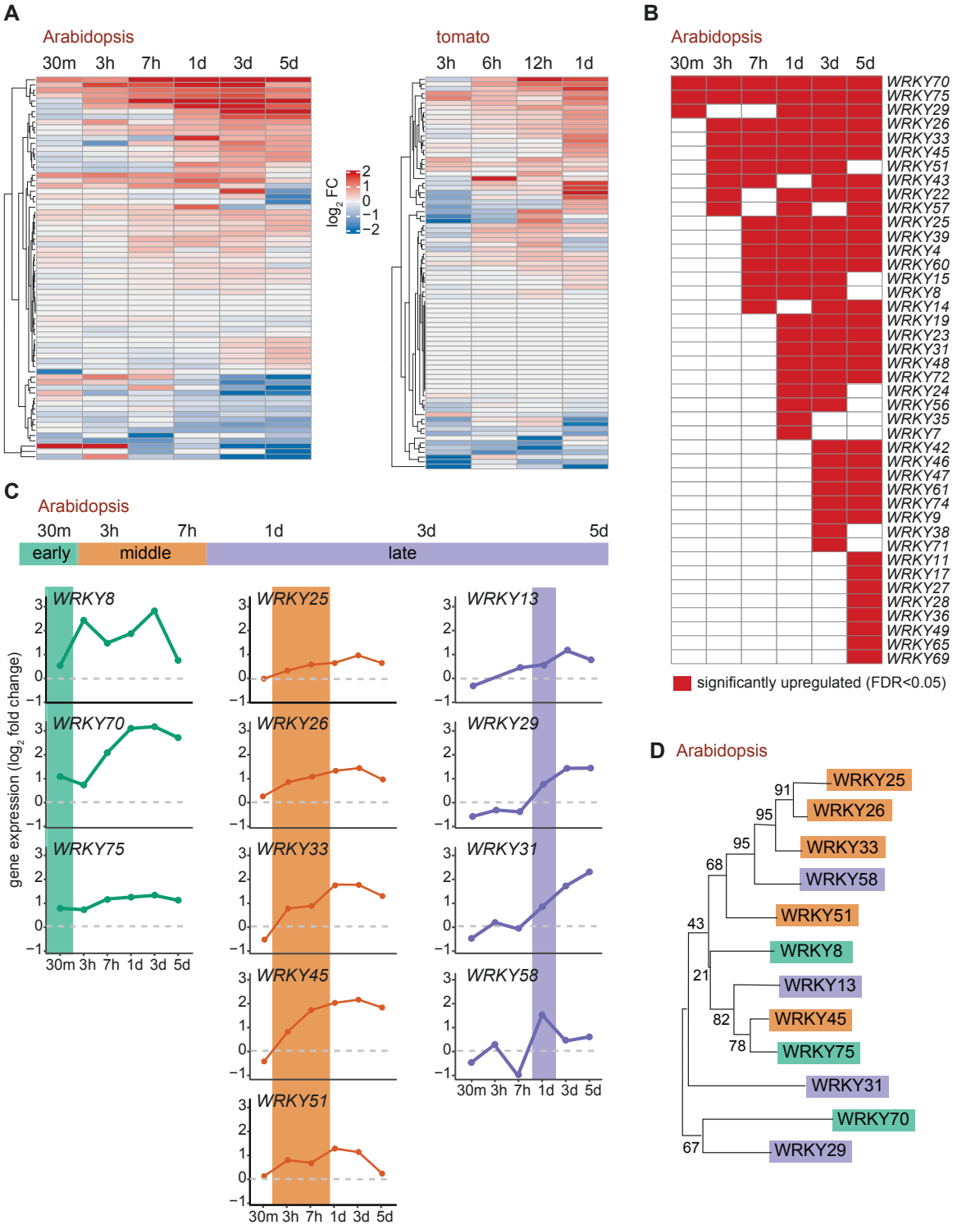
30m 3h 7h 1d 3d 5d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Arabidopsis
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tomato
3h 6h 12h 1d
log2 FC
2
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−2
B
Arabidopsis
30m 3h 7h 1d 3d 5d
WRKY70
WRKY75
WRKY29
WRKY26
WRKY33
WRKY45
WRKY51
WRKY43
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WRKY15
WRKY8
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WRKY74
WRKY9
WRKY38
WRKY71
WRKY11
WRKY17
WRKY27
WRKY28
WRKY36
WRKY49
WRKY65
WRKY69
significantly upregulated (FDR<0.05)
C
Arabidopsis
30m 3h 7h 1d 3d 5d
early
middle
late
gene expression (log2 fold change)
WRKY8
WRKY70
WRKY75
WRKY25
WRKY26
WRKY33
WRKY45
WRKY51
WRKY13
WRKY29
WRKY31
WRKY58
30m 3h 7h 1d 3d 5d
D
Arabidopsis
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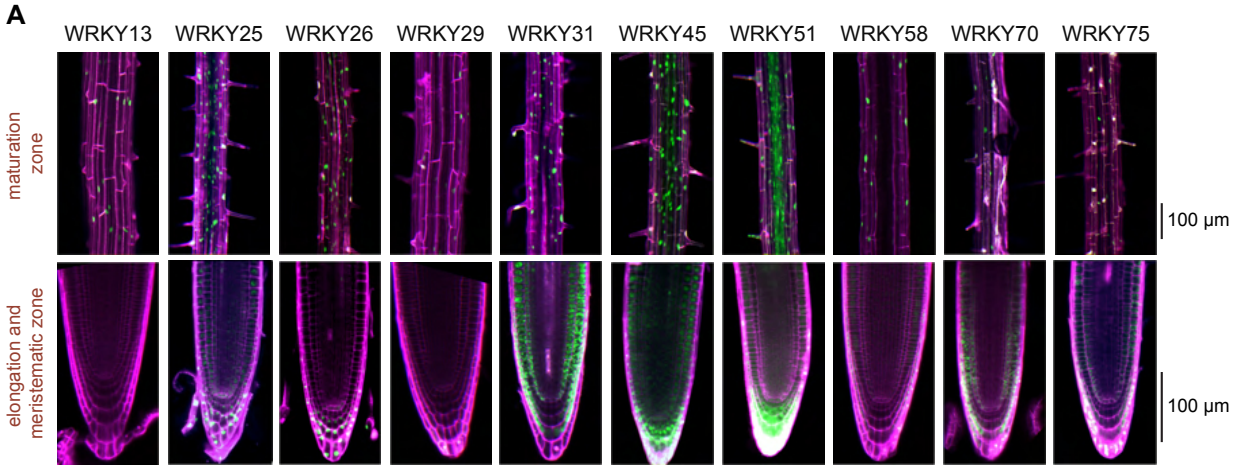
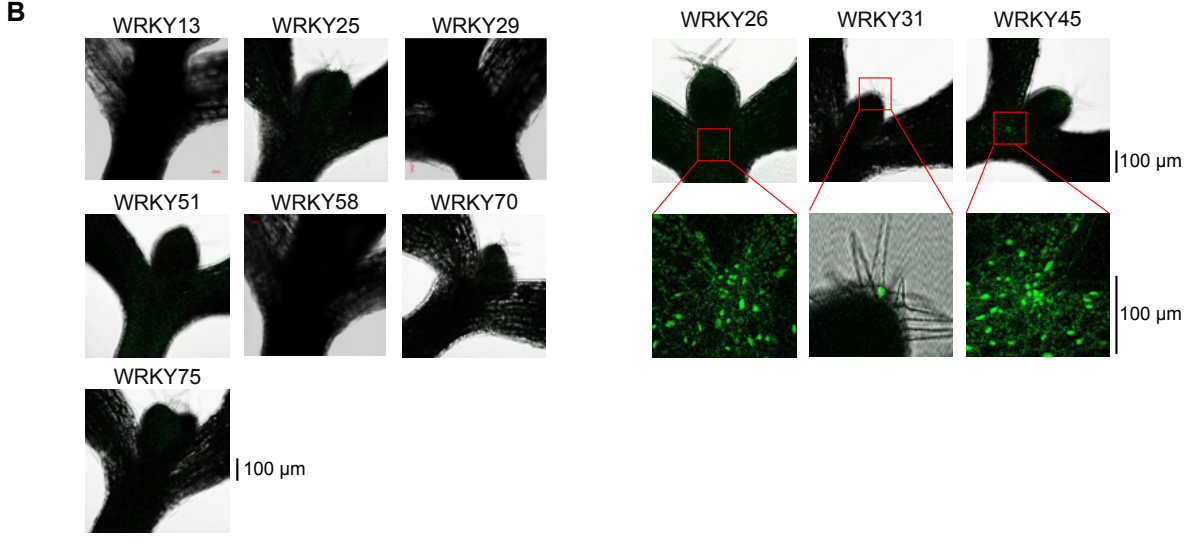

A
WRKY13
WRKY25
WRKY26
WRKY29
WRKY31
WRKY45
WRKY51
WRKY58
WRKY70
WRKY75
maturation zone
elongation and meristematic zone
100 µm
100 µm


B
WRKY13
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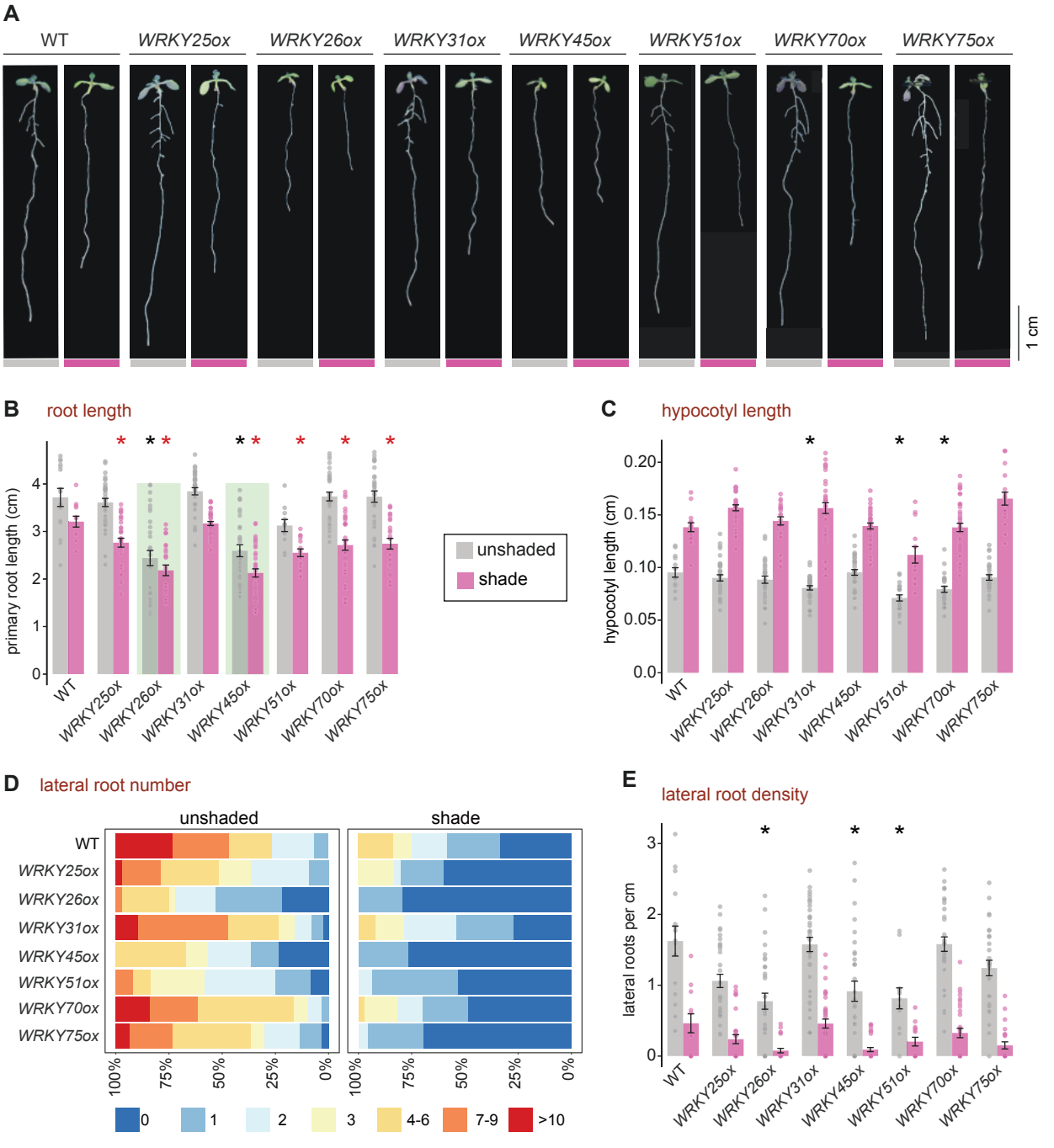
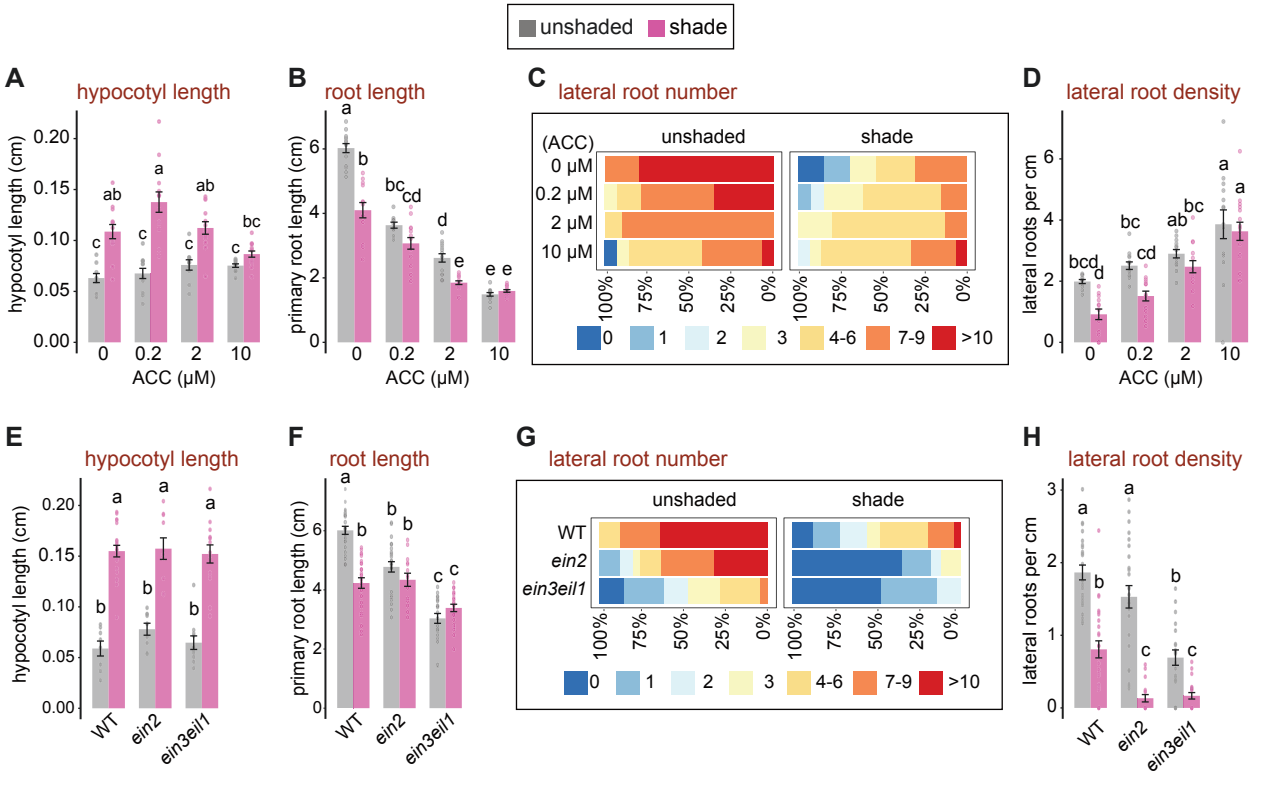
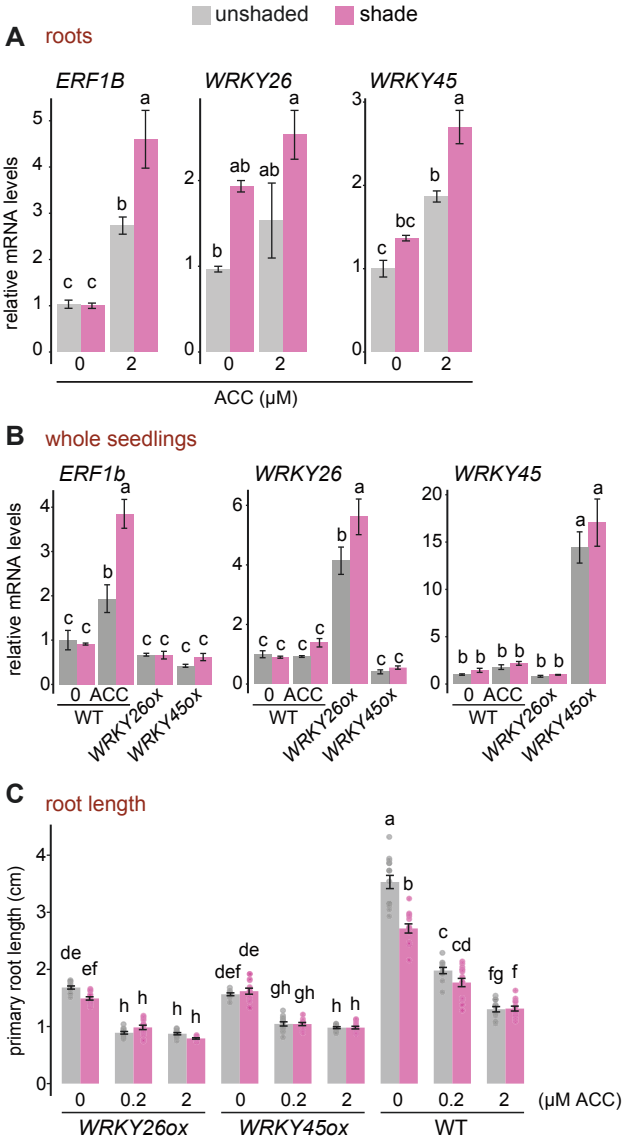
WRKY58
WRKY70
WRKY75
100 µm
100 µm
100 µm


A
WT
WRKY25ox
WRKY26ox
WRKY31ox
WRKY45ox
WRKY51ox
WRKY70ox
WRKY75ox
1 cm
B
root length
primary root length (cm)
unshaded
shade
C
hypocotyl length
hypocotyl length (cm)
D
lateral root number
unshaded
shade
100%
75%
50%
25%
0%
0
1
2
3
4-6
7-9
>10
E
lateral root density
lateral roots per cm


unshaded
shade
A
hypocotyl length
hypocotyl length (cm)
ACC (μM)
B
root length
primary root length (cm)
ACC (μM)
C
lateral root number
(ACC)
0 μM
0.2 μM
2 μM
10 μM
unshaded
shade
100%
75%
50%
25%
0%
0
1
2
3
4-6
7-9
>10
D
lateral root density
lateral roots per cm
ACC (μM)
E
hypocotyl length
hypocotyl length (cm)
WT
ein2
ein3eil1
F
root length
primary root length (cm)
G
lateral root number
WT
ein2
ein3eil1
H
lateral root density
lateral roots per cm


unshaded
shade
A
roots
ERF1B
WRKY26
WRKY45
relative mRNA levels
ACC (μM)
B
whole seedlings
ERF1b
WRKY26
WRKY45
relative mRNA levels
0 ACC
WT
WRKY26ox
WRKY45ox
C
root length
primary root length (cm)
(μM ACC)
WRKY26ox
WRKY45ox
WT

Parsed Citations

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