UNIVERSITY OF CALIFORNIA, SAN DIEGO

Analyses of Abscisic Acid and Jasmonate crosstalk signaling via interaction of a PYR/PYL ABA receptor and the bHLH transcription factor MYC2

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by

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The Thesis of Melissa Lee is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Analyses of Abscisic Acid and Jasmonate crosstalk signaling via interaction of a PYR/PYL ABA receptor and the bHLH transcription factor MYC2

by

Melissa Lee

Master of Science in Biology

University of California, San Diego 2014

Professor Julian Schroeder, Chair

The phytohormone abscisic acid (ABA) plays several critical roles in plant development when induced by abiotic stresses such as drought, salinity, heavy metals, and extreme temperatures. Some of these responses include stomatal closure, inhibition

of seed germination, and regulation of root growth, and these responses likely occur due to interactions between ABA and other plant hormones. The crosstalk networks between ABA and other plant hormones are not yet fully understood although several studies have been underway in order to understand the mechanisms by which several signaling pathways respond to abiotic stresses. We have studied a recently discovered involvement of a PYR/PYL ABA receptor, PYL6, and a basic-helix-loop-helix (bHLH) transcription factor (TF), MYC2, in ABA and jasmonic acid (JA) signaling. In order to gain insight on the developmental and spatial expression of PYL6, the β-glucuronidase (GUS) reporter system was utilized to analyze the activity of the promoter and full length gene of PYL6 in plant tissue. GUS expression was detected in the anther of adult transgenic *pyl6* mutant plants but not in wild type background plants. Protein-protein-interaction analyses were performed to characterize a potential structural basis of interaction between PYL6 and MYC2. It was seen that several amino acid point mutations in the PYL6 protein sequence have the potential to establish a constitutive interaction between PYL6 and MYC2 with and without enhancement by ABA. Phenotypical assays with two different *pyl6* T-DNA insertion mutants displayed ABA and JA hypersensitivity during germination and cotyledon greening but no significantly altered response during root growth.

1. INTRODUCTION

Abiotic stress responses are crucial for plants to survive complex environmental conditions such as high and low temperatures, drought, salinity, and heavy metals, all of which threaten crop yield worldwide. With the current and prospected global climate change, understanding abiotic stress responses is of high priority in plant biology (Hirayama et al., 2010). In order to understand how stress responses are elicited, studying the roles and mechanisms of the hormones that are directly involved in these responses is critical. The plant hormone ABA plays a key role in mediating physiological responses to abiotic stresses by regulating rapid gene expression in different tissues and developmental phases (Seki et al., 2002; Finkelstein et al., 2002).

The ABA receptor family PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) is comprised of 14 members and these receptors can be identified by their oligomeric states, as well as other distinguishing properties (Park et al., 2009; Nishimura et al., 2010). PYR1, PYL1, PYL2, and PYL3 are more likely to form stable dimers in solution, while PYLs 4-12 are the monomeric members of the family. The dimeric receptors display a lower ABA affinity than the monomeric receptors (Okamoto et al., 2013; Hao et al., 2011; Dupeux et al., 2011; Brandt et al., 2012). PYL13 has been found to be ABA irresponsive and its function remains ambiguous (Li et al., 2013). In the presence of ABA, the PYR/PYL/RCAR proteins bind to ABA and recruit group A type 2C protein phosphatases (PP2Cs) inhibiting their function. When ABA is not present, the PP2Cs are negative regulators of ABA signaling by inhibiting SNF1-related protein kinase 2s (SNRK2s) which phosphorylate downstream transcription factors, triggering downstream ABA signal transduction (Lackman et al., 2011; Gonzalez-Guzman et al., 2012; Dupeux

et al., 2011). The ABA receptor PYL6/RCAR9 belongs to subgroup II of the PYR/PYL/RCAR family that includes PYL4 and PYL5, and these members exhibit a high intrinsic affinity for ABA and can form low-affinity complexes with PP2Cs in the absence of ABA (Dupeux et al., 2011).

Jasmonates such as jasmonic acid (JA) and methyl jasmonate (Me-JA) play crucial roles in plant defense and development by inducing the expression of specific plant genes (Staswick et al., 1992). They are synthesized via the octadecanoid pathway and regulate several physiological processes such as pollen maturation, tendril coiling, senescence, and responses to pathogen attack and wounding (Munemasa et al., 2011). JA is important for proper development, and it is also an important stress-signaling molecule in plants (Munemasa et al., 2011; Staswick et al., 1992). In regards to JA's role in reproductive development, it has been found to be a key hormone in the coordination of processes such as anther dehiscence, filament elongation, and pollen maturation (Song et al., 2011; Ito et al., 2007; Turner et al., 2002). The basic-helix-loop-helix (bHLH) transcription factor (TF) MYC2 has emerged as a key conductor of JA signaling by regulating the expression of numerous JA-responsive genes (Kazan and Manners, 2013). JA responses are inhibited by a group of proteins called JASMONATE-ZIM-DOMAIN (JAZ) repressor proteins that recruit the NINJA-TOPLESS co-repressor complex to inhibit MYC activity (Chini et al., 2007). When JA or Me-JA is applied to plants or produced endogenously, it is converted to the biologically active form (+)-7-isoJasmonoyl-L-isoleucine (JA-Ile) and binds to the Skp-Cullin-F-box (SCF)-CORONATINE INSENSITIVE1 (COI1) complex (Yi et al., 2009). This hormone-receptor interaction leads to degradation of the JAZ repressor proteins, releasing activator

proteins such as MYC2, MYC3, and MYC4 to activate JA response genes (Abe et al., 2003; Lackman et al., 2011). It has been found that MYC2 is a positive regulator of responses such as insect defense, wounding, flavonoid metabolism, and oxidative stress tolerance during JA signaling. MYC2 is involved in several signaling pathways including JA and other hormones such as ABA, salicylic acid (SA), gibberellins (GAs), and auxin (IAA). (Kazan and Manners, 2013).

Several studies support the idea that crosstalk occurs among plant signaling pathways within a complex network of interactions, which allow the activation of an appropriate spectrum of responses depending on the stimuli (Fernandez-Arbaizar et al., 2012; Anderson et al., 2004). MYC2 was originally described as a transcriptional activator of ABA signal transduction pathways before being discovered to play a critical role in JA signaling (Lackman et al., 2011). Under drought stress, MYC2 is a positive regulator of ABA-inducible genes such as RD22 and ADH1 (Gangappa et al., 2010; Abe et al., 2003). ABA has been shown to play an important role in inhibition of seed germination and growth arrest under stressed environmental conditions, and a synergistic effect has been seen when ABA and JA was combined to inhibit germination (Fernandez-Arbaizar et al., 2012). On the other hand, an antagonistic interaction between ABA and JA signaling pathways has been observed in the *jasmonic acid resisitant* (*jar1*) and *jasmonic acid insensitive4* (*jin4*) mutants, which showed hypersensitivity to ABA inhibition of seed germination and cotyledon expansion (Staswick et al., 1992; Anderson et al., 2004). Other studies have revealed several key signal components involved in Me-JA-induced stomatal closure, which suggest some type of crosstalk mechanism between ABA and Me-JA in guard cells (Kim et al., 2010; Munemasa et al., 2011). It is critical to

understand potential interactions between ABA and other hormonal signal transduction pathways in order to develop plants resistant to different types of stresses without compromising other critical stress response pathways.

An interaction between the ABA receptor PYL6 and the transcription factor MYC2 was identified to be a part of a mechanistic link between ABA and JA signaling. In collaboration with Dr. Joseph Ecker at the Salk Institute, we carried out a high-density protein array where 12,000 *Arabidopsis* proteins were synthesized on glass slides and tested for interactions with 38 TF proteins that function in several phytohormone regulatory pathways. An interaction was found between PYL6 and MYC2, and was confirmed in yeast two-hybrid analyses (Aleman et al., 2014 in preparation). This interaction in yeast was enhanced in the presence of ABA in the media. Also, PYL6 and MYC2 were found to interact *in planta* in the nucleus based on bimolecular fluorescence complementation (BiFC) experiments in *N. benthamiana* leaves. In an effort to further understand the mechanism by which PYL6 and MYC2 participate in ABA and JA crosstalk signaling, several studies were conducted to follow the results obtaianed in our lab. In order to determine tissue expression pattern of PYL6, the β-glucuronidase (GUS) reporter system was utilized to detect the activity of the promoter region and full gene of PYL6 in plant organs. GUS expression was detected in the anther of adult transgenic *pyl6* mutant plants but not in wild type background plants. Protein-protein-interaction studies using the yeast two-hybrid system were performed to characterize a potential structural basis of protein interaction between PYL6 and MYC2. Six amino acid point mutations in the PYL6 protein sequence were found to have the potential to establish a constitutive interaction between PYL6 and MYC2 without the requirement of ABA for enhancement.

Establishing this constitutive interaction between a mutant *pyl6* and MYC2 can support a future construction of a protein structure-function relationship between PYL6 and MYC2. Phenotypical assays with *pyl6* T-DNA insertion mutants displayed hypersensitivity to ABA during seed germination and cotyledon greening, and the effect was increased when Me-JA was also in the media. *myc2* mutants displayed hyposensitivity to ABA as well as ABA and Me-JA in combination during cotyledon greening, but not a significantly altered response during seed germination. Furthermore, none of the mutants displayed a significantly altered response for root growth assays. ABA and Me-JA in combination displays a synergistic negative effect on wild type plants during seed germination, cotyledon greening, and root growth, as seen in a previous study (Fernandez-Arbaizar et al., 2012). This synergistic negative effect was more identifiable in *pyl6* mutant plants than in wild-type plants during seed germination and cotyledon greening, suggesting ABA and JA crosstalk signaling to occur *in planta* through the PYL6 gene.

2. RESULTS

2.1. New research in our laboratory show that PYL6 and MYC2 interact in yeast two-hybrid system and *in planta*

Dr. Fernando Aleman et al. (in preparation) carried out a series of experiments following a high-density protein array in which the ABA receptor PYL6 was found to interact with the transcription factor MYC2 on glass slides. The interaction between PYL6 and MYC2 was further investigated by yeast two-hybrid analyses (Fig 1). When ABA was present in the triple drop out yeast media, the interaction between PYL6 and MYC2 was enhanced, and growth was observed in 3 days (Fig 1C). In the absence of ABA, an interaction between PYL6 and MYC2 was observed after 10 days of growth. The specificity of this interaction was tested by using the closest homologue of PYL6, PYL5, and the closest homologue of MYC2, MYC3. An interaction was not seen between MYC3 and PYL6, MYC3 and PYL5, or between MYC2 and PYL5 in triple dropout media with or without ABA (Fig 1B, C), indicating a specific interaction between PYL6 and MYC2. As a positive control, the PP2C, ABI1 was used to interact with PYL6, and this interaction was observed in the presence and absence of ABA.

To analyze the subcellular localization of PYL6 and MYC2, PYL6 and MYC2 were tagged with Venus fluorescence protein in plant compatible vectors and then transiently expressed by *Agrobacterium* infiltration into epidermal leave cells of *N. benthamiana*. PYL6 was localized in the cytoplasm and nucleus (Fig 2A), as are other ABA receptors (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Szostkiewicz et al., 2010) and MYC2 was localized exclusively in the nucleus (Fig 2B). In a control bimolecular fluorescence complementation (BiFC) experiment, the C-terminal half of construct YFP-PYL6 interacted with the N-terminus half of construct YFP-ABI1 in the

cytoplasm and nucleus (Fig 2C). The C-terminal half of construct YFP-PYL6 was found to interact with the N-terminus half of YFP-MYC2 exclusively in the nucleus (Fig 2D).

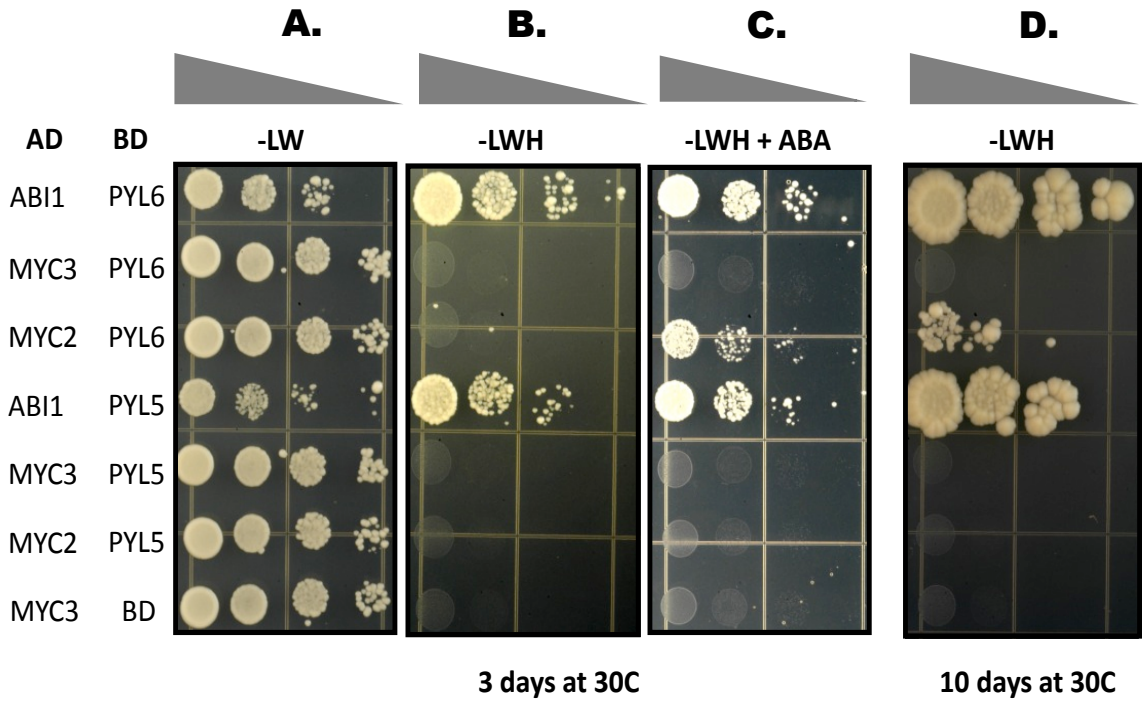

Figure 1. PYL6 interacts with MYC2 in yeast and the interaction is enhanced in the presence of ABA. Decreasing 10-fold dilution series show growth of all constructs of yeast after 3 days on control double dropout media lacking leucine and tryptophan (Panel A). In triple dropout media lacking leucine, tryptophan, and histidine, only positive control constructs that included ABI1 grew after 3 days (Panel B). When the triple dropout media was supplemented with 10 μM ABA, the interaction of PYL6-MYC2 was seen after 3 days. Interactions were not found in the combinations of the closest homologues of MYC2 and PYL6, which are MYC3 and PYL5, suggesting a highly specific interaction. After 10 days of growth, PYL6-MYC2 interaction was observed in the absence of ABA. Figure provided by Dr. Fernando Aleman.

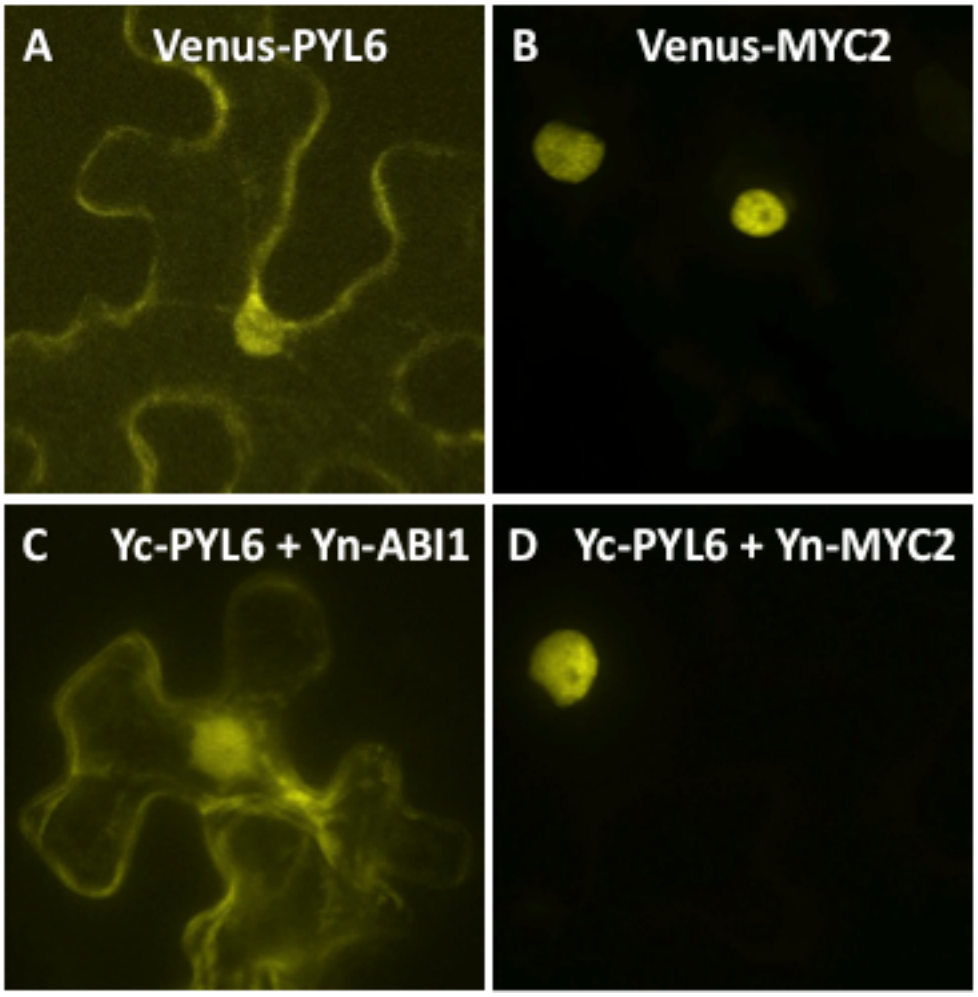

Figure 2. PYL6 interacts with MYC2 in the nucleus. A, B) Subcellular localization of PYL6 and MYC2 tagged with Venus fluorescence protein *N. benthamiana* leaves cells show that PYL6 is localized in the cytoplasm and nucleus while MYC2 is localized exclusively in the nucleus. C, D) BiFC experiments show that PYL6 interacts with ABI1 in the cytoplasm and nucleus while PYL6 interacts with MYC2 exclusively in the nucleus. Figure provided by Dr. Fernando Aleman.

2.2. Tissue expression of PYL6 in wild type and mutant *pyl6* plants

In order to identify the location of expression of the PYL6 gene in plant tissue, the promoter of PYL6 and the full PYL6 gene were cloned and the β-glucuronidase (GUS) reporter system was utilized. It was also of interest to investigate any changes in gene expression after ABA and Me-JA treatments. The promoter region alone and promoter with the full gene of PYL6 were cloned into a plant compatible vector containing the β-glucuronidase gene. Constructs were transformed in wild type Col-3 and *pyl6-2* (Col-3 background) adult plants by *Agrobacterium* infiltration resulting in the following transgenic plant lines*: pPYL6::GUS-Col-3*, *pPYL6::GUS-pyl6-2, pPYL6::PYL6-GUS-Col-3*, and *pPYL6::PYL6-GUS-pyl6-2*. Seeds were selected by Hygromycin B resistance and then transferred to 0.5 MS plates for 1 and 2 week old seedling analyses, or to potted soil for 6 week old adult plant analyses. The seedlings or plants were treated with 100 μM ABA, 50 μM Me-JA, 100 μM ABA and 50 μM Me-JA in combination, or 0.2% ethanol as a control condition for 5 hours prior to GUS staining. In 1 week old seedlings, GUS expression was not detected in any of the transgenic lines per treatment condition (Fig 3). Similarly in 2 week old seedlings, GUS expression was not detected in any of the transgenic lines per treatment condition (Fig 4). In 6 week old plants, GUS expression was detected in the anther of *both pPYL6::GUS-pyl6-2* and *pPYL6::PYL6-GUS-pyl6-2* transgenic lines (Fig 5). Any changes in PYL6 expression were not found among different treatment conditions.

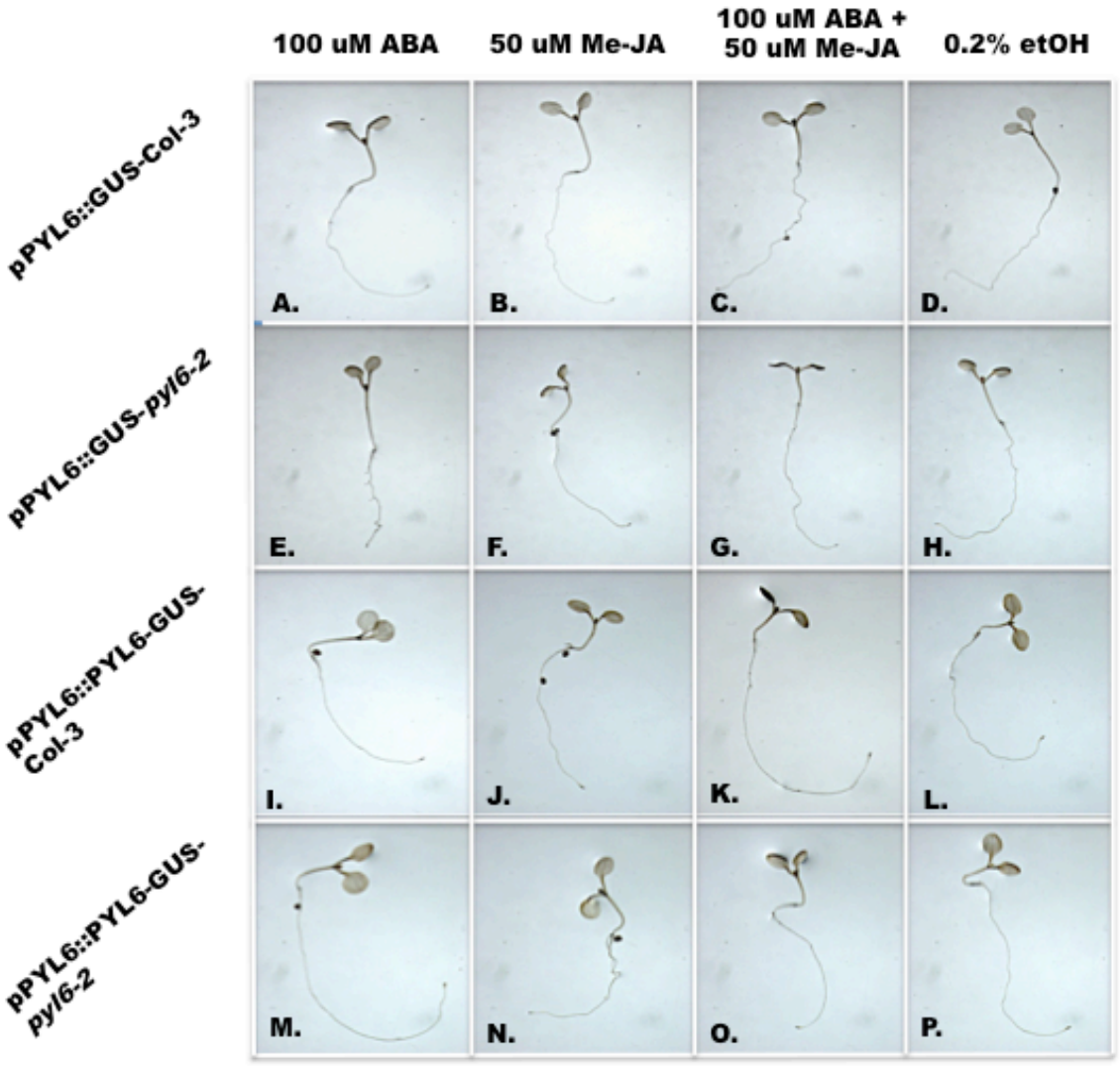

Figure 3. pPYL6 and pPYL6::PYL6 expression cannot be detected via GUS reporter system in 1 week old seedlings. 1 week old seedlings were sprayed with 100 µM ABA, 50 µM Me-JA, 100 µM ABA and 50 µM Me-JA in combination, or 0.2% ethanol as a control condition and then incubated in growth room conditions for 5 hours prior to GUS staining. GUS stained seedlings were washed with an ethanol series of 20%, 40% and 60% the following day and then left in 70% ethanol for microscope analyses and storage.

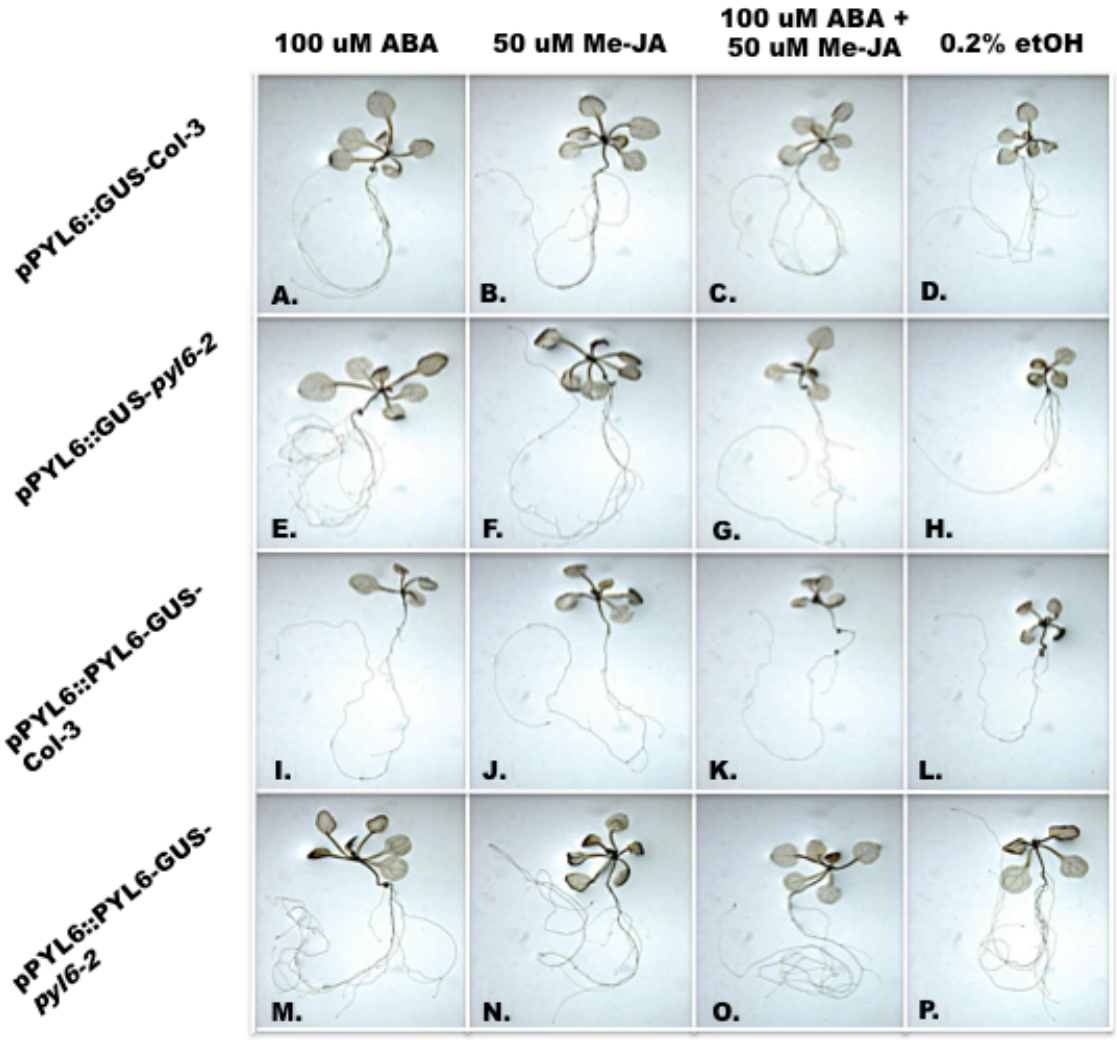

Figure 4. pPYL6 and pPYL6::PYL6 expression cannot be detected via GUS reporter system in 2 week old seedlings. 2 week old seedlings were sprayed with 100 µM ABA, 50 µM Me-JA, 100 µM ABA and 50 µM Me-JA in combination, or 0.2% ethanol as a control condition and then incubated in growth room conditions for 5 hours prior to GUS staining. GUS stained seedlings were washed with an ethanol series of 20%, 40% and 60% the following day and then left in 70% ethanol for microscope analyses and storage.

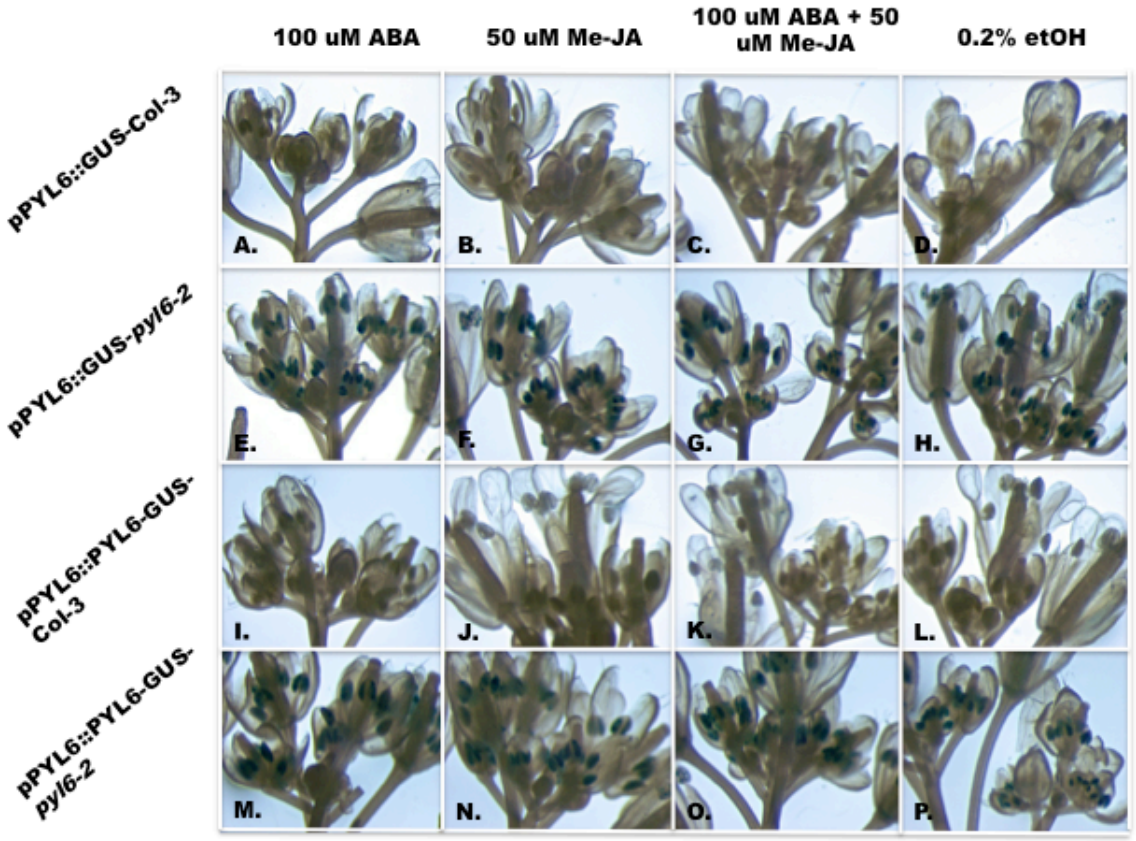

Figure 5. pPYL6 and pPYL6::PYL6 expression detected in *pyl6* mutant plants but not in wild type background plants via GUS reporter system in 6 week old plants. 6 week old flowering adult plants were sprayed with 100 µM ABA, 50 µM Me-JA, 100 µM ABA and 50 µM Me-JA in combination, or 0.2% ethanol as a control condition and then incubated in growth room conditions for 5 hours prior to GUS staining. GUS stained plants were washed with an ethanol series of 20%, 40% and 60% the following day and then left in 70% ethanol for microscope analyses and storage. **E-H,** *pPYL6::GUS-pyl6-2* plants displayed GUS expression in the anther of the stamens in all treatment conditions. **M-P,** *pPYL6::PYL6-GUS-pyl6-2* plants also displayed GUS expression in the anther of the stamens in all treatment conditions. Transgenic plants with wild type Col-3 background **(A-D, I-L)** did not display GUS expression in the anther of the stamens nor in other parts of plant tissue.

2.3. Random Mutagenesis of PYL6 for protein-protein interaction analyses

The interaction of PYL6 and MYC2 was previously confirmed in a yeast two-hybrid experiment where the interaction was enhanced with the addition of ABA in the media (Fig 1). In order to obtain a structure-function understanding of the interaction of PYL6 and MYC2, we attempted to isolate a mutated *pyl6* gene product that constitutively interacts with MYC2 without enhancement by ABA. Random mutagenesis of PYL6 was performed by error-prone PCR in which the concentrations of nucleotides dATP and dGTP were lowered and $MnCl_2$ was added to the PCR reactions to induce errors (Mohan et al., 2011). The resulting mutated BD-*pyl6* products were eventually co-transformed with AD-MYC2 in yeast. Roughly 3000 yeast colonies were screened for constitutive interaction on triple dropout media lacking leucine, tryptophan, and histidine, and supplemented with and without ABA. Figure 6 describes the screening process of determining potential *pyl6* mutants that constitutively interact with MYC2. 26 transformants were found to grow on triple dropout media with and without ABA, and 6 of those 26 transformants were found to have a point mutation in the PYL6 gene that resulted in an amino acid substitution. The 6 mutant *pyl6* genes are denoted with letters a-f and Figure 7 lists each of the six nucleotide point mutations and its corresponding amino acid substitution. With the known nucleotide mutations, site-directed mutagenic PCR was performed to recreate the mutations in the PYL6 gene and the mutant BD-*pyl6* plasmid was co-transformed again with AD-MYC2 in yeast to confirm the random mutagenesis results. In order to obtain a quantitative value of growth, the BD-*pyl6* and AD-MYC2 constructs were grown for five days in liquid triple dropout media supplemented with and without ABA and the optical density (O.D.) was measured at 600

nm for 5 days. For negative controls, each of the mutant BD-*pyl6* plasmids was co-transformed with empty AD plasmid and empty BD plasmid was co-transformed with AD-MYC2. For positive controls, BD-PYL6 and AD-ABI were co-transformed and BD-PYL6 and AD-MYC2 were co-transformed as a positive control in media with ABA.

The O.D. growth test in liquid triple dropout media with and without ABA was performed with three colonies per construct in order to obtain an average O.D. value for each construct. For several constructs, the O.D. values were very inconsistent among colonies, resulting in large standard errors, deeming the average values not statistically representative (Figs 8 and 9). There appeared to be greater variation in O.D. values between colonies of a construct in the growth test with ABA (Fig 9). In the growth test without ABA, mutant BD-*pyl6*-F92L appeared to have the highest average O.D. value of all of the other candidate BD-*pyl6* mutants at day 5 (Fig 8), although it cannot be confidently reported that this mutation results in constitutive interaction with MYC2 due to a similar O.D. value in the negative control construct of that candidate BD-*pyl6* and empty AD. Further analyses with a more precise and repeatable assay must be performed in order to confidently test the candidate mutant *pyl6*s for constitutive interaction with MYC2.

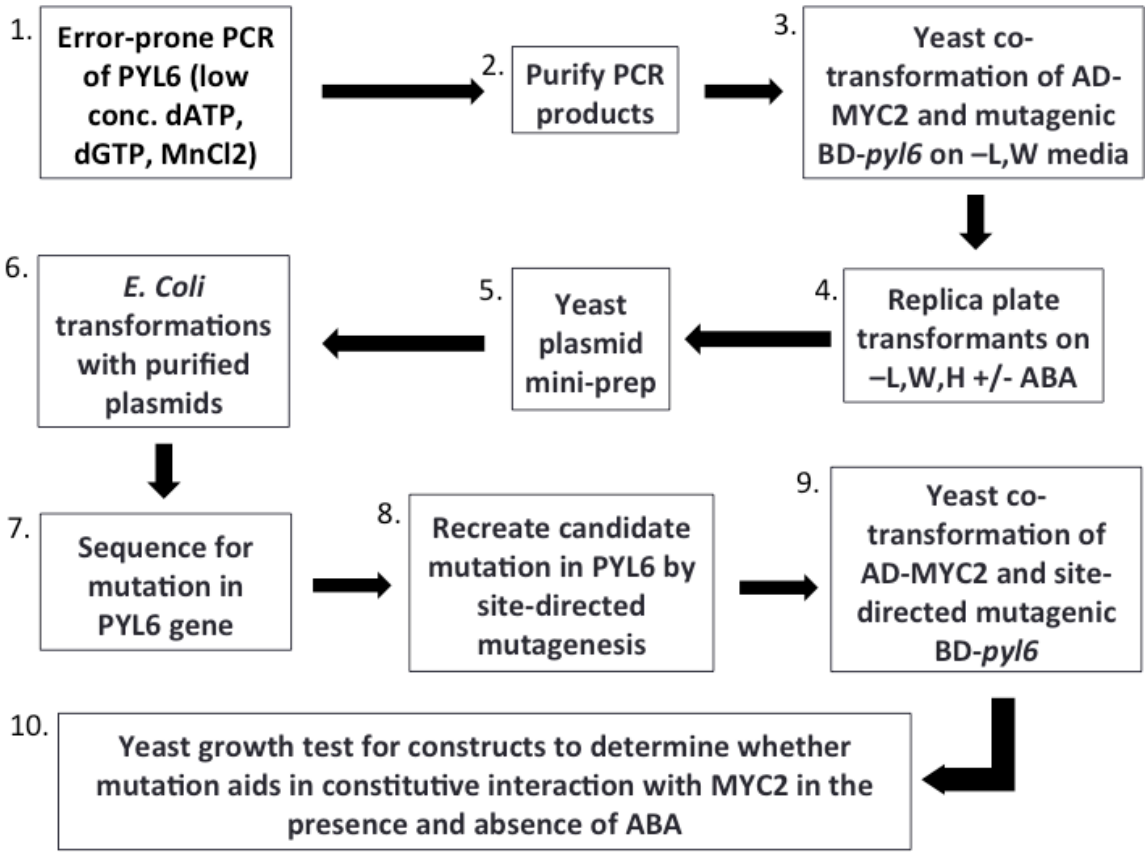

Figure 6. Screening process of *pyl6* mutant that interacts constitutively with MYC2 without ABA. Random mutagenesis was used to produce candidate mutant *pyl6*s that can potentially interact with MYC2 in the absence of ABA. Site-directed mutagenesis was used to recreate mutations that occurred from the random mutagenesis. The *pyl6*-MYC2 constructs were tested for constitutive interaction by growth in liquid dropout media with and without ABA.

	Nucleotide sequence		Protein sequence	
PYL6	gccGAGaacACTtct	Pos. 1526	A E N T S	Pos. 211
pyl6-a	gccGAGaacGCTtct		A E N A S	
PYL6	tcgGAGgagGACtcc	Pos. 1377	S E E D S	Pos. 161
pyl6-b	tcgGGgagGACtcc		S G E D S	
PYL6	catCAGaaaCAGgtt	Pos. 983	H Q K Q V	Pos. 30
pyl6-c	catCAGgaaCAGgtt		H Q E Q V	
PYL6	ttcGTGaaaAGCtgc	Pos. 1169	F V K S C	Pos. 92
pyl6-d	ctcGTGaaaAGCtgc		L V K S C	
PYL6	gcgGGTaacGATaag	Pos. 1452	A G N D K	Pos. 186
pyl6-e	gcgGGTaacGATagg		A G N D R	
PYL6	gtgGAGcttTCCcac	Pos. 1040	V E L S H	Pos. 49
pyl6-f	gtgAAGcttTCCcac		V K L S H	

Figure 7. Six candidate point mutations in PYL6 gene were found after performing error-prone PCR. Each of the six mutations (*pyl6-a-f*) is aligned with the wild type PYL6 nucleotide sequence (left) at the position of mutation and the corresponding amino acid substitution and position is shown (right).

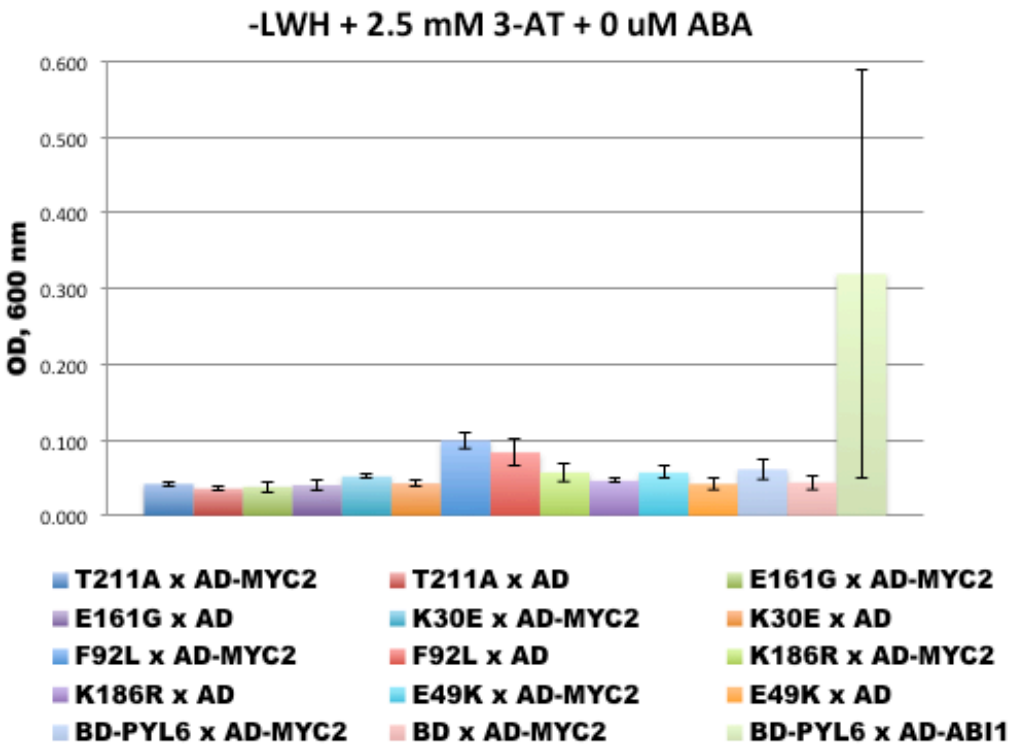

Figure 8. Liquid culture growth test in triple dropout media without ABA. The candidate mutant BD-*pyl6*s are denoted above with the corresponding amino acid substitution and were co-transformed with AD-MYC2 and empty AD plasmid. Constructs that include empty AD or empty BD were used as negative controls. BD-PYL6/AD-ABI1 construct was used as a positive control. At day 5 of growth, the BD-*pyl6* (F92L)/AD-MYC2 construct had the highest average O.D. value, although the negative control of BD-*pyl6* (F92L)/empty AD had a similarly high O.D. value. Values are based on the average O.D. values of three colonies per construct ± s.e.

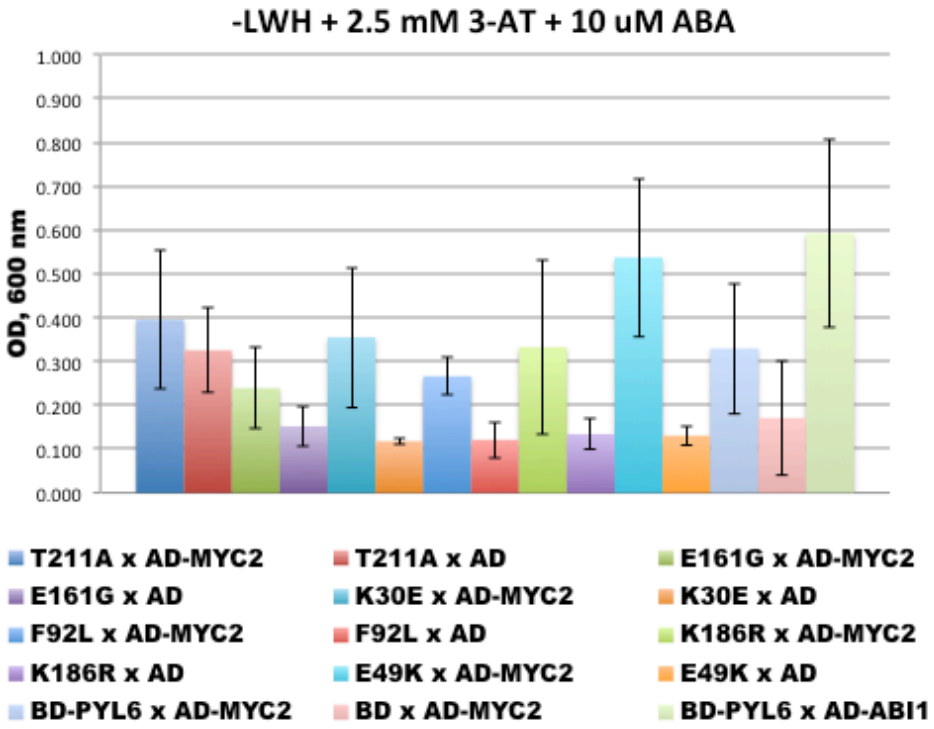

Figure 9. Liquid culture growth test in triple dropout media with ABA. The candidate mutant BD-*pyl6*s are denoted above with the corresponding amino acid substitution and were co-transformed with AD-MYC2 and empty AD plasmid. Constructs that include empty AD or empty BD were used as negative controls. BD-PYL6/AD-MYC2 and BD-PYL6/AD-ABI1 constructs were used as positive controls. Values are based on the average O.D. values of three colonies per construct ± s.e. The high error bars seen for several of the constructs were due to inconsistent values between colonies of a construct, resulting in standard deviations greater than the average O.D. value.

2.4. *pyl6* T-DNA mutants are hypersensitive to ABA and JA at germination and cotyledon greening

It was crucial to characterize the phenotype for *pyl6* mutants in ABA responses, but it was also of interest to understand PYL6's role in JA responses. Two different *pyl6* T-DNA mutants, referred to as *pyl6-1* and *pyl6-2,* were analyzed for germination, post-germination, and root growth assays. The *pyl6-1* T-DNA is inserted at the C-terminus of the PYL6 gene in the Col-0 background, while the *pyl6-2* T-DNA is inserted at the N-terminus of the PYL6 gene in the Col-3 background. Previous studies have reported that *MYC2* overexpressing plants and *myc2* mutant plants show increased and reduced ABA sensitivity, respectively (Kazan and Manners, 2013; Abe et al., 2003; Lorenzo et al., 2004), and in our studies, a *myc2* T-DNA mutant in the Col-0 background was used and is referred to as *myc2-2* (Shin et al., 2012). Seed germination (radical emergence) and cotyledon greening (cotyledon expansion) rates were recorded for *pyl6*-1, *pyl6*-2, and *myc2-2* mutants in 0.5 MS media and 0.5 MS media supplemented with 0.5 μM ABA, 1 μM ABA, 10 μM Me-JA, or 0.5 μM ABA and 10 μM Me-JA in combination (Fig 10).

In control media without ABA or Me-JA, germination and cotyledon greening was not affected in any mutant genotype (Fig 10A, F; Fig 11A). Analyses of *pyl6-1* and *pyl6-2* mutants at germination displayed a hypersensitivity to 1 μM ABA with a germination rate between 60-70% compared to 90% germination for Col-0 and *myc2-2* at day 3 of analysis (Fig 10C). The *pyl6* mutants did not display a significantly altered response to 0.5 μM ABA, revealing a concentration dependent response (Fig 10B). When 0.5 μM ABA and 10 μM Me-JA was combined, *pyl6-2* mutants displayed hypersensitivity with a germination rate of 40% compared to that of Col-0, *pyl6-1,* and

myc2-2 mutants, which had a 60% germination at day 2 of analysis (Fig 10E). In a post-germination analysis of cotyledon greening, *pyl6-1* and *pyl6-2* displayed hypersensitivity to 1 μM ABA with cotyledon greening rates of 30% and 40%, respectively, compared to Col-0 and *myc2-2* that had 70% and 80% cotyledon greening, respectively (Fig 10F). *pyl6-1* and *pyl6-2* mutants displayed slight hypersensitivity to 0.5 μM ABA and 10 μM Me-JA in combination as these mutants exhibited 40% green cotyledons compared to 60% green cotyledons in Col-0 (Fig 10F). *myc2-2* mutants displayed earlier cotyledon greening compared to Col-0 in 1 μM ABA as well as in 0.5 μM ABA and 10 μM Me-JA combination. The synergistic effect of ABA and JA during seed germination and cotyledon greening is visible in Col-0 and *pyl6* mutants as inhibition by ABA is enhanced when Me-JA is added to the media, compared to inhibition by ABA alone (Fig 11).

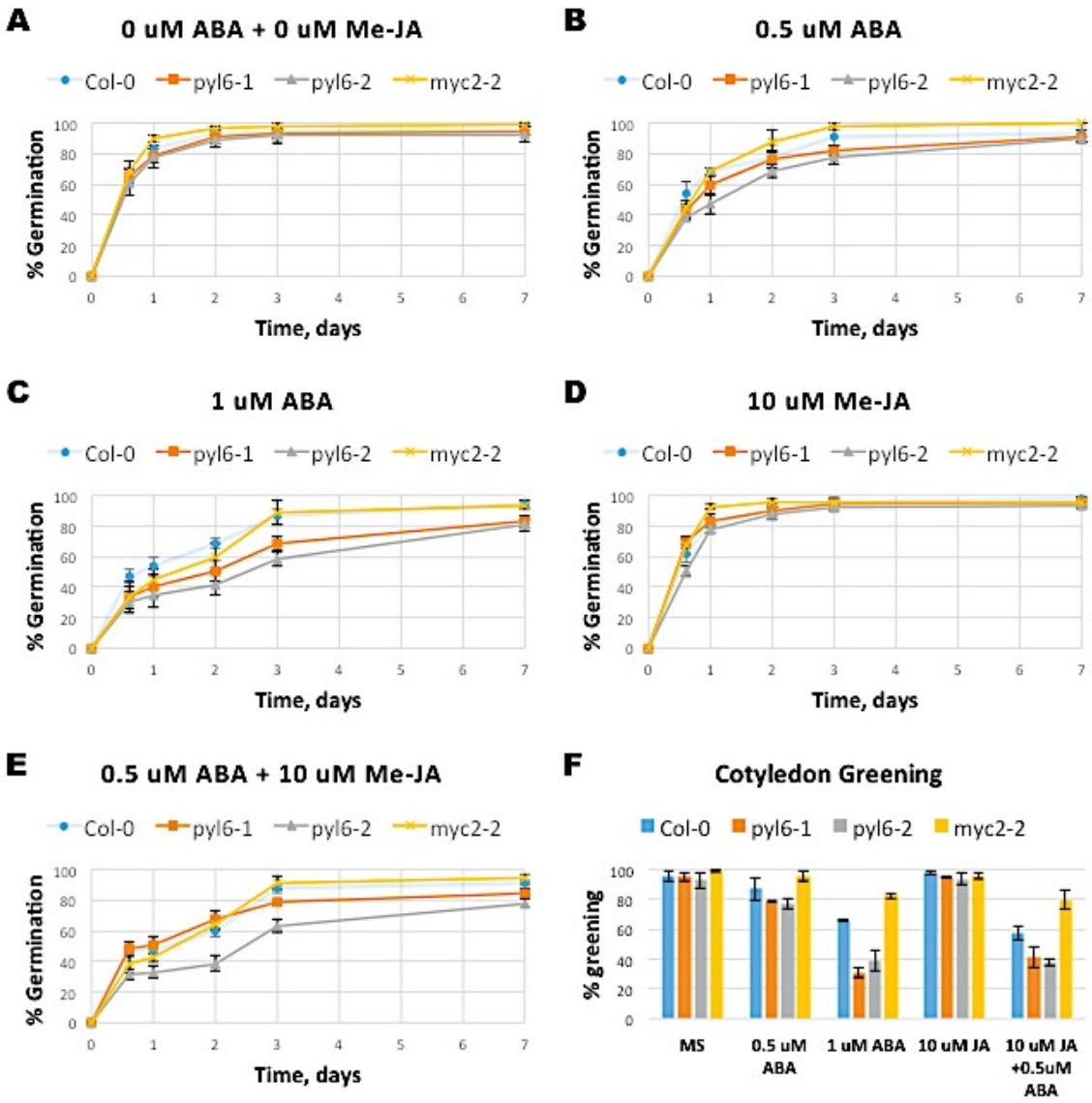

Figure 10. *pyl6* T-DNA mutants are slightly hypersensitive to ABA and JA during seed germination. A-E, Seed germination on media with 0.5 MS (**A**) or supplemented with 0.5 µM ABA (**B**), 1 µM ABA (**C**), 10 µM Me-JA (**D**), or 0.5 µM ABA and 10 µM Me-JA in combination (**E**) was recorded on days 1, 2, 3, and 7 and cotyledon greening was recorded on day 7 (**F**). Date represents average values ± s.d. This experiment was repeated three times with similar results. At 1 µM ABA, *pyl6-1* and *pyl6-2* mutants display ABA hypersensitivity (**C**). At 0.5 µM ABA and 10 µM Me-JA in combination, *pyl6-2* mutants displays hypersensitivity compared to that of Col-0, *pyl6-1,* and *myc2-2* mutants (**E**). Compared to wild type Col-0 and *myc2-2* T-DNA mutants, *pyl6-1* and *pyl6-2* mutants display hypersensitivity to 1 µM ABA and a slight hypersensitivity to 0.5 µM ABA and 10 µM Me-JA in combination during cotyledon greening (**F**).

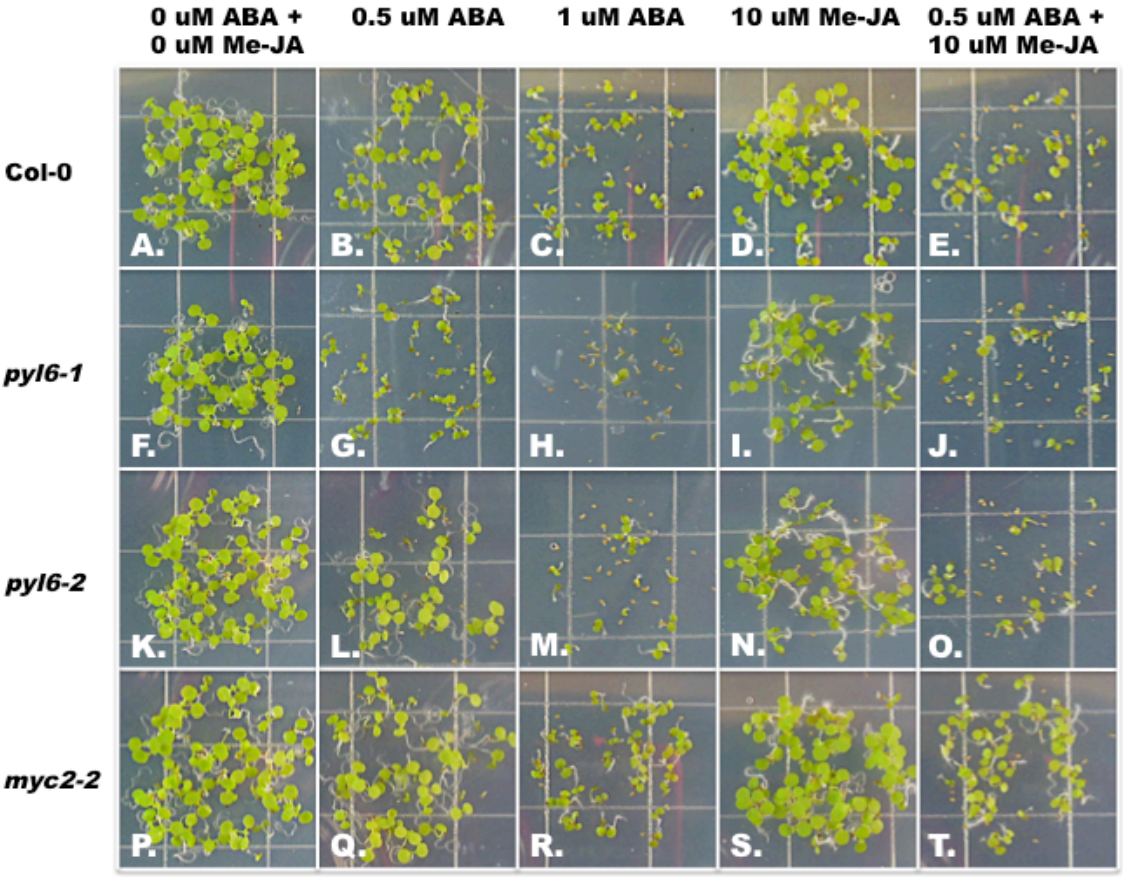

Figure 11. *pyl6-1* and *pyl6-2* T-DNA mutants are more sensitive to ABA and JA during cotyledon greening. A-E, Cotyledon greening was measured on day 7 of germination on media with 0.5 MS (**A**) or 0.5 MS supplemented with 0.5 µM ABA (**B**), 1 µM ABA (**C**), 10 µM Me-JA (**D**), or 0.5 µM ABA and 10 µM Me-JA in combination (**E**). At 1 µM ABA, *pyl6-1* and *pyl6-2* mutants are more sensitive to ABA inhibition than wild type Col-0 and *myc2-2* mutants (**C**). At 0.5 µM ABA and 10 µM Me-JA in combination, *pyl6-1* and *pyl6-2* mutants are slightly more sensitive than wild type Col-0 and *myc2-2* mutants (**E**).

Root growth analyses were conducted on *pyl6* and *myc2* mutants to observe root growth inhibition by ABA and JA. It has been reported than under certain plant growth conditions and the concentration, ABA has an inhibitory role in root growth (Antoni et al., 2013; Fernandez-Arbaizar et al., 2012). Jasmonates are also known to inhibit root growth although they do not have an inhibitory role in seed germination (Staswick et al., 1992; Berger et al., 1996; Huang et al., 2010). Four day old seedlings of genotypes, Col-0, *pyl6-1*, *pyl6-2*, and *myc2-2* grown on 0.5 MS media were transferred to 0.5 MS media or 0.5 MS media supplemented with 10 μM ABA, 10 μM Me-JA, or 5 μM ABA and 5 μM Me-JA in combination and grown for 10 days (Fig 12). In control media without ABA or Me-JA, root growth was not affected in any mutant genotype (Fig 12). *pyl6-1* and *pyl6-2* mutants did not display hypersensitivity or hyposensitivity to any of the experimental conditions of 10 μM ABA, 10 μM Me-JA, or 5 μM ABA and 5 μM Me-JA in combination (Fig 12B-D). *myc2* mutants are reported to be defective in JA-mediated root growth inhibition (Lorenzo et al., 2004; Nakata et al., 2013) and that was confirmed in root growth analyses with the *pyl6* mutants. *myc2-2* mutants displayed slight hyposensitivity to root growth inhibition in 10 μM Me-JA, as well as 5 μM ABA and 5 μM Me-JA in combination (Fig 12C-D).

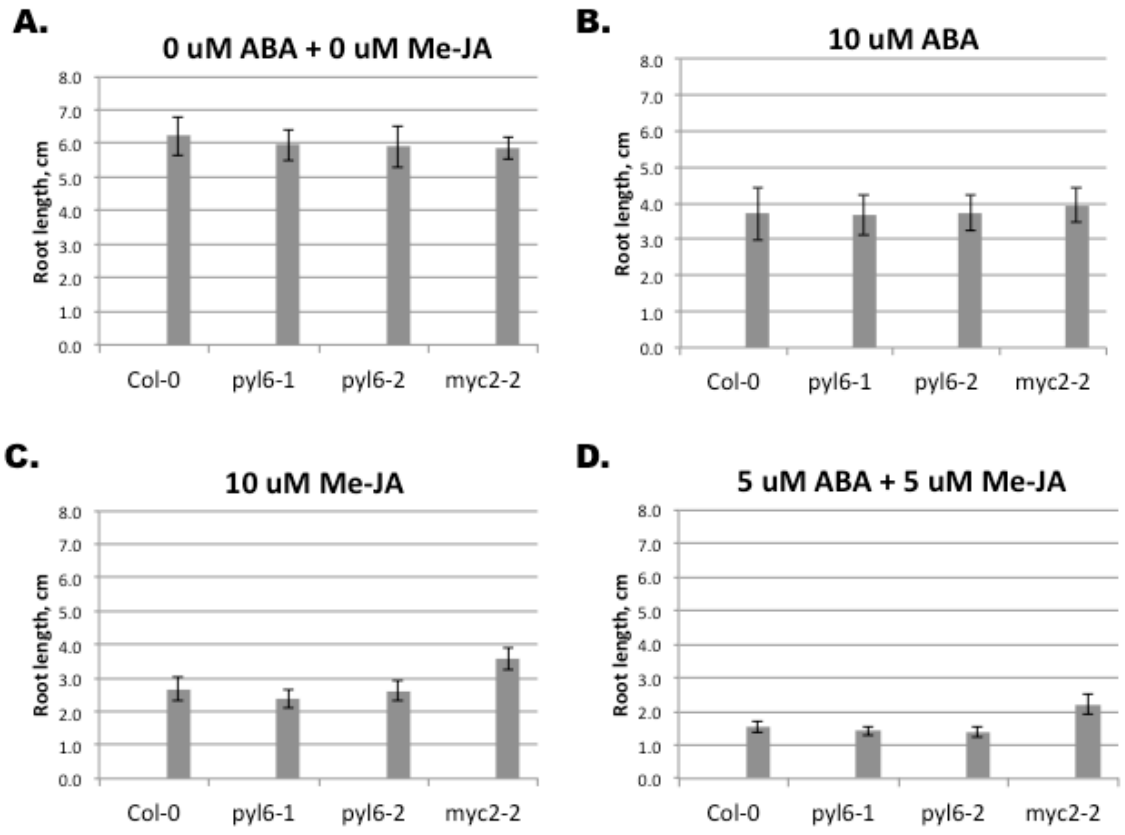

Figure 12 part I. Root growth is not significantly altered in *pyl6-1* and *pyl6-2* T-DNA mutants while *myc2-2* mutants display slight hyposensitivity to ABA and JA. Four day old seedlings of indicated genotypes were transferred to 0.5 MS media (**A**) or 0.5 MS supplemented with 10 μM ABA (**B**), 10 μM Me-JA (**C**), or 5 μM ABA and 5 μM Me-JA in combination (**D**) and grown for additional ten days after seedling transfer. Data represents average values ± s.d. This experiment was repeated three times with similar results. *pyl6-1* and *pyl6-2* mutants display root growth inhibition by ABA and Me-JA similar to that of Col-0 (**B-D**). *myc2-2* mutants display slight insensitivity to root growth inhibition by Me-JA as well as inhibition by ABA and Me-JA in combination (**C, D**).

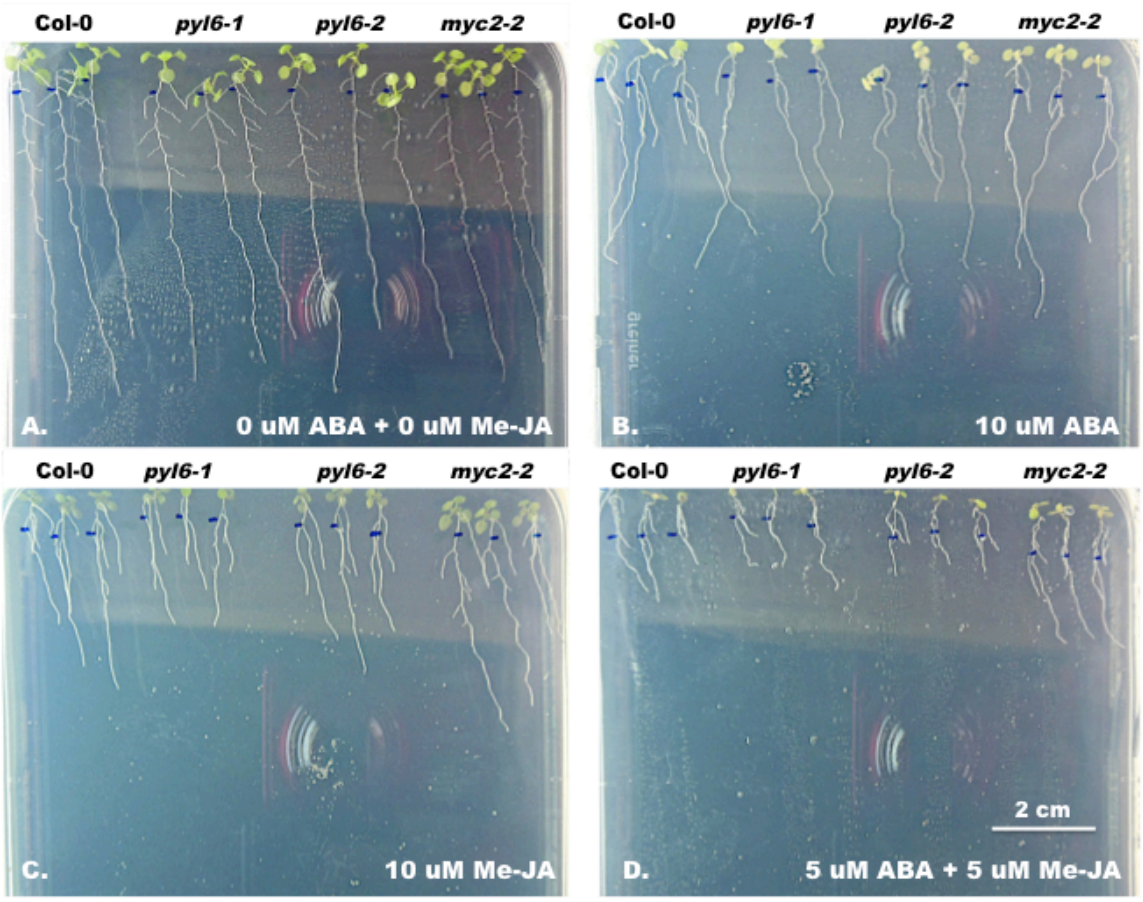

Figure 12 part II. Root growth is not significantly altered in *pyl6-1* and *pyl6-2* T-DNA mutants while *myc2-2* mutants display slight hyposensitivity to ABA and JA. Above is a visual representation of data shown in Figure 12 part I.

3. DISCUSSION

3.1. Tissue expression of PYL6 and repression of PYL6 in the anther-stamen region of wild type plants

PYL6 is reported to be expressed at various developmental stages in several plant structures such as the carpel, cotyledon, flowers, guard cell, hypocotyl, petal, plant embryo, pollen, root, sepal, and vascular leaf (Arabidopsis.org). Previous studies have conducted GUS reporter analyses of several PYR/PYL ABA receptors. Root expression of GUS driven by the promoter of PYL6 in 5 day old seedlings was reported to be almost undetectable in control conditions (Antoni et al., 2013). To analyze PYL6 localization in tissue at different developmental stages, the GUS reporter system was utilized in wild type and *pyl6* mutant plants. Several questions of PYL6 expression were explored; 1) Is there a difference between *pPYL6* and *pPYL6::PYL6* driven GUS expression? 2) Is there a difference in GUS expression between wild type and *pyl6* mutant plants? 3) Can ABA and Me-JA treatment affect *pPYL6* and *pPYL6::PYL6* driven GUS expression? It was found that GUS expression could not be detected in 1 week and 2 week old seedlings in any of the transgenic lines per treatment condition (Fig 3, 4) while using a positive control of an unrelated gene driven by GUS expression to confirm proper GUS staining. In 6 week old adult plants, GUS expression was detected in the anther of both *pPYL6::GUS* and *pPYL6::PYL6-GUS* transgenic *pyl6-2* mutant plants, but not in those of wild type Col-3 background (Fig 5). GUS expression in the anther of transgenic *pyl6-2* mutant plants was present in all treatment conditions (Fig 5E-H, M-P), therefore in future studies, it would be of interest to increase the ABA and Me-JA concentrations used in this experiment and modify exposure to treatments in order to up or down regulate PYL6 expression.

The result of GUS expression in *pyl6-2* mutant plants but not in wild type Col-3 background suggests that there is indirect repression or inhibition of PYL6 in the anther of wild type plants. JA is well known to play an important role in stamen development and is considered to be central in coordination of processes such as anther dehiscence, filament elongation, and pollen maturation (Song et al., 2011; Ito et al., 2007; Turner et al., 2002). Several studies acknowledge that JA plays an important role in stamen development, and this was revealed through the characterization of the *CORONATINE INSENSITIVE1 (CO1)* gene. *coi1* mutants are completely male sterile due to the retardation of stamen development with characteristics of short filaments, delayed anther dehiscence, and unviable pollen (Song et al., 2011). The lack of GUS expression in the anther of *pPYL6::GUS* and *pPYL6::PYL6-GUS* transgenic plants of Col-3 background may be indicative of an indirect PYL6 repression in wild type plants for proper JA-regulated stamen development. A change in PYL6 expression was not seen after 5 hours of Me-JA treatment, and this may be due to ineffectiveness of the method of treatment, which was a spray bottle method of approximately 1 mL of treatment solution per seedling or 3 mL of treatment solution per adult plant. Although a change in expression was not seen, a recent study reported that loss of function in two specific PYL genes alters the JA response in transgenic *Arabidopsis* mutants (Lackman et al., 2011). *pyl4* and *pyl5* knockout mutants were found to be hypersensitive to JA after prolonged growth, and biomass and anthocyanin production decreased in response to JA exposure. In the same study, the expression of PYL4, PYL5, and PYL6 was reported to be able to be regulated up and down by JA (Lackman et al., 2011).

Although the absence of GUS expression in the anther of transgenic plants of wild type background is not direct evidence of a relationship between PYL6 and JA-regulated stamen development, it does suggest some type of inhibitory effect upon functional PYL6 in the anther-stamen region of normal wild type plants. Analyses by Lackman et al. support the notion that PYL ABA receptors are involved in crosstalk between JA and ABA signaling pathways in order to regulate metabolism and growth. Reasoning for possible PYL6 repression in wild type anther has yet to be investigated experimentally, but based on the studies of this project, the interaction between PYL6 and MYC2, a known JA regulator, may the basis of the mechanism for this repression. Future analyses of JA-regulated stamen development in *pyl* mutant plants can reveal important information about the role of ABA and JA crosstalk in plant reproduction through the identification of inhibitory components of PYL gene expression and its association with MYC2.

3.2. Structural analysis of PYL6-MYC2 interaction by random mutagenesis

Experimental evidence of a physical interaction between PYL6 and MYC2 has been found in vitro, in a yeast two-hybrid analysis and *in planta* (Aleman et al., 2014 in preparation) (Fig 1, 2). To understand a structural basis of this interaction, it was of interest to isolate a mutant *pyl6* gene that constitutively interacts with MYC2 with and without the enhancement by ABA. Random mutagenesis via error-prone PCR was performed and the resulting mutant BD-*pyl6* products were co-transformed with AD-MYC2 in the yeast two-hybrid system. After roughly 3000 colonies were screened for constitutive interaction with and without ABA, 6 transformants were found to have the following individual amino acid point mutations in the PYL6 protein sequence: T211A, E161G, K30E, F92L, K186R, E49K (Fig 7). After recreating each of these mutations via site-directed mutagenic PCR, the mutant *pyl6*-MYC2 interaction was tested for constitutive interaction in liquid triple dropout media with and without the enhancement by ABA where the O.D. value was measured at 600 nm for several days. For several constructs, the O.D. values were extremely variable among colonies resulting in large values of standard error, deeming the average values statistically unrepresentative. Some negative control constructs had O.D. values similar to that of the experimental constructs (Fig 9), indicating leakiness in this interaction method, and in the future, a more precise and repeatable method of testing interaction should be used.

Random mutagenesis is a powerful tool that can highlight a relationship between the structure and function of a protein. It has the potential to introduce new functionality, and alter topology and substrate specificity. A randomly mutagenized protein can be further used for protein engineering by repeating the mutagenic and screening processes

in a manner mimicking Darwinian evolution and this process of direct evolution is a core method for biomolecular engineering (Fujii et al., 2004). Although the liquid O.D. growth test was not a successful method for determining whether any of the six established amino acid point mutations of the PYL6 protein led to constitutive interaction with MYC2, the position of each candidate mutation may provide information about the functionality of PYL6. The carboxylate group of ABA establishes interactions with conserved residues E125, E172, S153, and Y151 of PYL6 through hydrogen bonds facilitated by three internal water molecules. K90 of PYL6 establishes direct contact between the amine group and the carboxylate group of ABA (Santiago et al., 2012). None of the six mutations studied in this project, T211A, E161G, K30E, F92L, K186R, E49K, are in a position that is involved in ABA binding to the receptor, although the F92 residue (F61 in PYR1) is a conserved residue of *Arabidopsis* PYR/PYL proteins and is a part of the hydrophobic pocket at the center of PYR/PYL ABA receptor-PP2C interface (Dupeux et al., 2011). The F92L mutation established in PYL6 is also the mutant construct that had the highest O.D. at day 5 of the growth test without the enhancement of ABA (Fig 8). Although the results of the O.D. liquid growth test are not statistically significant due to high errors, it is still preliminary insight of the effect of each mutation on the PYL6-MYC2 interaction. The residues of PYL6 that directly interact with MYC2 have yet to be studied, but uncovering those residues would be a pivotal step in understanding the structural basis of PYL6 and MYC2 interaction and the role of ABA in the interaction.

3.3. Functional roles of PYL6 in ABA and JA response

ABA plays an important physiological role in plants as it inhibits seed germination and promotes seed dormancy (Fernandez-Arbaizar et al., 2012; Staswick et al., 1992), and high concentrations of ABA can inhibit root growth (Antoni et al., 2013). Me-JA has an inhibitory effect on root growth even in concentrations as low as 0.1 μM for wild type *Arabidopsis* (Staswick et al., 1992). Here mutant *pyl6* and *myc2* plants were analyzed for ABA and JA responses during germination, cotyledon greening, and root growth. At 1 μM ABA, *pyl6-1* and *pyl6-2* mutants displayed hypersensitivity, particularly at day 3 of germination. While Col-0 and *myc2-2* approached 90% germination, *pyl6* mutants germinated at a rate between 60% to 70% (Fig 10C). 0.5 μM ABA and 10 μM Me-JA alone did not have a significant inhibitory on any genotype (Fig 10B), but in combination, *pyl6-2* maintained 20% more inhibition than Col-0 at days 2 and 3 of germination (Fig 10E). These results carried over into analysis of cotyledon greening at day 7 of germination (Fig 10F) in which *pyl6-1* and *pyl6-2* mutants displayed approximately 40% more inhibition than Col-0 at 1 μM ABA. *myc2-2* displayed slight hyposensitivity at this concentration. When 0.5 μM ABA and 10 μM Me-JA was combined, both *pyl6* mutants displayed about 20% more inhibition than Col-0, while *myc2-2* displayed 20% less inhibition than Col-0. During root growth, *pyl6* mutants did not display an altered response to ABA or Me-JA. *myc2-2* displayed slight hyposensitivity to 10 μM Me-JA as well as 5 μM ABA and 5 μM Me-JA in combination.

It has been previously reported that the *myc2* mutant displays reduced ABA sensitivity (Kazan and Manners, 2013; Abe et al., 2003; Lorenzo et al., 2004; Yadav et al., 2005), and the result of the germination and cotyledon greening assays in this study

support those previous studies. As MYC2 is a known positive transcriptional activator of ABA signaling, *myc2* mutants display decreased ABA sensitivity, resulting in decreased expression of ABA induced genes such as RD22 and ADH1 (Abe et al., 2003). While both *pyl6* mutants displayed slight hypersensitivity to 1 μM ABA during seed germination, it did not display a significant response to 0.5 μM ABA, indicating a concentration dependent sensitivity. It was unexpected that disruption of an ABA receptor gene resulted in ABA hypersensitivity, as opposed to hyposensitivity, possibly indicating that wild type PYL6 is a negative regulator of ABA-mediated germination and cotyledon greening inhibition. *pyl6* mutants did not display a phenotype to ABA and Me-JA-mediated root growth inhibition, which may suggest that PYL6 negatively regulates ABA signaling specifically at the germination stage, but not during the root elongation stage. The *pyl6-1* T-DNA is inserted at the C-terminus of the PYL6 gene while the *pyl6-2* T-DNA is inserted at the N-terminus, and both mutants allow for positive ABA signaling during germination and cotyledon greening. This indicates that disruption at either terminus may lead to positive ABA signaling during early developmental phase, but further studies need to be conducted to understand the biochemical basis for this hypersensitive effect.

A synergistic effect between ABA and JA on inhibition of seed germination has been reported in previous studies (Staswick et al., 1992; Fernandez-Arbaizar et al., 2012). This synergistic effect was observed in germination, cotyledon greening, and root growth assays in this study. When 0.5 μM ABA and 10 μM Me-JA were combined, the germination rate of Col-0 was drastically reduced in comparison to its germination rate on 0.5 μM ABA and 10 μM Me-JA alone (Fig 10B, D, E). This inhibitory effect was also

seen during cotyledon greening. At 0.5 μM ABA and 10 μM Me-JA alone, the cotyledon greening rate for Col-0 was approximately 90% and 100%, respectively, and in combination, Col-0 had a cotyledon greening rate of less than 60% (Fig 10F). This joint effect of ABA and Me-JA is seen more clearly in *pyl6* mutants during germination and cotyledon greening. During root growth, the combination of ABA and Me-JA also had a stronger inhibitory effect than ABA and Me-JA alone for Col-0 and *pyl6* mutants. ABA and JA crosstalk may be possible through JA's role as a positive regulator of ABA signaling, although both synergism and antagonism between ABA and JA has been reported (Staswick et al., 1992; Fernandez-Arbaizar et al., 2012; Ellis and Turner, 2002). Binding of PYL6 to MYC2 may be modifying the DNA binding affinity of MYC2, promoting the expression of genes to respond to a certain stress signal through synergistic or antagonistic effects of the ABA and JA pathways. The possible involvement of PYL6 and MYC2 in ABA and JA crosstalk signaling leads to investigation of the mechanism by which the protein-protein interaction leads to up or down regulation of plant stress response. Future studies may provide crucial information about the mechanism of crosstalk between ABA and JA signaling that will support the engineering of crops resilient to various abiotic stresses.

4. MATERIALS AND METHODS

4.1. T-DNA genotyping

T-DNA lines used in this project were SALK_206633 (*pyl6-1*), SAIL_1179_D01 (*pyl6*-2), and SALK_130877.22.15.X (*myc2-2*). Genomic DNA was isolated by grinding plant tissue using a disposable pestle with 10 µL of 0.5N NaOH followed by 495 µL of 0.1M Tris-HCl pH 8. Samples were vortexed and kept on ice or at 4 °C until use. T-DNA insertions were confirmed by PCR and sequencing of the left- and right-borders using genomic DNA. Specific primers used are listed in Table S1.

4.2. Plant growth and phenotypic analyses

Seeds were surface sterilized in 70% ethanol followed by 10 minutes of inversion in solution containing 70% ethanol, 0.1% SDS, and 20% bleach. The seeds were then washed four times in 100% ethanol and suspended in 0.1% agarose. After four days of stratification at 4 °C in the dark, the suspended seeds were sown on 0.5 Murashige and Skooge (Sigma) (pH 5.6) supplemented with 1% Phyto Agar (RPI) and 1% sucrose. Plants were grown in a growth room in long day conditions (16 hr light/8 hr dark). 5-7 day old seedlings were transferred to pots for further growth.

For seed germination assays, after four days of stratification in 0.1% agarose, seeds were sown on 0.5 MS Phyto agar plates lacking or supplemented with 0.5 µM ABA, 1 µM ABA, 10 µM Me-JA, or 0.5 µM ABA and 10 µM Me-JA in combination. Seed germination (radicle emergence) and cotyledon greening (cotyledon expansion) were recorded for a time period of 7 days. Analyses represent % germination of at least 50 seeds per genotype (average ± s.d.) and each experimental condition was comprised of triplicate plates. Experiment was repeated three times.

For root growth assays, four-day old seedlings were transferred to 0.5 MS Phyto agar plates lacking or supplemented with 10 μM ABA, 10 μM Me-JA, or 5 μM ABA + 5 μM Me-JA and grown vertically in growth room. Images were acquired three, seven, and ten days after seedling transfer. Root length was measured using Fiji (http://fiji.sc.Fiji). Root length was analyzed as averages of at least seven seedlings per genotype ± s.d. The experiment was repeated three times.

4.3. Cloning of PYL6 and GUS expression in plant tissue analyses

In order to obtain the promoter region of PYL6 and the promoter and full gene of PYL6, the same genomic DNA extraction method was used as mentioned in section 4.1. The promoter region and promoter and full gene of PYL6 were amplified by PCR on genomic DNA and sequencing of the left- and right-primers. A detailed list of primers is provided in Table S1. The products were run on a 1% agarose gel via gel electrophoresis and excised from the gel based on the corresponding molecular weight. The PCR products were purified using Gel Extraction Kit (Thermo Scientific).

The plasmid vector hygII-MCSII-Uid-A was provided by Dr. Fernando Aleman and isolated using Plasmid Miniprep Kit (Thermo Scientific) and made USER compatible by ligation with USER cassette. The PCR purified pPYL6 region and full pPYL6::PYL6 region was cloned by USER cloning in USER compatible hygII-MCSII-Uid-A vector backbone (Nour-Eldin, 2010). The plasmid was transformed into *Escherichia coli* by heat-shock method in which competent cells were thawed on ice for 3 minutes without DNA and 2 minutes with DNA, followed by a 1 minute incubation at 42 °C and 5 minutes on ice. The cells incubated at 37 °C in 2% Lennox Broth media shaking for one

hour and then plated and selected on 50 μg/mL Kanamycin media. The plasmid of transformants were isolated using Plasmid Miniprep Kit (Thermo Scientific) and confirmed by sequencing that they were in frame with the GUS gene. The positive constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90; Koncz and Schell, 1986) via electroporation.

For plant transformations, Col-0 and *pyl6-2* seeds were sown in pots and grown to flowering adult plants. Inflorescences were trimmed 7 days before transformation and each *A. tumefaciens* construct was spread on selection media supplemented with Rifampicin, Gentamicin, and Hygromycin B two days before transformation. At time of transformation, each *A. tumefaciens* construct was scraped and homogenized in 150 mL solution containing 5% sucrose and 0.03% Silwet L-77. All formed siliques were removed from Col-0 and *pyl6-2* plants prior to dipping. Each pot was dipped into transformation media for 1 minute while ensuring that the inflorescences were in contact with the media. The pots were covered with plastic wrap and placed in the dark in the growth room overnight. The pots were uncovered the following day and maintained for further growth until time of seed harvest.

Seeds were selected on MS media supplemented with 1% sucrose and 30 μg/mL Hygromycin B and then transferred to MS media supplemented with 1% sucrose or soil pots for further growth. GUS staining was performed on 1 week and 2 week-old seedlings on plates and 6 week-old adult plants in pots. Seedlings and plants were treated with 100 μM ABA, 50 μM methyl jasmonic acid (MeJA), 100 μM ABA + 50 μM MeJA, or 0.2% ethanol as a mock treatment for 5 hours. Seedling or plant tissue was harvested in 90% acetone in glass scintillation vials and fixed at room temperature for 30 minutes.

The acetone was removed from the samples and samples were washed with Buffer I wash solution containing 50 mM Na_3PO_4, 0.2% Triton X-100, 2 mM potassium ferrocyanide, and 2 mM potassium ferricyanide. Buffer I was removed and samples were stained with Buffer II staining solution containing 50 mM Na_3PO_4, 0.2% Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, and 1 mg/mL X-gluc (Gold Biotechnology) that was dissolved in dimethylformamide. Samples were infiltrated under vacuum on ice for 1 hour and then left to incubate at 37 °C overnight. Following overnight incubation, samples were washed through an ethanol series of 20%, 40%, 60% for 30 minutes each and then left in 70% ethanol until microscope visualization.

4.4. Random mutagenesis and yeast two-hybrid analyses

The pGAD.GH plasmid (AD-MYC2) was transformed into yeast strain PJ69-4A (James et al., 1996) using the polyethylene glycol/lithium acetate method (Gietz et al., 2007). Random mutagenesis was performed on pGBT9.BS plasmid (BD-PYL6) via transition and transversion error-prone PCR (Muhlrad et al., 1992). Transition and transversion mutagenic *pyl6* PCR products were cloned into BD vector backbone previously digested at restriction site BbvCI by the vector gap repairing method in yeast (Alemen et al., 2014).

Mutagenic BD-*pyl6* plasmids were transformed into AD-MYC2 yeast using the polyethylene glycol/lithium acetate method (Gietz et al., 2007) and 2970 transformants were selected on CSM double dropout media lacking leucine and tryptophan and then incubated for 2-3 days at 28 °C. The transformants were replica plated using sterile velveteen on CSM triple dropout media lacking leucine, tryptophan, and histidine

supplemented with 2.5 mM 3-amino-1,2,4-triazole (3-AT) for selection of positive interaction and also supplemented with and without 10 μM ABA. Yeast transformants were incubated for 5-7 days at 28 °C. The positively selected transformants on CSM triple dropout media supplemented with 3-AT without ABA were inoculated in CSM triple dropout liquid media supplemented with 3-AT without ABA and grown at 28 °C shaking for 2 days. The plasmids were isolated from each transformant using a yeast plasmid mini prep kit (Zymoprep) and the isolated plasmids were transformed into *E. coli* by heat-shock method. The plasmid of the *E. coli* transformants was isolated using Plasmid Miniprep Kit (Thermo Scientific) and the PYL6 gene was sequenced for mutations that occurred from the error-prone PCR. To confirm these results, the resulting point mutations were recreated in BD-PYL6 plasmid via site-directed mutagenesis using the USER reagent. A detailed list of primers used for site-directed mutagenesis is provided in Table S1.

Plasmids AD-MYC2 and site-directed mutated BD-*pyl6* were co-transformed into yeast strain PJ69-4A (James et al., 1996) and transformants were selected on CSM double dropout media lacking leucine and tryptophan and incubated for 2-3 days at 28 °C. Three colonies of each transformation was inoculated in CSM triple dropout liquid media lacking leucine, tryptophan, and histidine supplemented with 2.5 mM 3-AT and also supplemented with or without 10 μM ABA to obtain a final optical density (OD) of 0.01. The cultures were grown at 28 °C shaking for 5 days with OD measurements taken on day 5. Error bars represent standard error of each construct with n =3.

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6. SUPPLEMENTAL INFORMATION

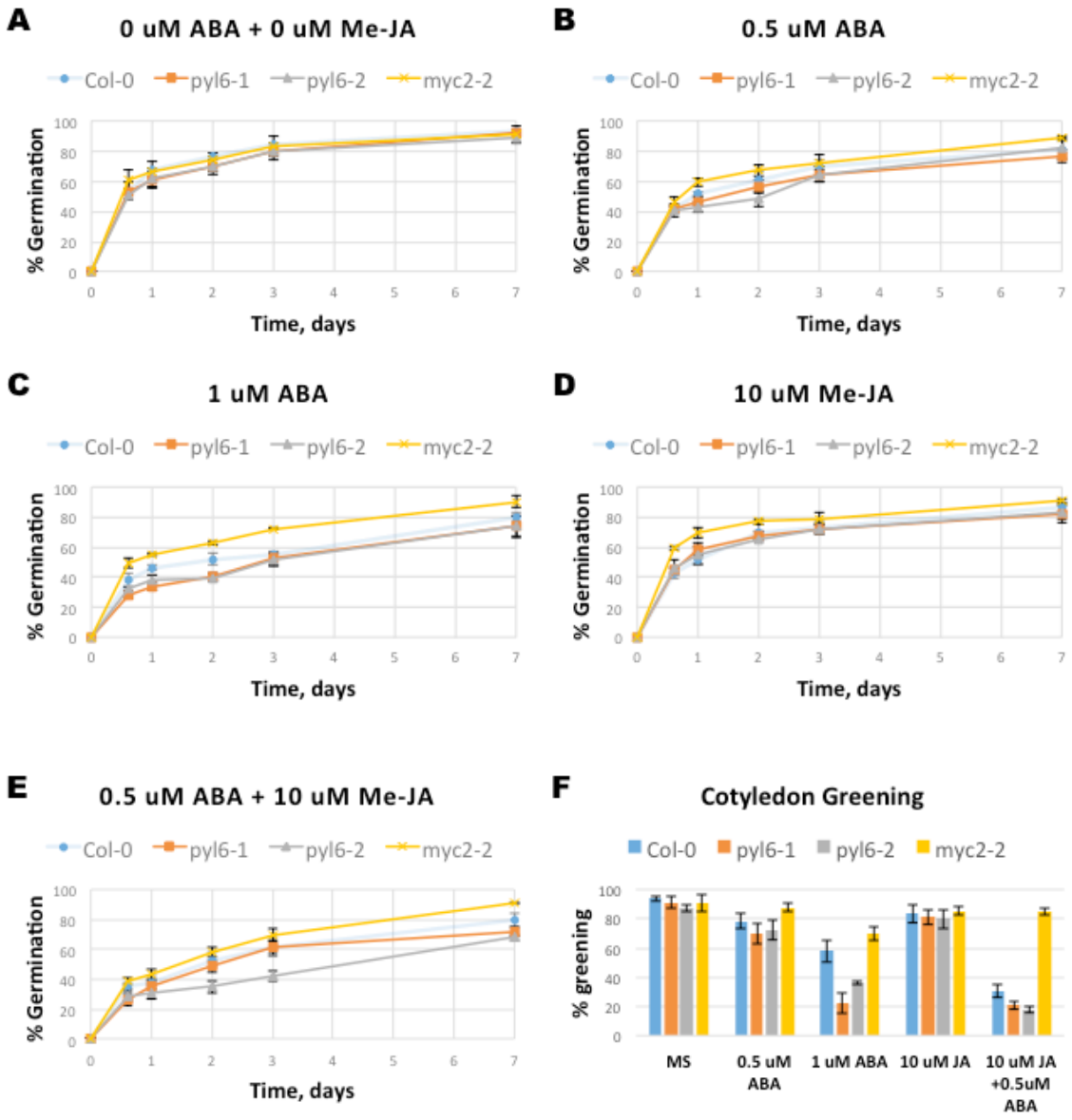


Figure S1. Repeated germination and cotyledon greening assay of Col-0, *pyl6-1*, *pyl6-2*, and *myc2-2*. See Figure 10 for description.

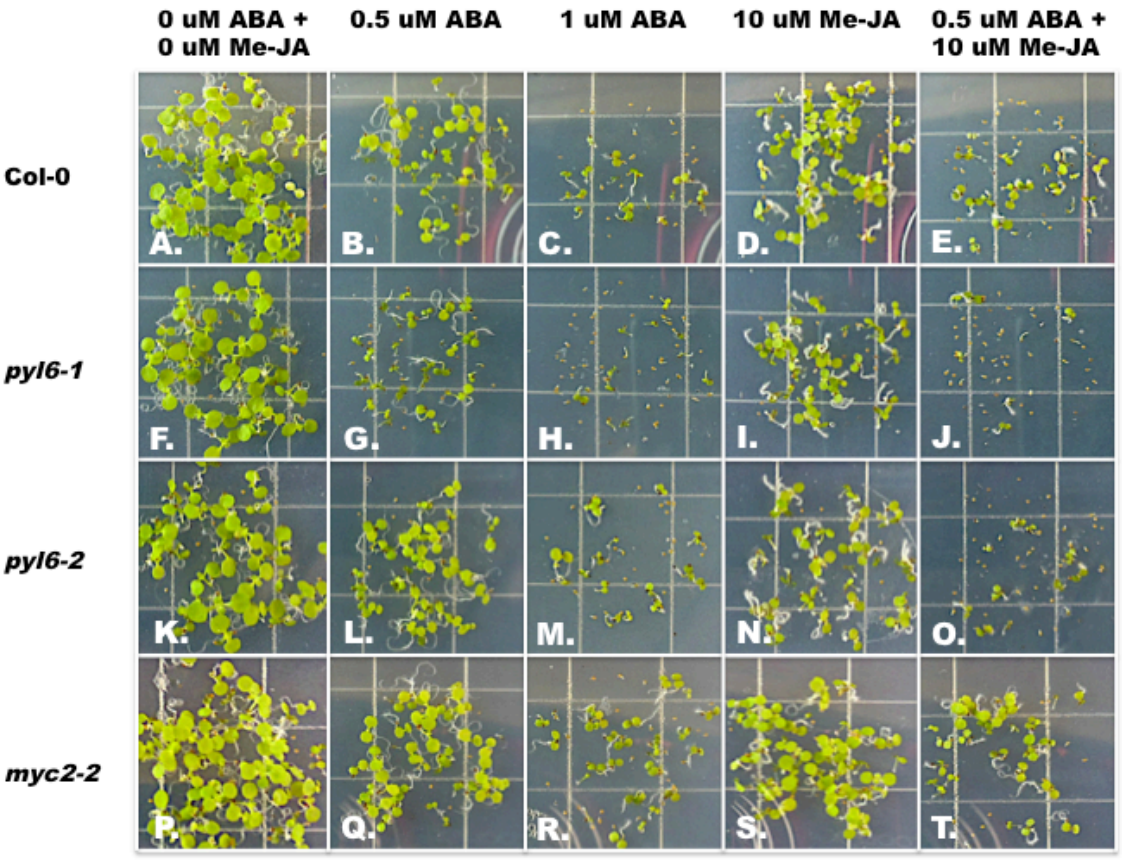


Figure S2. Repeated cotyledon greening assay of Col-0, *pyl6-1*, *pyl6-2*, and *myc2-2*. See Figure 11 for description.

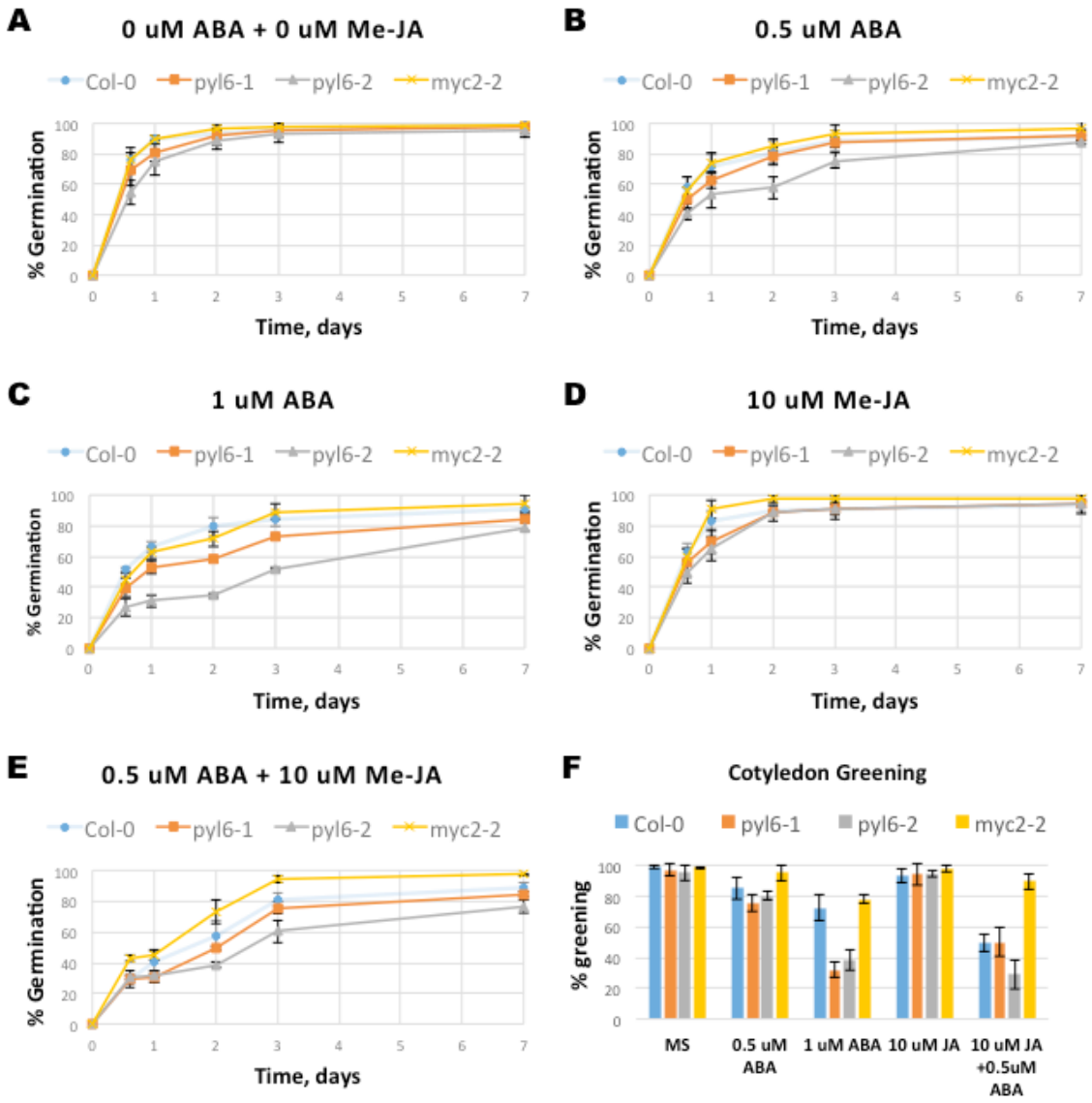


Figure S3. Repeated germination and cotyledon greening assay of Col-0, *pyl6-1*, *pyl6-2*, and *myc2-2*. See Figure 10 for description.

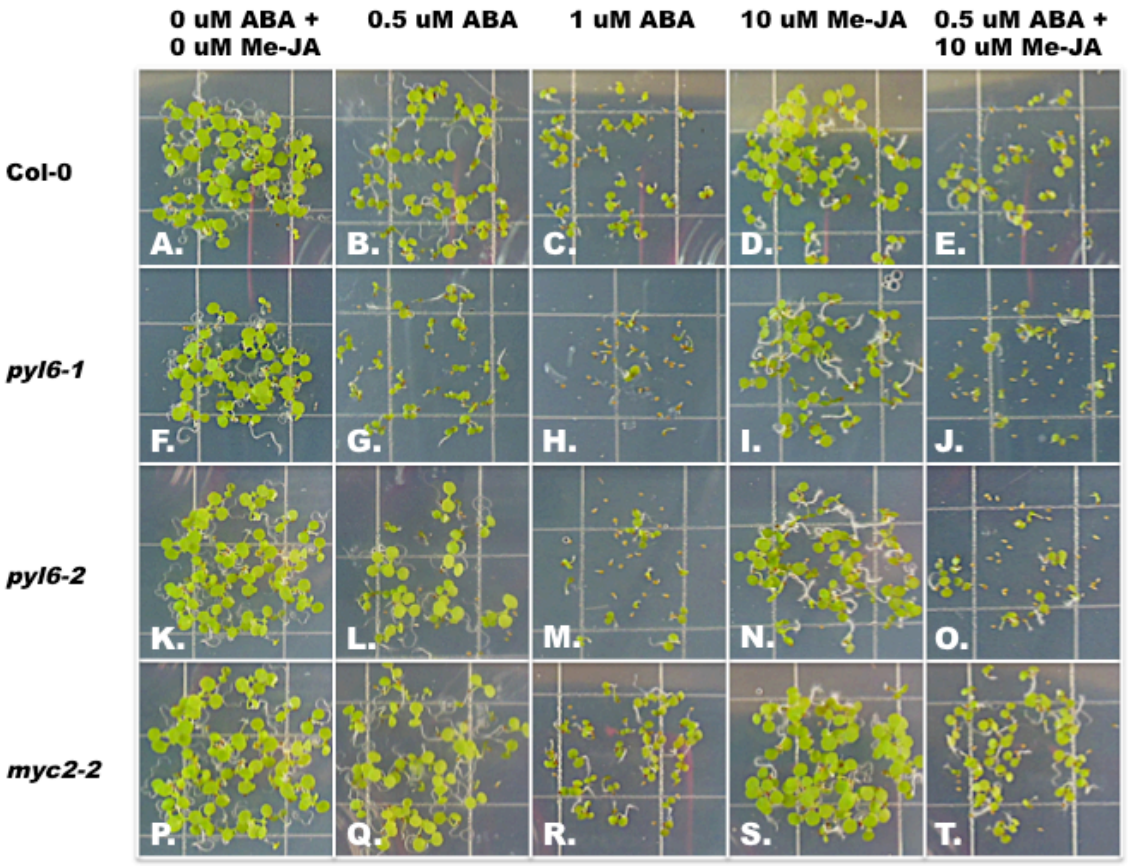


Figure S4. Repeated cotyledon greening assay of Col-0, *pyl6-1*, *pyl6-2*, and *myc2-2*. See Figure 11 for description.

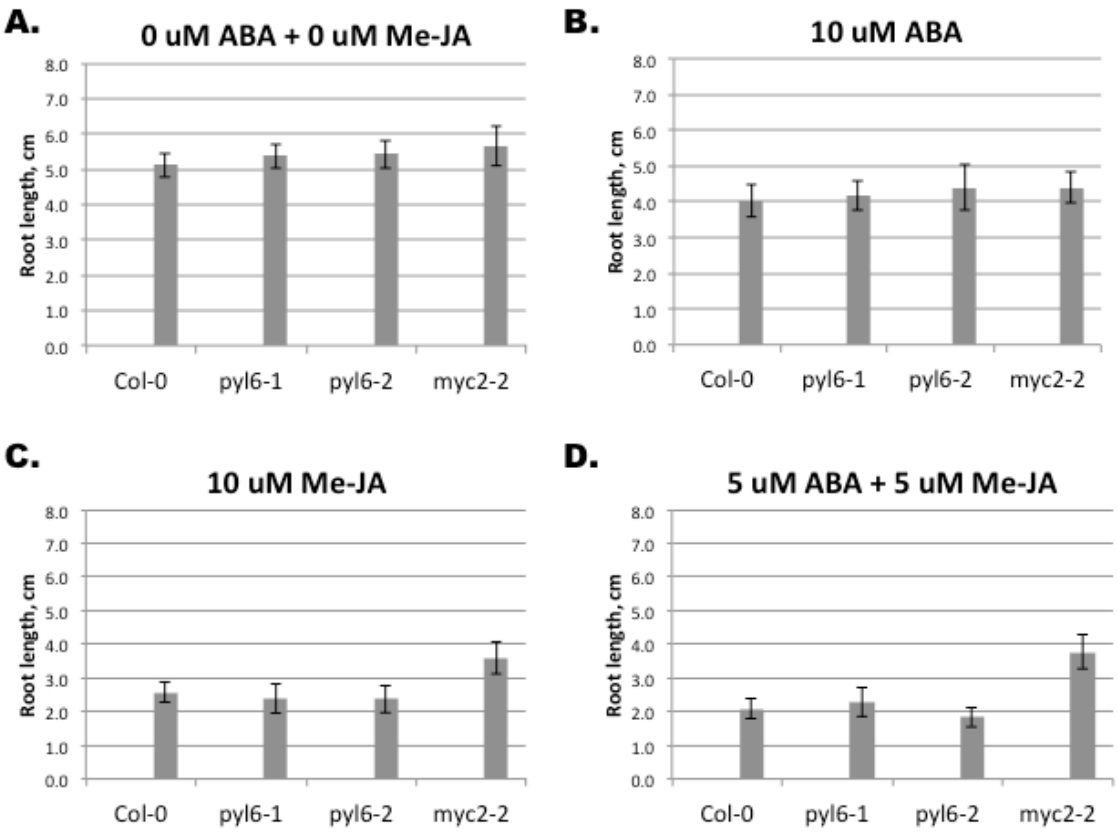


Figure S5. Repeated root growth assay of Col-0, *pyl6-1*, *pyl6-2*, and *myc2-2*. See Figure 12 for description.

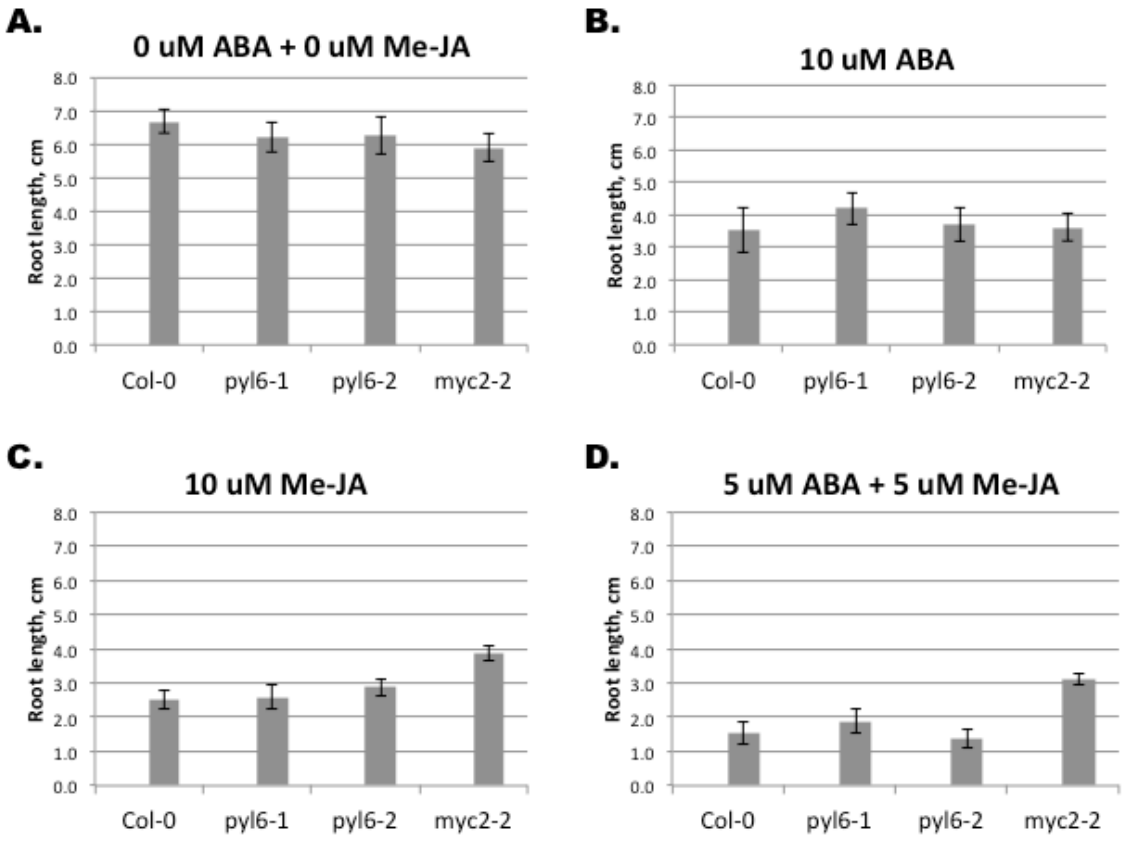


Figure S6. Repeated root growth assay of Col-0, *pyl6-1*, *pyl6-2*, and *myc2-2*. See Figure 12 for description.

Table S1. List of oligonucleotides used in this work.

Nr.	AGI Nr.	Gene Name	Primer Name	5'-3'-Sequence	Restri-ction site	Description
1	-	T-DNA	LB b1.3	ATTTTGCCGATTTCGGAAC	-	SALK T-DNA left border
2	-	T-DNA	SAIL_LB2	GCTTCCTATTATATCTTCCCAAATTACCAATACA	-	SAIL lines C/390-423 of pCSA110-pDAP101_T-DNA left border
3	AT2G40330	PYL6	PYL6_LP	TGTATGGGTTTTCTGATTGCC	-	SALK PYL6 left primer
4	AT2G40330	PYL6	PYL6_RP	GTTCAAAAAGTGAGCCTCACG	-	SALK PYL6 right primer
5	AT2G40330	PYL6	PYL6-ATGu	GGCTTAAUatgccaacgtcgatacagtttc	-	PYL6-ATG for USER cloning
6	AT2G40330	PYL6	PYL6-STOPu	GGTTTAAUTTACGAGAATTTAGAAGTGTTC	-	PYL6-STOP for USER cloning
7	AT2G40330	PYL6	pPYL6uFW	GGCTTAAUCCTTTGACAATGTGTCCTCTTCTCC	-	PYL6 promoter forward for USER cloning
8	AT2G40330	PYL6	pPYL6-Seq1	GTTCCAACAAAATTAAAAATCCACC	-	PYL6 promoter forward for sequencing
9	AT2G40330	PYL6	pPYL6_Antoni-Rv	GGTTTAAUGGATCTCTGAAACTGTATCGACG	-	Antoni 2013 PYL6 translational fusion reverse
10	AT2G40330	PYL6	PYL6-NoSTOPu	GGTTTAAUCGAGAATTTAGAAGTGTTCTCGG	-	PYL6 reverse no STOP for USER cloning
11	AT1G32640	MYC2	MYC2u-141FW	GGCTTAAUGAGCTTAACTCGTTGATCTCC	-	MYC2 forward from Glu141 for USER cloning
12	AT1G32640	MYC2	MYC2-530u	GGTTTAAUTTAACTAGCACTCGCTTTTCTTCCG	-	MYC2 reverse and stop (530 amino acids) for USER cloning
13	AT1G32640	MYC2	MYC2-seq	ATCTGATTCUCCGGCGGTTTT	-	MYC2 reverse USER sequence
14	-	USER	SpeI-USER-Fw	ctagtgggctgaggcttaattaaacctcagcaggc	SpeI	USER cassette forward
15	-	USER	XmaI-USER-Rv	CCGGGGCCTGCTGAGGTTTAATTAAGCCTCAGCCCA	XmaI	USER cassette reverse
16	-	Vector	GBK-rev	TAAATCATAAGAAATTCGCCCG	-	PGBKT-7 reverse for sequencing (ADH1 terminator binding)
17	-	Vector	BD-for	tcatcggaagagagtag	-	Forward primer for sequencing pGBT9.BS
18	-	Vector	BD-Fw	tggagactgatatgcctctaac	-	BD primer for random mutagenesis
19	-	Vector	AD-fw	gcctcctctaacgttcatgat	-	pGAD.GH forard primer
20	-	Vector	pGPTV-for	gctttctctttgcgcttgcg	-	GUS vector forward
21	-	Vector	GUS_Rv	CGAACTGATCGTTAAAACTGCC	-	GUS vector reverse for sequencing
22	AT2G40330	*pyl6*	L6-T211A	GGTTTAAUTTACGAGAATTTAGAAGcGTTC	-	Site-directed T211A mutated PYL6 with STOP

Table S1. Continued

Nr.	AGI Nr.	Gene Name	Primer Name	5'-3'-Sequence	Restri-ction site	Description
23	AT2G40330	*pyl6*	L6-E161G-Fw	acggtgcaUgagtcggGggagg	-	Site-directed E161G mutated PYL6 forward
24	AT2G40330	*pyl6*	L6-E161G-Rv	ATGCACCGuCGTCACCG ACTTG	-	Site-directed E161G mutated PYL6 reverse
25	AT2G40330	*pyl6*	L6-K30E-Fw	AGTGAGCCuCACGCGCG	-	Site-directed K30E mutated PYL6 forward
26	AT2G40330	*pyl6*	L6-K30E-Rv	AGGCTCACuTTTTGAACC	-	Site-directed K30E mutated PYL6 reverse
27	AT2G40330	*pyl6*	L6-F92L-Fw	ACACcTCGuGAAAAGCT GCCACGTG	-	Site-directed F92L mutated PYL6 forward
28	AT2G40330	*pyl6*	L6-F92L-Rv	ACGAgGTGuTTGTACGC TTGAGG	-	Site-directed F92L mutated PYL6 reverse
29	AT2G40330	*pyl6*	L6-K186R-Fw	ACCGGCGGGuAACGATA gGGAAGAG	-	Site-directed K186R mutated PYL6 forward
30	AT2G40330	*pyl6*	L6-K186R-Rv	ACCCGCCGGuACGTCAA CGACG	-	Site-directed K186R mutated PYL6 reverse
31	AT2G40330	*pyl6*	L6-E49K-Fw	ACGTGaAGCuTTCCCAC ACG	-	Site-directed E49K mutated PYL6 forward
32	AT2G40330	*pyl6*	L6-E49K-Rv	AGCTtCACGuGCTCCGG CACATC	-	Site-directed E49K mutated PYL6 reverse

7. APPENDIX

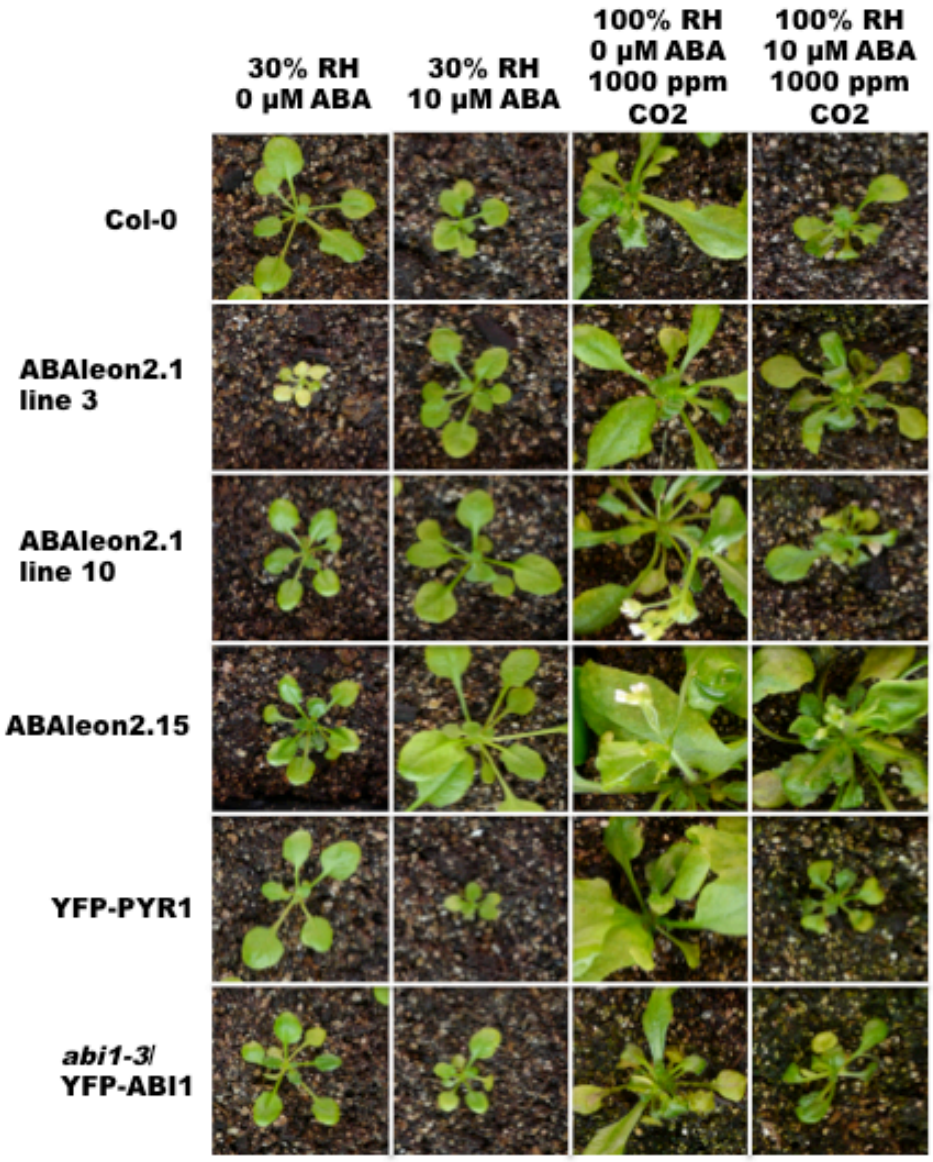
7.1. Growth inhibition of ABAleon expressing plants can be rescued with ABA treatment or high humidity

ABAleons are ABA specific optogenetic reporters that send a fluorescence resonance energy transfer (FRET) signal in response to the ABA dependent interaction of PYR/PYL ABA receptors and PP2C-type phosphatases. ABAleons can be used to map ABA concentration changes in plant tissues, and high ABAleon expression has been reported to partially repress Arabidopsis ABA responses (Waadt et al., 2014). It was observed that at low humidity levels, ABAleon expressing plants experience difficulty growing in potted soil after transplanting from MS media plates and the plants die within the first 2 weeks of growth. This may be due to the ability of ABAleon expressing plants to sequester endogenous ABA, resulting in growth inhibition. In this preliminary study, the growths of ABAleon expressing plants were compared in a low humidity chamber and in a high humidity chamber, and the effect of exogenous ABA treatment was tested as well.

Three lines of ABAleon expressing plants were used which were ABAleon 2.1 line 3, ABAleon 2.1 line 10, and ABAleon 2.15, and they were compared to wild type Col-0, YFP-PYR1, and *abi1-3*/YFPABI1. Seeds were sterilized and then sowed on 0.5 MS media and stratified at 4 °C in the dark for four days. The plates were transferred to a growth room in long day conditions (16 hr light/ 8 hr dark) with 50-80 mE m^{-2} s^{-1} light intensity and seedlings were grown for six days. Six day old seedlings were transferred to soil for the growth assay. Each genotype was transplanted in 3 pots of soil, and each pot contained five plants, resulting in 15 plants per genotype per condition. The different conditions tested were as follows: 30% relative humidity with no ABA treatment, 30%

relative humidity with 10 μM ABA treatment, 100% relative humidity with no ABA treatment, and 100% relative humidity with 10 μM ABA treatment. The plants were grown for 4 weeks in either the low humidity or high humidity growth chamber in a tray and a transparent plastic cover. Twice a day, the ABA treated plants were sprayed with approximately 10 mL of 10 μM ABA in deionized water, and the non-ABA treated plants were sprayed with approximately 10 mL of 0.01% ethanol mock treatment.

It was seen that at 30% relative humidity, the growth of ABAleon lines suffered and they grew smaller than wild type Col-0 plants, particularly ABAleon 2.1 line 3. At the same humidity, the growth inhibition of ABAleon lines was recovered when treated with ABA for the four weeks of growth, while the growth of Col-0, YFP-PYR1, and *abi1-2*/YFP-ABI1 suffered with ABA treatment. At 100% relative humidity, ABAleon expressing plants had similar growth to Col-0. The addition of 10 μM ABA treatment at 100% relative humidity negatively affected growth of all of the lines except ABAleon2.15 (see Appendix Figure 1).



Appendix Figure 1. Preliminary studies of ABAleon expressing plants show that the growth inhibition at low relative humidity can be overcome by exogenous ABA treatment or high humidity. ABAleon2.1 line 3, 10, and ABAleon 2.15 were grown for 4 weeks in growth chambers with 30% relative humidity or 100% humidity with 0 µM ABA ethanol-mock treatment or 10 µM ABA treatment. Col-0, YFP-PYR1, and *abi1*/YFP-ABI1 were used as controls. At 30% relative humidity, ABAleon expressing plants grew smaller than Col-0, but the growth inhibition was slightly rescued with 10 µM ABA treatment at the same humidity. When relative humidity was increased to 100%, ABAleon expressed plants had similar growth to Col-0. The addition of 10 µM ABA treatment at 100% relative humidity negatively affected growth of all of the lines except ABAleon2.15. Images provided by Dr. Rainer Waadt. ABAleon design and expression is described by Waadt et al. (2014).