이학석사 학위논문

Investigation of Transcription Factors that Fine-tune the Secondary Growth and Environmental Responses in Arabidopsis Roots

애기장대 뿌리에서 환경에 대한 반응과 이차 생장을 조율하는 전사 인자에 대한 연구

2019년 6월

서울대학교 대학원

생명과학부

허 재 령

Abstract

Investigation of Transcription Factors that Fine-tune the Secondary Growth and Environmental Responses in Arabidopsis Roots

Jae Ryung Hur
School of Biological Sciences
The Graduate School
Seoul National University

The essential nutrients with optimum concentrations are required for normal plant growth and development. However, change in the concentration of these elements may cause stress and can alter the growth pattern. Nonetheless, plants have evolved with the ability to adapt to the changes in environmental conditions and accordingly

regulate their growth. Previously, stress and growth usually have been studied separately. Hence, this study aims to find a connection between plant growth and stress response. We analyzed tissue-specific transcriptome profiles from two radish lines, which showed a distinct difference in growth. As a result, we discovered numbers of stress-related transcription factors in the radish cambium.

Further, we tried to identify the role of these transcription factors by analyzing the Arabidopsis perturbation lines. We found that Ethylene Responsive Factor (ERF1) and C_2H_2-type zinc finger transcription factor (STZ/ZAT10) may have a role in mediating the secondary growth and plant responses to environmental changes, mainly, abiotic stress. Furthermore, we analyzed the common stress conditions of the selected stress-related transcription factors through meta-analysis and confirmed that various types of stress are shared among selected factors

Keywords : Secondary Growth, Cambium, Plant Stress, *Arabidopsis thaliana*, *Raphanus sativus*

Student Number: 2017-26034

Table of Contents

List of Figures

List of Tables

I. Introduction

1. Environmental components influencing plant growth and development

Plants enhance their growth via photosynthesis, which requires not only water, carbon dioxide, and light, but also essential elements, such as nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, iron, zinc, manganese, copper, chlorine, molybdenum, and boron, which are mainly derived from soil (Uchida et al., 2000). However, they can become a stress to plants if their supply is excessive or limited.

Plant stress is largely divided into two groups, biotic and abiotic stress. Biotic stress means the damage from living organisms such as fungi, bacteria, and insects, while abiotic stress is caused by non-living factors such as drought, light, salinity, heat, cold and chemical toxicity (Wang et al., 2003). Abiotic stresses mainly related to the changes in a plant growth environment can act as a critical limiting component for plant growth and yields production (Zhu, 2016) (Fig 1). For example, light is one of the critical factors that control multiple physiological responses of plants, which is called photomorphogenesis (Kendrick et al., 1994; Kami et al.,

2010). Drought is known to affect plant cell wall biosynthesis and destabilize microtubules (McFarlane et al., 2014; Feng et al., 2016). Most of the responses to plant stresses are regulated by phytohormones, to switch the regulatory program from growth to survival under stress conditions (Wolters et al., 2009). Under drought, abscisic acid (ABA) has a role against drought stress, inducing expression of stress-related transcription factors (Shinozaki et al., 2006).

Thus, when various abiotic stresses (or environmental changes) incur, plants should turn on the program that enhances their survivability at the expense of their growth.

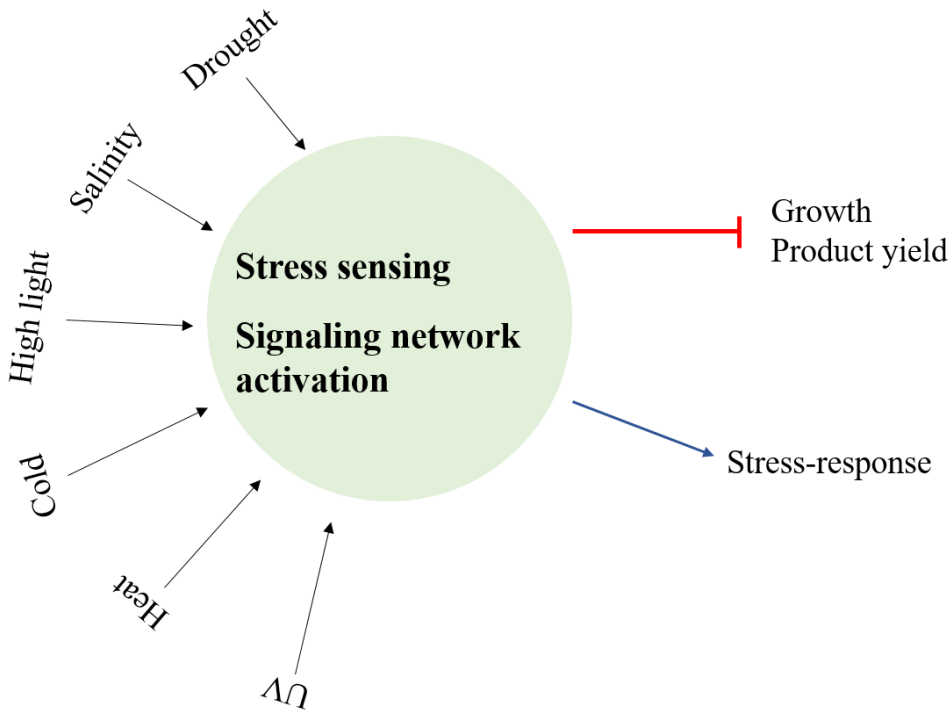

Figure 1. Dual role of plants under abiotic stresses.

Abiotic stresses negatively affect plant growth and activate stress signaling network to respond against stresses. (This figure is adapted and modified from Taiz et al., 2015)

2. Secondary growth and the cambium

Vascular plant development is categorized into two distinct growth phases depending on the origin of cell proliferation for growth: the primary and secondary growth (Eames et al., 1947). The primary growth is driven by cell proliferation of the apical meristems in the shoot and root, which mainly makes a plant grow in an apical direction. During the primary growth, subsets of cells in the vascular bundles (procambium) and neighboring cells in stems and roots are reprogrammed to become stem cells and turn into cambium. This post-embryonically derived meristematic cell population, or cambium, produces cells in the lateral direction via asymmetric cell divisions, making plants grow in girth. Because of postembryonic origin of cambium, the cambium-driven growth, is called as the secondary growth (Eames et al., 1947). One major regulator known for the secondary growth is *PHLOEM INTERCALATED WITH XYLEM* (*PXY*). PXY/TDR, a leucine-rich repeat receptor kinase expressed in the cambium tissue, binds to peptides called CLAVATA3/ESR-RELATED 41 (CLE41) and CLE44, which is produced in the phloem (Fisher et al., 2006, Hirakawa et al., 2008). *PXY*-CLE41/44 signaling promotes the expression of *WUSCHEL-RELATED HOMEOBOX 4 (WOX4)*, a

homolog of *WUS*, and *WOX14*, thereby promoting vascular stem cell proliferation in the cambium (Hirakawa et al., 2010, Etchells et al., 2013). This secondary growth contributes significantly to the biomass increase in roots and stems, especially of perennial woody species (Miyashima et al., 2013). Therefore, understanding of the secondary growth mediated cambium is essential for understanding plant growth and yields in agriculture. Recently, it was shown that cell proliferation in the cambium is critical for the growth and yield of a radish storage tap root (Jang et al., 2015).

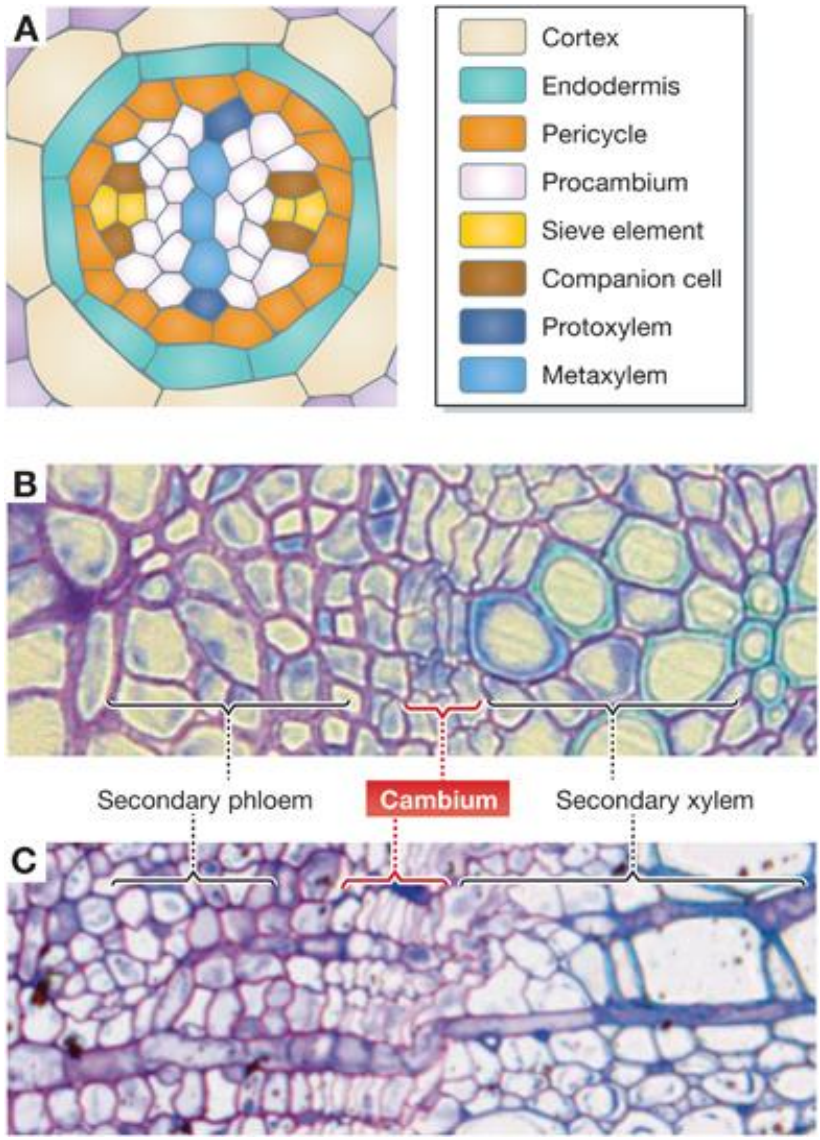

Figure 2. Vascular tissues and secondary growth.

A. A root cross section shows the vascular organization during the primary growth. B, C. Cross section images of Arabidopsis root (B), and the Populus stem (C) during the secondary growth. (Miyashima et al., 2013).

3. Crosstalk between growth and stress responses

A major question of this thesis is to understand how plants balance between upcoming stress and their growth. Despite many independent studies that investigated molecular mechanisms underlying stress responses or growth regulation, how plants coordinate these two is not well understood. Most of the studies about stress responses focused on enhancing survivorship of plants under severe stresses (Boyer et al., 1982; Ron Mittler, 2006).

Considering more than one environmental factors affect plant growth throughout their life cycles, we need to understand how multiple stress response factors are integrated and affect the growth, and ultimately predict yields or quality of crops. For example, over the past two-decade crop yields have shown that climate change, especially high temperatures due to global warming, has lowered overall crop yields (Mishra et al., 2010). Therefore, we should find a way to sustain the yield of crops against the various stresses triggered by environmental changes. Especially, it is necessary to find the key regulators that govern the responses to environmental stimuli of plants and to find design schemes that might help to maintain plant growth and yield.

While investigating the regulation of cambium activities in the

storage tap root of radish (*Raphanus sativus* L.), we found the potential role of stress-related transcription factors in the secondary growth (Choe et al., manuscript in preparation). Specifically, two inbred radish lines 216 and 218, which have shown distinct growth difference, were used for this study. We previously reported that the secondary growth of radish inbred line 216 had occurred actively than that of radish inbred line 218 because of the difference in cell proliferation activities of cambium (Fig 3a, Jang, et al., 2015). To understand the transcriptional regulatory programs responsible for the secondary growth and its differences, tissue-specific transcriptome profiles in line 216 and 218 were generated by using Laser Capture Microdissection (LCM) with RNA sequencing at three different time points, 5, 7, and 9 weeks after seed planting (Fig 3b). Differentially Expressed Genes (DEGs) were identified by comparison of expression levels of each gene in the cambium against those in the neighboring tissues, parenchyma, and cortex (Fig 3c). These genes were clustered into those with similar expression patterns, and Gene Ontology (GO) analysis was performed for each cluster. We found that many stress-related transcription factors are expressed in the higher levels in the cambium of small radish inbred line, 218 than in line 216 (Fig 4).

Because stress-related transcription factors were enriched in

the cambium of radish inbred line 218, we hypothesized that they have a role both in the secondary growth via the cambium and their defense mechanism against environmental stimuli. To test this hypothesis, I set up the experiments to analyze the secondary growth under the perturbation of cambium-enriched stress-related transcription factors, and to find their transcriptional regulatory relationships with *PXY-WOX4/14* pathway and others.

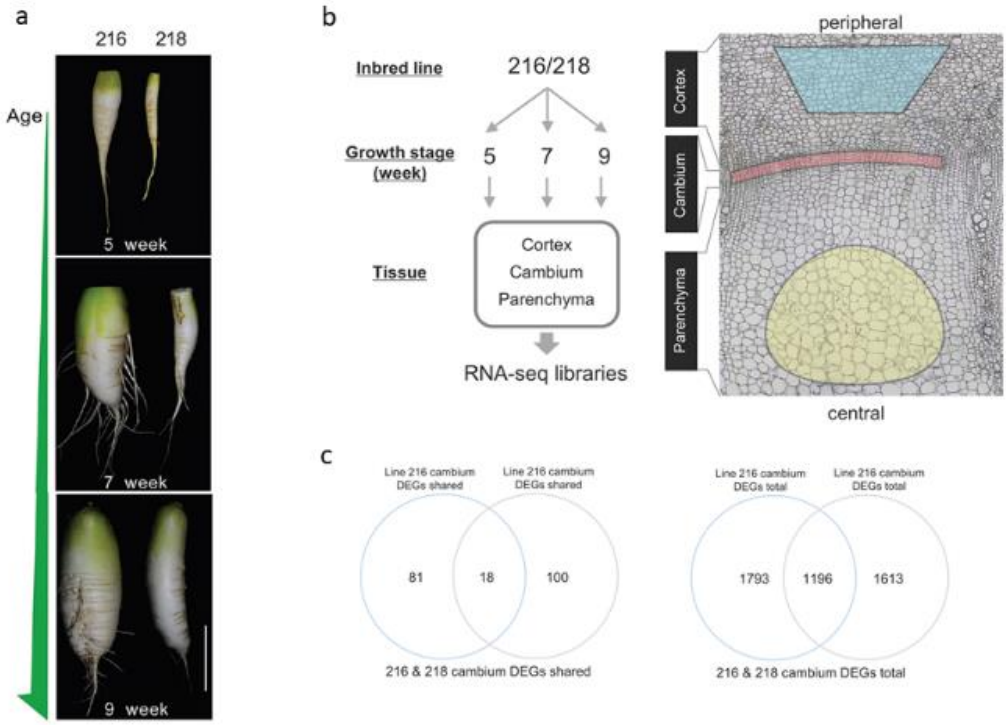

Figure 3. The procedure of Transcriptome Analysis. (a) Two radish inbred lines, 216 and 218 in different growth stages (Jang et al., 2015) (b) Sampling procedure for RNA-seq libraries (left) and tissue areas for sampling (right) (Choe et al., unpublished) (c) DEG analysis. Cambium DEGs shared (left) and cambium DEGs total (right) between radish inbred lines 216 and 218 (Choe et al., manuscript in preparation)

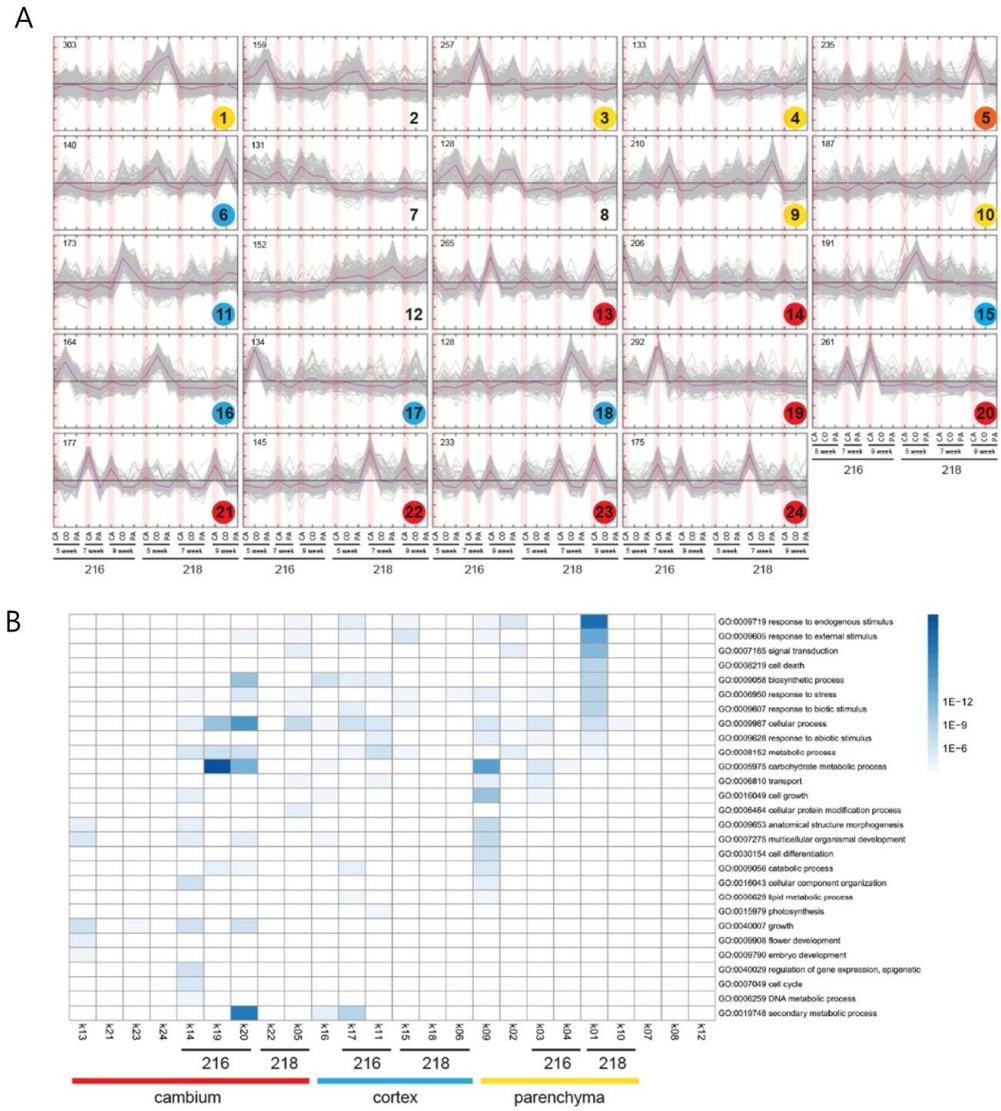

Figure 4. K−means clustering and Gene Ontology analysis. A. 24 clusters were grouped from 4,602 of total DEGs with colored marks which are showing tissue−specific expression patterns. B. A heatmap of Gene Ontology (GO) categories from co−expressed clusters (p−value cutoff 0.001). Color indication: red (cambium), blue (cortex), and yellow (parenchyma). (Choe et al., manuscript in

preparation)

II. Materials and Methods

1. Plant Materials and Growth Conditions

All Arabidopsis seeds were surface-sterilized, plated on Murashige and Skoog (MS) agar medium with 1% sucrose, cold-treated for two days at 4℃, and then grown under long-day condition (16-h-light and 8-h-dark cycle) in a plant growth chamber. For long-term experiments, seeds were planted on 32 cell tray filled with soil (Sun Gro® soil), cold-treated, and grown under the same light conditions as described above. For estradiol induction experiments, 5 mM estradiol (Sigma-Aldrich) stock solution prepared in DMSO was added to MS agar medium to a final concentration of 5 μM. Then, seedlings grown for 9 days on the regular MS agar medium were transferred to the medium with estradiol to incubate 36 hours. For GR induction, 10 μM of dexamethasone (DEX; Sigma-Aldrich) contained MS medium was used, and seedlings grown for 9 days on the regular MS agar medium were transferred and inducted for 24 hours or 5 days for long-term induction. DEX inducible Arabidopsis transgenic lines, *35S:: STZ-GR, 35S:: ERF1-GR*, and *erf1, stz* mutant lines were obtained from Dr. Dirk Inzé's group at Ghent

University (Broeck et al., 2017). Other mutants' information is described in Table 1. Estradiol inducible lines, *35S:: XVE−WOX4, 35S:: XVE−WOX14*, and *35S:: XVE−SCL7*, were provided by Dr. Ykä Helariutta's group.

Table 1. Arabidopsis mutants used in this study.

Mutants	Stock Name	Locus	References
erf1	SALK_036267	AT4G17500	Broeck et al., 2017
wrky33	SALK_006602	AT2G38470	
stz	SALK_054092	AT1G27730	Broeck et al., 2017
myb15	SALK_151976	AT3G23250	
erf2	FLAG_314D04	AT5G47220	
lhw	SALK_079402	AT2G27230	Ohashi-Ito et al., 2014
asl9	CRISPR-Cas9 mutant	AT1G16530	Zhang et al., 2019
pxy	SALK_009542	AT5G61480	Hirakawa et al., 2008
wox4	GABI462G01	AT1G46480	Hirakawa et al., 2010

2. Quantitative RT-PCR analysis

Quantitative RT-PCR analysis was performed with Dr. Hoang van Nam. Samples for qRT-PCR were collected from the thickest parts of roots. 1cm long primary root segments just below the hypocotyl were collected after removing lateral roots. Total RNA was extracted with RNeasy plant mini-prep kit (Qiagen). Then, reverse transcript reaction was performed for making cDNA, by using 1 μg of total RNA and Superscript III reverse transcriptase (Invitrogen). The cDNA template was diluted 25 fold, and then 1 μL was used for 20 μL of quantitative RT-PCR reaction. For that, SYBR Green supermix (Bio-Rad) was used and fluorescence detection were performed by using CFX96 Real-Time PCR machine (Bio-Rad), normalized using GAPDH. Primers used for this study are described in Table 2.

Table 2. A list of qRT-PCR Primers used this study.

Primer Name	Sequence
ERF1_AT4G17500.1_F	AACGGAGCTAGGGTTTGGTT
ERF1_AT4G17500.1_R	TCAACAACCTCGCACTTCAC
GAPDH_F	TTGGTGACAACAGGTCAAGCA
GAPDH_R	AAACTTGTCGCTCAATGCAATC
MYB15_AT3G23250.1_F	AGGACCATGGACACCTGAAG
MYB15_AT3G23250.1_R	CTGCAATCGCTGACCATCTA
STZ_AT1G27730.1_F	GGTCCACTAGCCACGTTAGC
STZ_AT1G27730.1_R	TTTGACCGGAAAGTCAAACC
WRKY33_AT2G38470.1_F	GTGGTGGAAGCAAGACAGTGAG
WRKY33_AT2G38470.1_R	TGTGCACTTGTAGTAGCTTCTTGG
ASL9_AT1G16530.1_F	TTGTGCCGGCTGTAAACTTC
ASL9_AT1G16530.1_R	GCTCCTGCAACATCTTATTGAC
WOX4_AT1G46480.1_F	TCACGACCACTGGTGTCTTT
WOX4_AT1G46480.1_R	CCCAGCTCCTACATGTCCTC
WOX14_AT1G20700.1_F	TACGCCGTTATTTGTGACCA
WOX14_AT1G20700.1_R	GTCGATTCGGTGTTCCACTT
PXY_AT5G61480.1_F	AAAAGCATGGGTAGGTCGTG
PXY_AT5G61480.1_R	AACATCACCAACGACGATCA
LHW_AT2G27230.1_F	CGAGCTGCATTTACTGGACA
LHW_AT2G27230.1_R	AGAGGAACCGAGCTGAACAA
SCL7_AT3G50650.1_F	CAAAGAATCCGTGTCCGAAT

SCL7_AT3G50650.1_R	GGACATGCGTCGTTTAAGGT

3. Histology

Sampling procedure of roots samples was as the same as described above. Root samples were fixed in 4% paraformaldehyde (PFA) prepared in 1X PBS buffer for overnight at 4℃. Samples were then dehydrated in ethanol series (25, 50, 75, and 100% (v/v) in PBS buffer). The samples were further dehydrated for one more time with 100% ethanol for overnight at 4℃. Samples were then infiltrated in Technovit 8100 I resin series diluted in 25, 50, 75 and 100% (v/v) in absolute ethanol for at least 1 hour each time and then in 100% Technovit 8100 I resin for overnight at 4℃. Polymerization steps were performed according to the manufacturer's instructions (Technovit® 8100). Sections (each 6 μm) were performed with a microtome (RM2255, Leica), and then stained with 0.05% Toluidine Blue solution. All images were taken with a Nikon eclipse Ni light microscope.

4. Meta-analysis

Microarray datasets were extracted from Genevestigator (https://www.genvestigator.com) (Hruz et al., 2008). Further, through the condition search tools, stress conditions were selected which showed more than 2-fold changes of *STZ* with significant p-

value <0.05.

III. Results

1. Validation of transcription factors in the *Arabidopsis* model to find a relationship between stress regulation and the secondary growth.

First, a group of stress-related transcription factors which showed high expression in the cambium of radish inbred line, 218, was selected (Table 1). These are ETHYLENE RESPONSE FACTOR1 (*ERF1*), *ERF2, WRKY33*, MYB transcription factor *MYB15*, and C_2H_2-type zinc finger transcription factor, *STZ/ZAT10*.

ERF family contains an AP2 DNA-binding domain and has 122 transcription factors in Arabidopsis (Stockinger et al., 1997; Nakano et al., 2006). *ERF1* is a crucial regulator of ethylene signaling (Lorenzo et al., 2003), and has a role in mediating abiotic stress responses (Cheng et al., 2013).

Containing a functional ERF-associated amphiphilic repression (EAR) domain, *STZ* is known as a protein that gives tolerance to various stress conditions including salinity, osmotic and heat stresses (Sakamoto et al., 2004, Kazan et la., 2006).

MYB transcription factors contain a conserved MYB DNA binding domain with three imperfect repeats (R1, R2, and R3), forming a helix-turn-helix motif (Stracke et al., 2001; Chen et al.,

2006). Also, a study showed that overexpression of *MYB15* enhances drought and salt tolerance in *Arabidopsis* by modulating ABA sensitivity (Ding et al., 2009)

Interestingly, these stress-responsive transcription factors found differentially enriched both in the cambium of radish inbred lines and in the early stage of cambia in the Arabidopsis roots (Fig. 5; Zhang et al., 2019)

As a group of growth regulators, we selected *PXY*, *WOX4*, *WOX14*, *ASL9, LHW,* and *SCL7*, to analyze their relationships with the aforementioned stress-related transcription factors in the normal condition. All selected group used for this study is described in Table 3. We confirmed the role of *PXY, WOX4* as a positive regulator of the secondary root growth by examining their knockout mutants in our plant growth conditions. We also included *SCL7, ASL9*, and *LHW* as newly identified cambium enriched transcription factor in our cell type-specific root expression map (Zhang et al., 2019). *SCL7* and *WOX4* also showed increased root secondary growth when their expression was induced under the 35S promoter (Fig. 6). The knockout mutant of *LHW* showed reduced secondary growth, again consistent with our transcriptome data.

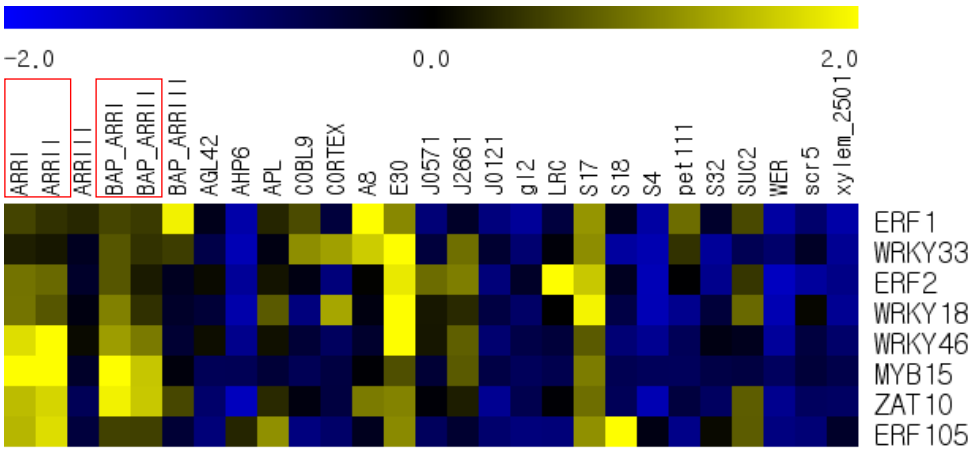

Figure 5. A heatmap showing that the stress responsive transcription factors found in the radish cambium profile is enriched in the early cambium in the Arabidopsis root (boxed in red: ARRI, ARRII, BAP_ARRI, BAP_ARRII).

Table 3. A list of the stress related transcription factors and the growth regulators that were used in this study.

A group of stress-related TFs	A group of growth regulators
ERF1 (AT4G17500)	PXY (AT5G61480)
ERF2 (AT5G47220)	WOX4 (AT1G46480)
WRKY33 (AT2G38470)	WOX14 (AT1G20700)
STZ (AT1G27730)	SCL7 (AT3G50650)
MYB15 (AT3G23250)	ASL9 (AT1G16530)
	LHW (AT2G27230)

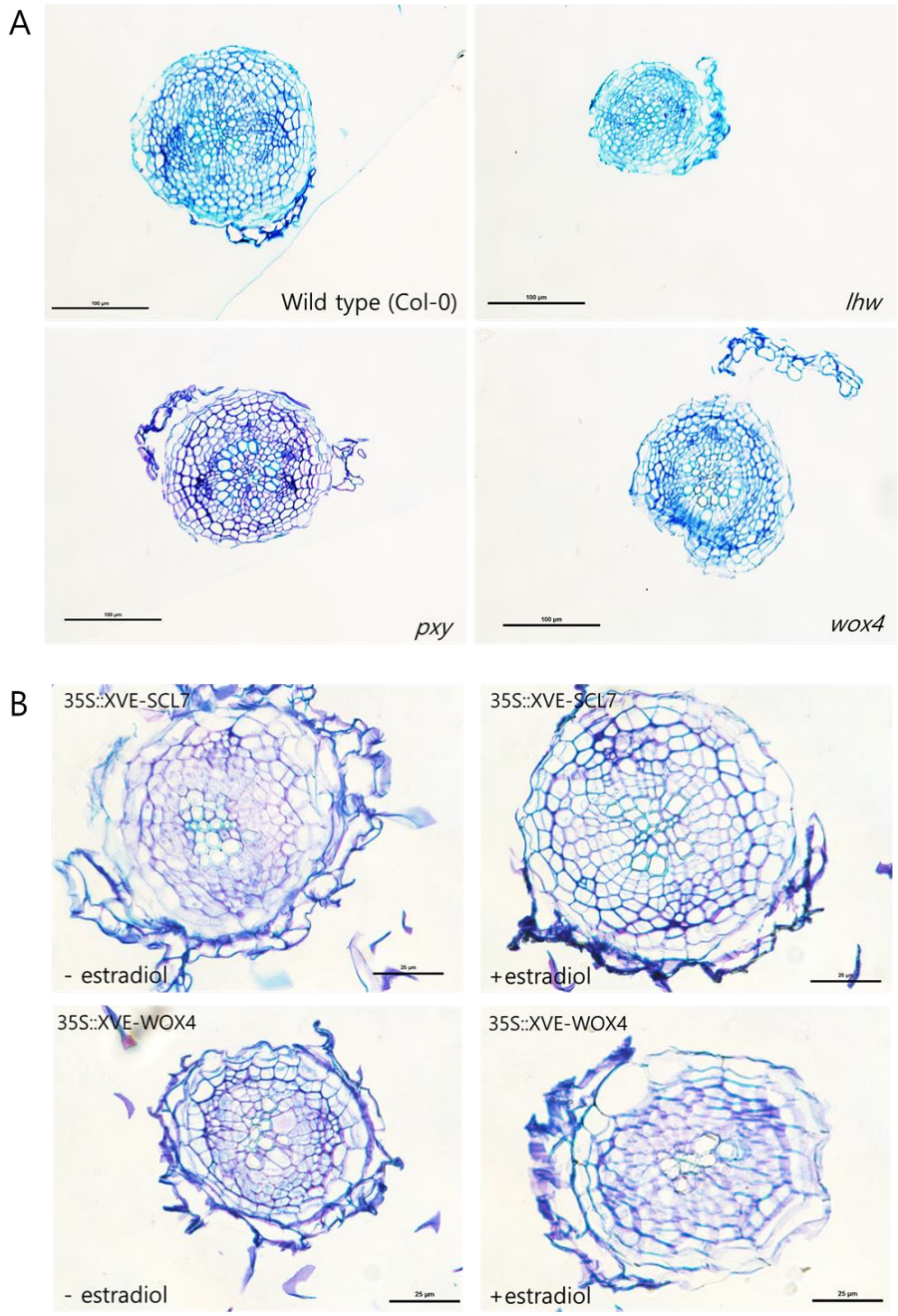

Figure 6. Root secondary growth in perturbation lines of selected growth regulators. A. 12 Day-after-transfer (DAT) of *lhw*, *pxy* and *wox4* mutant root samples. *lhw*, *pxy* and *wox4* showed smaller diameter than wild type (Col-0). 200X magnitude and scale bars

are 100 μm. B. Estradiol inducible lines of *SCL7* and *WOX4* under 35S promoter. After induction of *SCL7* and *WOX4*, root diameter increased faster than the one measured without induction. 600X magnitude and scale bars are 100 μm

2. Identification of the regulatory network among the selected cambium-enriched transcription factors

To analyze the role of stress-related transcription factors in the secondary growth, several *Arabidopsis* T-DNA insertion knockout plants were used. Seeds of each knockout mutant lines were germinated on MS media and grown for ten days. Next, we harvested the 1cm long root segments right below hypocotyls. At this stage, these segments contain cambia already established, and start the secondary growth. By examining this early stage of cambium, we tried to minimize indirect regulation among the selected regulators.

First, we collected expression patterns of selected genes in the perturbation lines that we have (knockout mutants and inducible line for *SCL7*) by using qRT-PCR. Expression changes beyond 1.5 fold of genes measured in all perturbation lines against controls are shown in Table 4. Most of them showed dynamic changes both in the growth regulator group and the stress-related transcription factors. To visualize connections between the stress-related group and the growth regulator group, a network was drawn based on the result of these qRT-PCR data in Table 4 (Fig. 7). In this network, we found that *STZ* and *ERF1* are nodes located in the middle of the network with the highest connections. Also, they were located

upstream of *PXY* and *WOX4,* suggesting they may have a role in modulating cambium function. Thus, *ERF1* and *STZ* were selected for further analysis.

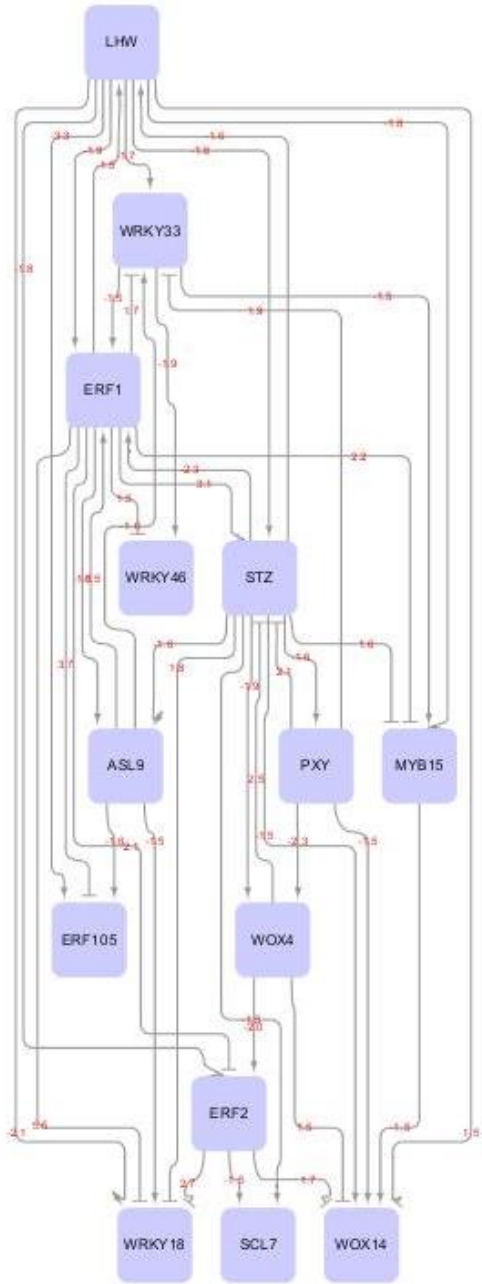

Figure 7. A transcriptional regulatory network among cambium enriched transcription factors.

CYTOSCAPE tool was used for this network. A data set for this network is shown in table 4.

Table 4. Relative expression changes of target gene expression when the source genes were perturbed. Those with more than 1.5 fold are shown.

source (mutant)	Target	Fold change	Activation / Repression
ASL9	ERF1	−1.49715	ACTIVATION
ASL9	ERF105	−1.62253	ACTIVATION
ASL9	WRKY18	−1.51764	ACTIVATION
ASL9	WRKY33	−1.6307	ACTIVATION
ASL9	WRKY46	−1.29239	ACTIVATION
ASL9	WOX4	−1.36817	ACTIVATION
LHW	ERF1	−1.8764	ACTIVATION
LHW	ERF105	−3.30417	ACTIVATION
LHW	ERF2	−1.81543	ACTIVATION
LHW	MYB15	−1.8075	ACTIVATION
LHW	WRKY18	−2.06443	ACTIVATION
LHW	WRKY33	−1.69457	ACTIVATION
LHW	WRKY46	−1.34653	ACTIVATION
LHW	STZ	−1.59384	ACTIVATION
LHW	WOX14	1.53013	REPRESSION
PXY	ERF1	1.14755	REPRESSION
PXY	ERF2	1.32362	REPRESSION
PXY	WRKY18	1.20871	REPRESSION
PXY	WRKY33	1.94243	REPRESSION
PXY	WRKY46	1.37784	REPRESSION
PXY	STZ	2.10413	REPRESSION
PXY	CYC	−1.56305	ACTIVATION
PXY	WOX14	−1.47498	ACTIVATION
PXY	WOX4	−2.2821	ACTIVATION
WOX4	ERF105	1.39178	REPRESSION
WOX4	ERF2	−2.04836	ACTIVATION
WOX4	WRKY18	−1.44	ACTIVATION
WOX4	WRKY33	1.3071	REPRESSION
WOX4	WRKY46	1.14524	REPRESSION
WOX4	STZ	2.4687	REPRESSION

WOX4	ASL9	1.37354	REPRESSION
WOX4	LHW	1.41188	REPRESSION
WOX4	WOX14	1.46209	REPRESSION
ERF1	WRKY18	5.47813	REPRESSION
ERF1	ASL9	−1.62739	ACTIVATION
ERF1	ERF1	−1.83703	ACTIVATION
ERF1	ERF105	3.67514	REPRESSION
ERF1	ERF2	2.06451	REPRESSION
ERF1	LHW	−1.52452	ACTIVATION
ERF1	MYB15	2.23942	REPRESSION
ERF1	SCL7	−1.234	ACTIVATION
ERF1	WOX4	1.14988	REPRESSION
ERF1	WRKY33	1.72194	REPRESSION
ERF1	WRKY46	1.45408	REPRESSION
ERF1	STZ	3.05308	REPRESSION
ERF105	ASL9	−1.30481	ACTIVATION
ERF105	ERF2	2.51055	REPRESSION
ERF105	LHW	−1.42601	ACTIVATION
ERF105	MYB15	1.7784	REPRESSION
ERF105	PXY	−1.50053	ACTIVATION
ERF105	SCL7	−1.44109	ACTIVATION
ERF105	WRKY18	2.12952	REPRESSION
ERF105	WRKY33	2.2526	REPRESSION
ERF105	WRKY46	2.01912	REPRESSION
ERF105	STZ	2.34105	REPRESSION
ERF2	SCL7	−1.53836	ACTIVATION
ERF2	WOX14	1.65392	REPRESSION
ERF2	WOX4	1.28472	REPRESSION
ERF2	WRKY18	2.71106	REPRESSION
ERF2	WRKY33	1.14623	REPRESSION
MYB15	ERF1	−1.35949	ACTIVATION
MYB15	ERF105	−1.31201	ACTIVATION
MYB15	WOX14	−1.54907	ACTIVATION
STZ	ASL9	−1.59991	ACTIVATION
STZ	CYC	−1.83477	ACTIVATION
STZ	ERF1	−2.31765	ACTIVATION
STZ	ERF2	1.22417	REPRESSION

STZ	LHW	−1.60868	ACTIVATION
STZ	MYB15	1.62743	REPRESSION
STZ	PXY	−1.5729	ACTIVATION
STZ	SCL7	−1.47933	ACTIVATION
STZ	WOX14	−1.47712	ACTIVATION
STZ	WOX4	−1.89704	ACTIVATION
STZ	WRKY18	1.78268	REPRESSION
STZ	WRKY33	1.20987	REPRESSION
WRKY33	ERF1	−1.505	ACTIVATION
WRKY33	ERF105	−1.28207	ACTIVATION
WRKY33	ERF2	1.41079	REPRESSION
WRKY33	MYB15	−1.47617	ACTIVATION
WRKY33	WOX4	1.10416	REPRESSION
WRKY33	WRKY46	−1.93798	ACTIVATION

3. *STZ* and *ERF1*: balancers of secondary growth in response to the environmental changes?

To further investigate roles of *STZ* and *ERF1* in the secondary growth, glucocorticoid receptor (GR) inducible lines under 35S promoter (35S::TF-GR) and knockout mutants were used for qRT-PCR and phenotypic analysis. First, we performed qRT-PCR again in 14 DAT of mutant plants (Fig. 8). Both were down-regulating most transcription factors belonging to the stress and growth groups, except for *SCL7* (Fig. 8). The inducible line of ERF1 consistently showed down-regulation of the stress group and the growth group similar with the expression changes in the mutant (Fig. 8A), while the inducible line of *STZ* showed little changes only in *ERF1, SCL7*, and *WOX4*. Taken together, suppressing or enhancing the expression of *ERF1* and *STZ* did not result in contrasting gene expression and rather showed similar effects. These made us question whether those two transcription factors should maintain their expression levels.

Previously, *STZ* has been reported to be resistant to abiotic stress in both mutant and overexpressed transgenic lines, suggesting that *STZ* has a role as both positive and negative regulators against abiotic stress, which is consistent with our data

(Mittler et al., 2006). However, there was no report of how *Arabidopsis* root secondary growth is affected when *STZ* or *ERF1* were suppressed or enhanced. To address this further, the phenotypic analysis was also performed with both knockout mutants and ectopic expression lines (Fig. 10). 14DAT of *erf1* and *stz* mutants showed a decrease in root diameter compared to that of wild type (Col-0) (Fig 10A, Fig. 11A). Also, corresponding to qRT-PCR data, inducible lines of *ERF1* and *STZ* in 14DAT also showed a decrease of root diameter (Fig. 10B, Fig. 11A). It was also observed in the three weeks the stage when the secondary growth is quite active (Fig. 11B, C). In additon, what we could conclude through phenotypic data and qRT-PCR was that the GR inducible line of *STZ* has a "leakiness" problem. Despite there was no treatment on GR control lines, they showed a smaller root diameter than wild type (Fig. 11).

Nevertheless, our results support the previous study of *STZ* even though there was not any abiotic stress introduced. Furthermore, *ERF1* may have a similar role with *STZ*, acting as a "balancer" between stress conditions and secondary growth. When their expression level changes beyond a certain threshold, the consequence seems to suppress the secondary root growth, especially WOX4, meaning expression levels of *STZ* or *ERF1*

should be tightly controlled.

.

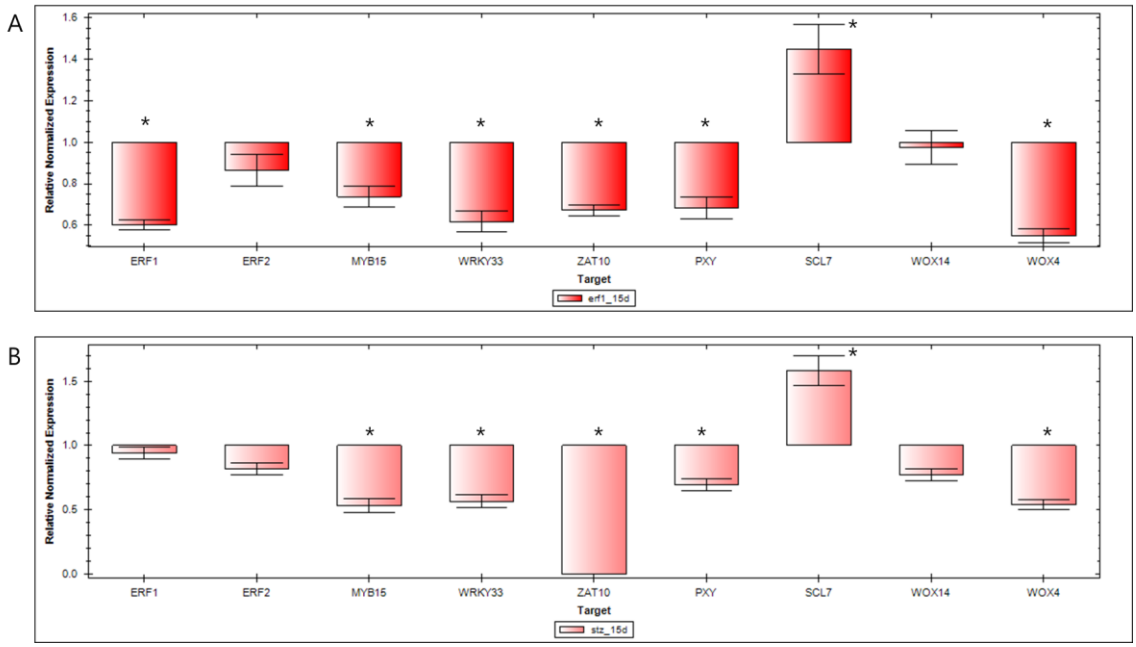

Figure 8. Expression Patterns of *erf1* and *stz* mutant lines (14DAT).

14DAT roots of *erf1* and *stz* were harvested and used for this analysis. A. *erf1* down-regulated most of selected stress-related transcription factors and also down-regulated *PXY* and *WOX4*. B. *stz* also showed similar expression pattern of *erf1*. The data represent the mean values, ±SE.

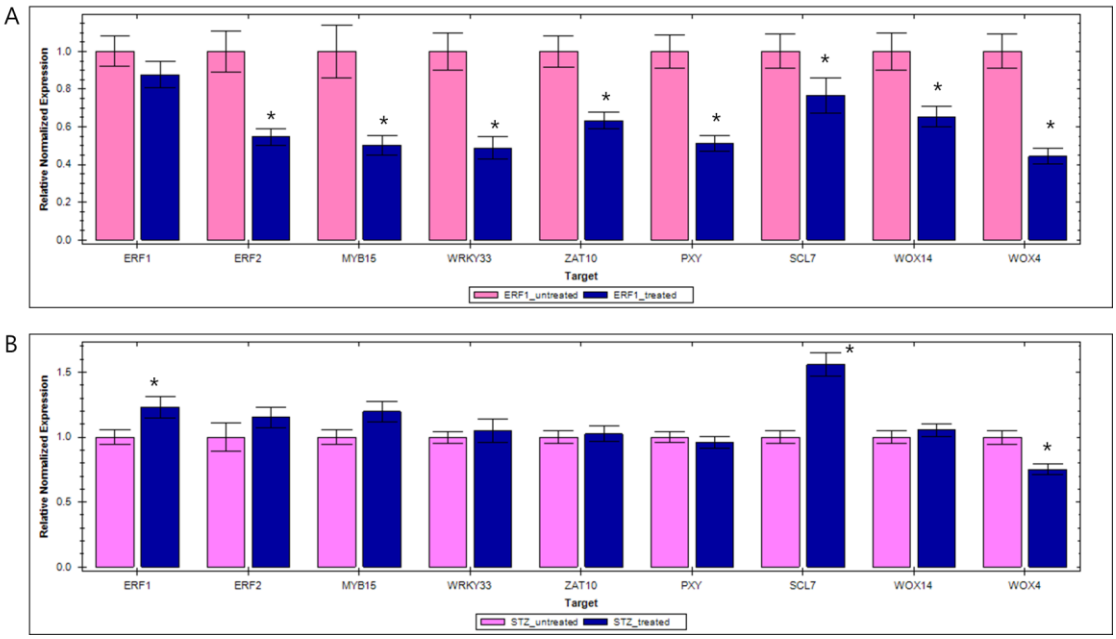

Figure 9. Expression patterns of *35S::ERF1-GR* and *35S::STZ-GR*.

DEX was treated on 9DAT samples for 5 days. After treatment, 14DAT of roots were collected and used for this analysis. (A) *35S::ERF1-GR* showed down-regulating all of two groups. (B) *35S::STZ-GR* showed down regulation of *WOX4*, up regulation of *ERF1* and *SCL7*. The data represent the mean values, ±SE.

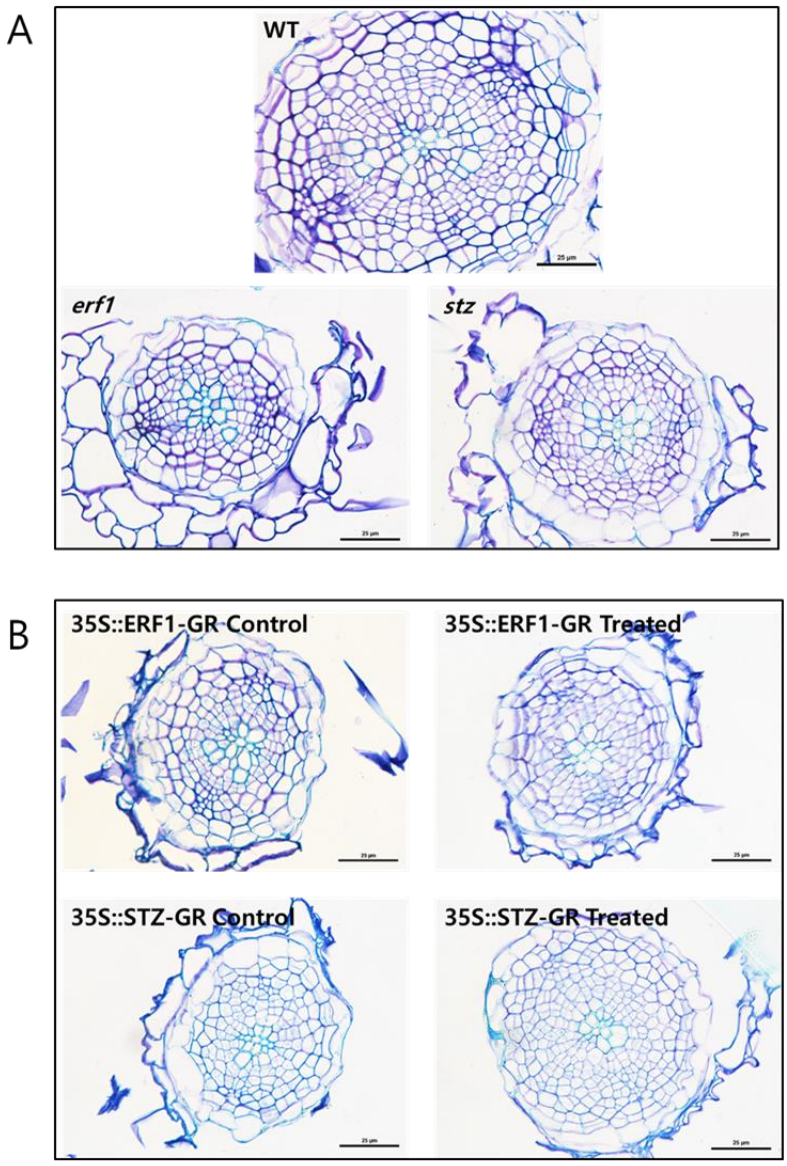

Figure 10. Phenotypes of *ERF1, STZ* Perturbation lines.

14DAT of roots were harvested and embedded for microsection with Technovit® 8100 solution. A. *erf1* and *stz* showed decreased root and cambium layers. B. Inducible lines of *ERF1* and *STZ*. DEX was treated for 5days.

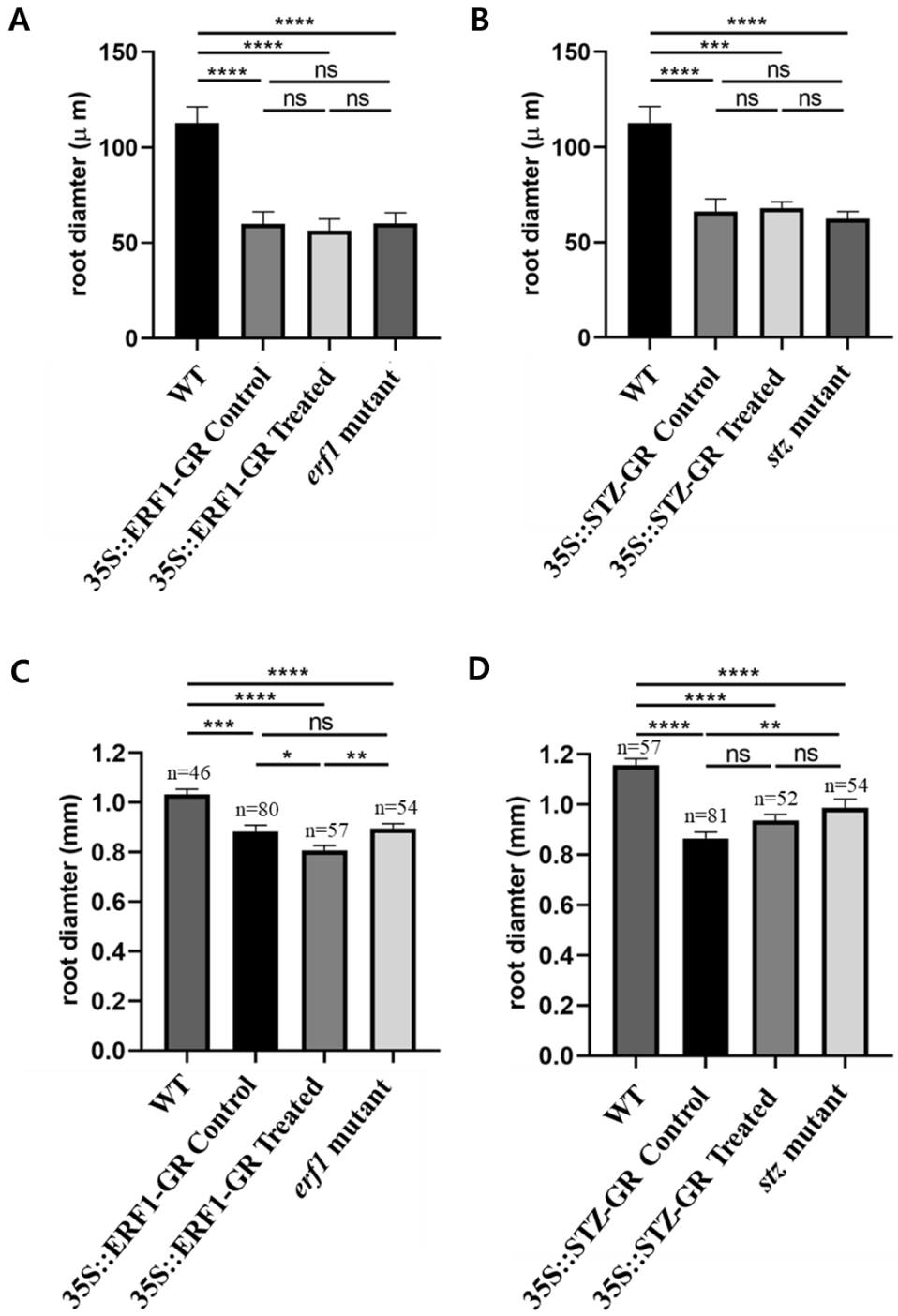
A
root diamter (μ m)
150
100
50
0
****
****
****
ns
ns
ns
WT
35S::ERF1-GR Control
35S::ERF1-GR Treated
erf1 mutant
B
root diamter (μ m)
150
100
50
0
****
***
****
ns
ns
ns
WT
35S::STZ-GR Control
35S::STZ-GR Treated
stz mutant
C
root diamter (mm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
****
****
***
ns
*
**
n=46
n=80
n=57
n=54
WT
35S::ERF1-GR Control
35S::ERF1-GR Treated
erf1 mutant
D
root diamter (mm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
****
****
****
**
ns
ns
n=57
n=81
n=52
n=54
WT
35S::STZ-GR Control
35S::STZ-GR Treated
stz mutant

Figure 11. Root diameters of *ERF1, STZ* Perturbation lines.

A, B. 14DAT of roots were harvested and measured by Image J (imagej.nih.gov/ij). All perturbation lines showed decreased root diameter than wild type (Col-0). C. 3 weeks of *ERF1* perturbation lines. All perturbation lines had smaller root diameter than wild type (Col-0). D. 3 weeks of *STZ* perturbation lines. All perturbation lines had smaller root diameter than wild type (Col-0). In all cases, differences between wild type (Col-0) roots were significant (Error bars indicate ± SE, *p<0.05, ** P < 0.01, *** P < 0.001), as determined by one-way ANOVA.

4. Finding common stress conditions that might affect both *STZ* and *ERF1*.

STZ and *ERF1* were positioned with the highest connection in the transcriptional network and seemed to regulate other stress-related transcription factors as well as well-established secondary growth regulators. Thus, we decided to investigate their roles in the secondary growth under real stress condition. To find the major stresses affecting selected transcription factors, conditions with upregulation of selected stress-related transcription factors were searched and sorted using Genvestivagtor. A meta-analysis of conditions with over 2-fold of *STZ* upregulation (p-value < 0.05) revealed that other stress transcription factors were also upregulated under the high-*STZ* condition (Fig. 12). A Venn diagram drawn to visualize stresses affecting selected stress transcription factors revealed 14 stress conditions shared by all the selected stress transcription factors (Fig. 13). These include salt, drought, hypoxia, and osmotic stresses.

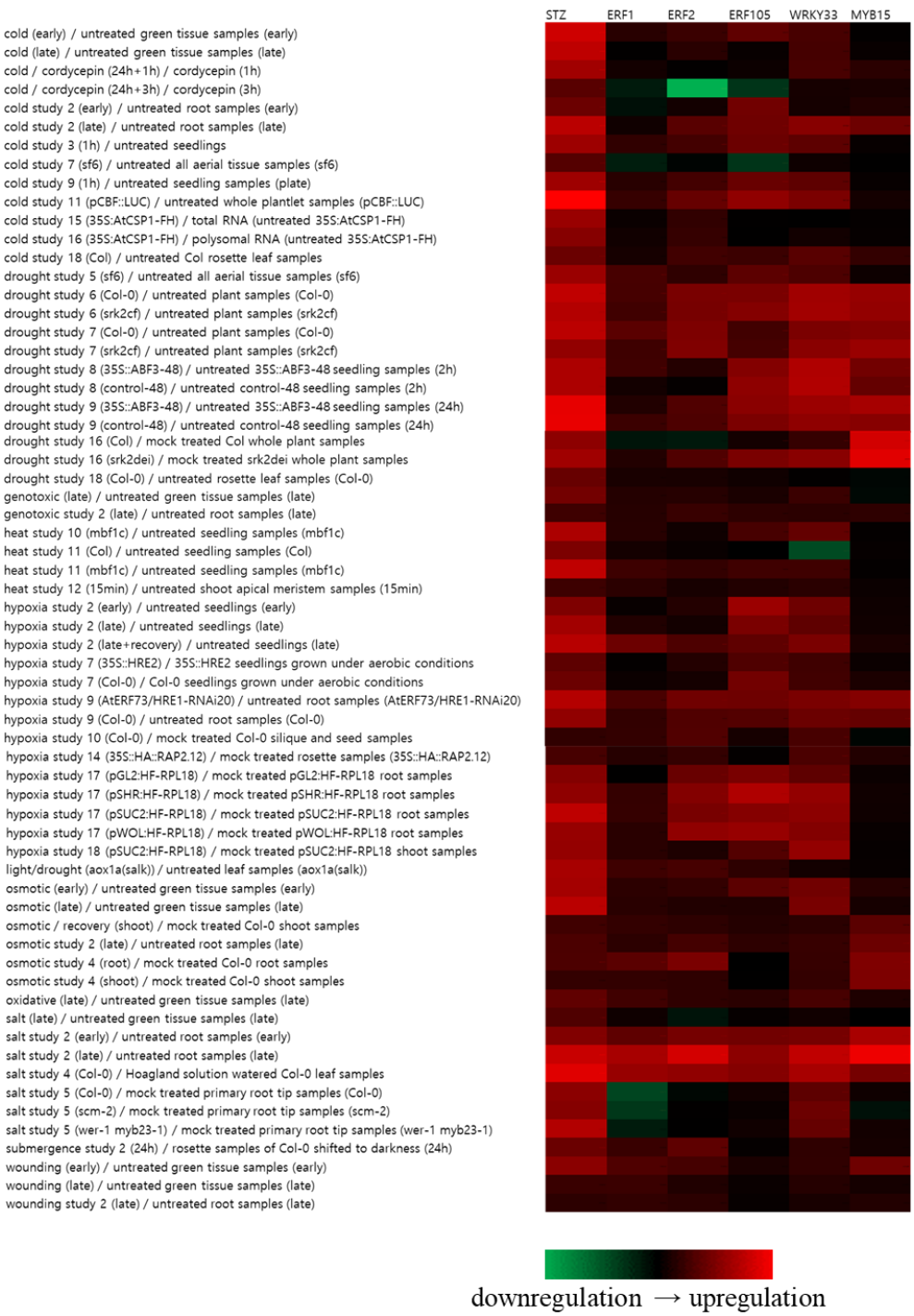

Figure 12. A Heatmap Showing *STZ*-upregulated Stress Conditions

Color indication: down-regulation in green, up-regulation in red.

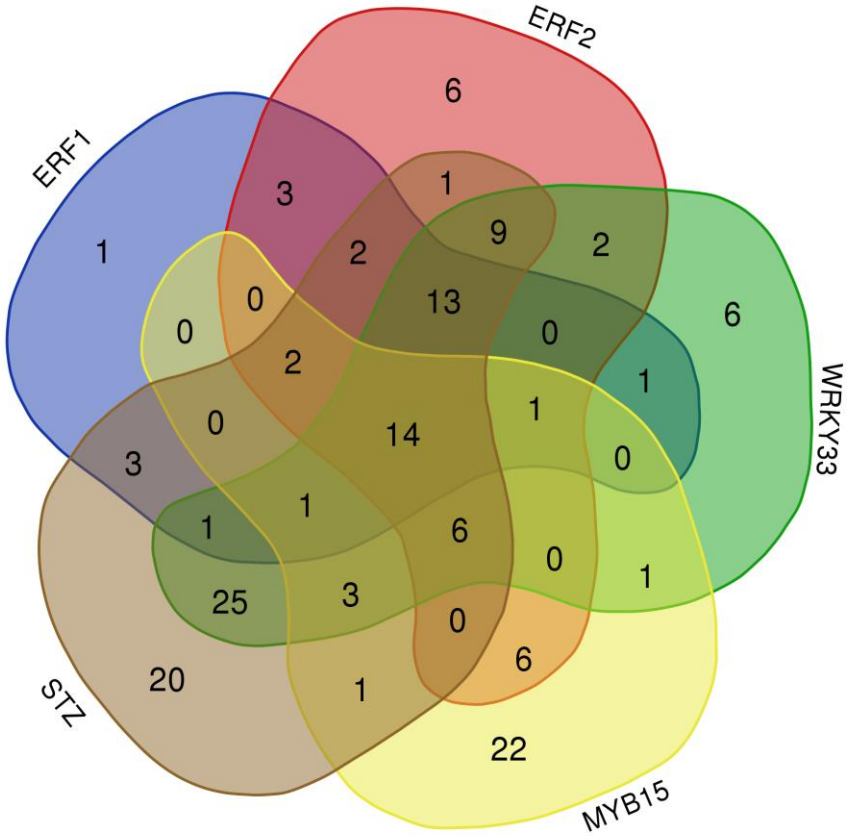

Figure 13. A Venn-diagram showing commonly shared stress conditions.

A number of commonly shared stress conditions are shown. This Venn-diagram was drawn by using a tool provided in the following link, bioinformatics.psb.ugent.be/webtools/Venn.

IV. Discussion

A potential role of *ERF1* and *STZ* as a "balancer" between stress response and the secondary growth

In this study, we analyzed tissue-specific genome-wide data obtained from two radish inbred lines and found the potential involvement of stress-responsive transcription factors in the secondary growth. A series of morphological and molecular analyses of selected transcription factors under perturbation further supported this finding. Thus, we quantified the changes in expression of selected 10 transcription factors under the perturbed condition and deduced the transcriptional network based on expression changes. Surprisingly, *ERF1* and *STZ* emerged as critical nodes in the network, and they were located upstream of *PXY* and *WOX4*, well-known pathway in the secondary growth. This indicates that *ERF1* and *STZ* might modulate the expression of cambium regulators, thereby the secondary growth. More interestingly, these two transcription factors seem to act as the balancer between plant stress responses and their growth. Both suppression and induction of *ERF1* and *STZ* expression resulted in a significant decrease in the secondary root growth. This means that the expression level of *ERF1* and *STZ* should be tightly

controlled to maintain the growth, and otherwise, the secondary growth slows down. The reduced secondary root growth in the perturbation lines of *ERF1* and *STZ* was found not only in 14 DAT the stage when Arabidopsis roots just start their secondary growth phase, but 21 DAT. This also means that the accurate balance of *ERF1* and *STZ* is essential for maintaining their growth throughout the secondary growth.

So far we only knew about ERF1 and *STZ* with their roles under the stress conditions. However, here, we found that ERF1 and *STZ* are also needed under normal condition. Suppression or induction of their expression in the normal condition changed the expression of other stress-related transcription factors as well as the other cambium-enriched transcription factors. This suggests these two transcription factors and other stress-responsive transcription factors might be the major part of the cambium function. Considering that the secondary growth is susceptible to the environmental changes, the network we discovered might contribute significantly to the cambium function, thereby plant growth and yields.

V. References

Berrocal-Lobo, M., and Molina, A. (2004). Ethylene Response Factor 1 Mediates Arabidopsis Resistance to the Soil borne Fungus Fusarium oxysporum. Molecular Plant-Microbe Interactions 17, 763-770.

Birkenbihl, R.P., Diezel, C., and Somssich, I.E. (2012). Arabidopsis WRKY33 Is a Key Transcriptional Regulator of Hormonal and Metabolic Responses toward Botrytis cinerea Infection. Plant Physiology 159, 266-285.

Bolt, S., Zuther, E., Zintl, S., Hincha, D.K., and Schmülling, T. (2017). ERF105 is a transcription factor gene of Arabidopsis thaliana required for freezing tolerance and cold acclimation. Plant, Cell & Environment 40, 108-120.

Brackmann, K., and Greb, T. (2014). Long- and short-distance signaling in the regulation of lateral plant growth. Physiologia Plantarum 151, 134-141.

Catinot, J., Huang, P.-Y., and Zimmerli, L. (2015). Ethylene response factors in Arabidopsis immunity. Journal of Experimental

Botany 67, 1231–1241.

Doust, A.N., Lukens, L., Olsen, K.M., Mauro–Herrera, M., Meyer, A., and Rogers, K. (2014). Beyond the single gene: How epistasis and gene–by–environment effects influence crop domestication. Proceedings of the National Academy of Sciences 111, 6178.

Eames, A. (1947). An Introduction to Plant Anatomy Second Edition (Tata Mcgraw–Hill Publishing Company, Bombay).

Etchells, J.P., Provost, C.M., Mishra, L., and Turner, S.R. (2013). WOX4 and WOX14 act downstream of the PXY receptor kinase to regulate plant vascular proliferation independently of any role in vascular organisation. Development 140, 2224.

Etchells, J.P., Provost, C.M., and Turner, S.R. (2012). Plant Vascular Cell Division Is Maintained by an Interaction between PXY and Ethylene Signalling. PLOS Genetics 8, e1002997.

Feng, W., Lindner, H., Robbins, N.E., and Dinneny, J.R. (2016). Growing Out of Stress: The Role of Cell– and Organ–Scale Growth Control in Plant Water–Stress Responses. The Plant Cell 28,

1769-1782.

Giuntoli, B., and Perata, P. (2018). Group VII Ethylene Response Factors in Arabidopsis: Regulation and Physiological Roles. Plant Physiology 176, 1143-1155.

Hirakawa, Y., Kondo, Y., and Fukuda, H. (2010). TDIF peptide signaling regulates vascular stem cell proliferation via the WOX4 homeobox gene in Arabidopsis. The Plant Cell 22, 2618.

Hirakawa, Y., Shinohara, H., Kondo, Y., Inoue, A., Nakanomyo, I., Ogawa, M., Sawa, S., Ohashi-Ito, K., Matsubayashi, Y., and Fukuda, H. (2008). Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system. Proceedings of the National Academy of Sciences 105, 15208.

Hu, Y., Zhao, L., Chong, K., and Wang, T. (2008). Overexpression of OsERF1, a novel rice ERF gene, up-regulates ethylene-responsive genes expression besides affects growth and development in Arabidopsis. Journal of Plant Physiology 165, 1717-1725.

Jang, G., Lee, J.-H., Rastogi, K., Park, S., Oh, S.-H., and Lee, J.Y. (2015). Cytokinin-dependent secondary growth determines root biomass in radish (Raphanus sativus L.). Journal of Experimental Botany 66, 4607-4619.

Ji, J., Strable, J., Shimizu, R., Koenig, D., Sinha, N., and Scanlon, M.J. (2010). WOX4 Promotes Procambial Development. Plant Physiology 152, 1346-1356.

Kami, C., Lorrain, S., Hornitschek, P., and Fankhauser, C. (2010). Chapter Two - Light-Regulated Plant Growth and Development. In Current Topics in Developmental Biology, M.C.P. Timmermans, ed. (Academic Press), pp. 29-66.

Kendrick, R.E., and Kronenberg, G.H. (2012). Photomorphogenesis in plants (Springer Science & Business Media).

McFarlane, H.E., Döring, A., and Persson, S. (2014). The Cell Biology of Cellulose Synthesis. Annual Review of Plant Biology 65, 69-94.

Mishra, V., and Cherkauer, K.A. (2010). Retrospective droughts in

the crop growing season: Implications to corn and soybean yield in the Midwestern United States. Agricultural and Forest Meteorology 150, 1030–1045.

Miyashima, S., Sebastian, J., Lee, J.Y., and Helariutta, Y. (2013). Stem cell function during plant vascular development. The EMBO Journal 32, 178–193.

Nakashima, K., Ito, Y., and Yamaguchi–Shinozaki, K. (2009). Transcriptional Regulatory Networks in Response to Abiotic Stresses in Arabidopsis and Grasses. Plant Physiology 149, 88.

Pirkkala, L., NykÄNen, P., and Sistonen, L.E.A. (2001). Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. The FASEB Journal 15, 1118–1131.

Ramegowda, V., and Senthil–Kumar, M. (2015). The interactive effects of simultaneous biotic and abiotic stresses on plants: Mechanistic understanding from drought and pathogen combination. Journal of Plant Physiology 176, 47–54.

Uchida, R. (2000). Essential nutrients for plant growth: nutrient

functions and deficiency symptoms. Plant nutrient management in Hawaii’s soils, 31−55.

Van den Broeck, L., Dubois, M., Vermeersch, M., Storme, V., Matsui, M., and Inzé, D. (2017). From network to phenotype: the dynamic wiring of an Arabidopsis transcriptional network induced by osmotic stress. Molecular systems biology 13, 961−961.

von Koskull−Döring, P., Scharf, K.−D., and Nover, L. (2007). The diversity of plant heat stress transcription factors. Trends in Plant Science 12, 452−457.

Zhang, J., Eswaran, G., Serra, JAA., Kucukoglu, M., Xiang, J., Yang, W., Elo, A., Nieminen, K., Damén, T., J−G Joung, J−Y Yun, J−H Lee, Ragni, L., PB de Reuille, Ahnert, SE., Lee, J.Y., AP, Mähönen and Helariutta, Y. (in press) Transcriptional regulatory framework for vascular cambium development in Arabidopsis roots. Nature Plants.

Zhao, H., Liu, K., Liu, K., Li, L., Chen, X., Li, Y., Han, S., and Wang, Y. (2018). ERF72 interacts with ARF6 and BZR1 to regulate hypocotyl elongation in Arabidopsis. Journal of Experimental Botany 69, 3933−3947.

국문 초록

애기장대에서 스트레스 환경과 뿌리 이차 생장을 미세조정하는 전사 인자에 대한 탐색

식물은 환경 조건의 변화에 적응하고, 그에 따라 성장을 조절할 수 있는 기능을 발달시켜왔다. 하지만, 기존의 연구들은 대체로 생장과 스트레스 반응을 나누어 연구하는 추세였기 때문에 이 두가지 반응의 연결점을 찾고자 하였다. 따라서 선행 연구로 비대 생장에 차이가 나는 두 무 육종 라인을 가지고 조직 특이적인 전사체 분석이 수행되었다. 여기서 비대 생장이 저해된 무의 형성층 조직에서 많은 수의 스트레스 관련 전사 인자들을 발굴할 수 있었다. 따라서 이들의 역할을 연구하기 위해 여러 애기장대 라인을 통해 분석하려 하였다. 애기장대 모델을 qRT-PCR과 뿌리 표현형 분석을 통해 관찰하였으며, 무에서 발굴한 전사 인자들 중 후보로 선정한 스트레스 관련 전사 인자들 중에서 특히 *ERF1*과 *STZ/ZAT10*가 환경 변화, 특히 비생물적 스트레스에 대한 이차 생장 및 식물 반응을 매개하는 역할을 가지고 있음을 발견하였다. 두 전사 인자 모두 일정한 값을 유지해야 식물의 이차 생장을 유지할 수

있는 스트레스와 이차 생장 사이의 조절자로서 역할을 할 것이라 예상할 수 있었다. 또한 선정된 두 인자의 공통적인 스트레스 조건을 메타 분석을 통해 분석하고 다양한 비생물적 스트레스 요인들이 선택된 인자들 사이에서 공유되고 있음을 확인할 수 있었다

Keywords : Secondary Growth, Cambium, Plant Stress, *Arabidopsis thaliana*, *Raphanus sativus*

Student Number: 2017-26034

Acknowledgements

First, and most of all, I would like to thank my supervisor Professor. Ji-Young Lee for giving me lots of support, wisdom, and guidance to make this study possible. Also, I would like to thank my committee members, Professor Yeonhee Choi and Professor Yuree Lee for their warm advices and supports.

I wish to express my thanks to all lab members. First, Dr. Goh Choe, now in KAIST, thank you so much for teaching me and giving advices. I also thank you to Dr. Muhammad Kamran for reviewing my thesis and kind supports. I hope your work is going well. Also I would like to thank you guys, Dr. Deepak Kumar, Dr. Nam van Hoang, Hyoujin Kim, Minji Seo, Jihyun Choi, and Eunju Kim for helping me a lot during over two years.

Last but not the least, I would like to thank my family for supporting me throughout my life.