

# **PYR/PYL/RCAR receptors play a major role for quantitative regulation of stomatal aperture and transcriptional response to abscisic acid**

Miguel Gonzalez-Guzman<sup>a1</sup>, Gaston A. Pizzio<sup>a1</sup>, Regina Antoni<sup>a</sup>, Francisco Vera-Sirera<sup>a</sup>, Ebe Merilo<sup>b</sup>, George W. Bassel<sup>c</sup>, Maria A. Fernández<sup>a</sup>, Michael J. Holdsworth<sup>c</sup>, Miguel Angel Perez-Amador<sup>a</sup>, Hannes Kollist<sup>b</sup> and Pedro L. Rodriguez<sup>a2</sup>

<sup>a</sup>Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas-Universidad Politécnica de Valencia, ES-46022 Valencia, Spain (M. G.-G., G.A.P., R.A., F.V.-S., M.A.P.A., P.L.R.)

<sup>b</sup>Institute of Technology, University of Tartu, Nooruse 1, Tartu 50411, Estonia (E.M., H.K.)

<sup>c</sup>Centre for Plant Integrative Biology (CPiB), University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, UK, LE12 5RD (G.W.B, M.J.H.)

<sup>1</sup>M.G.-G. and G.A.P. contributed equally to this work

<sup>2</sup>To whom correspondence should be addressed;

e-mail [prodriguez@ibmcp.upv.es](mailto:prodriguez@ibmcp.upv.es); phone: 34 963877860

Running title: ABA signaling through PYR/PYL receptors

Estimate of the length of the published article: 12 pages

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the instructions for authors ([www.plantcell.org](http://www.plantcell.org)) is Pedro L. Rodriguez ([prodriguez@ibmcp.upv.es](mailto:prodriguez@ibmcp.upv.es))

## **Synopsis**

A mutant lacking six ABA receptors and ABA-mediated activation of SnRK2.2/2.3/2.6 kinases shows an extreme ABA-insensitive phenotype, even though other branches for ABA perception remain functional. ABA perception through PYR/PYL/RCAR receptors plays a major role to regulate seed germination and establishment, vegetative and reproductive growth, stomatal aperture and transcriptional response to ABA.

## ABSTRACT

Absciscic acid (ABA) is a key hormone for plant growth, development and stress adaptation. Perception of ABA through four types of receptors has been reported. We show here that impairment of ABA perception through the PYR/PYL/RCAR branch reduces vegetative growth and seed production, and leads to a severe open stomata and ABA insensitive phenotype, even though other branches for ABA perception remain functional. An *Arabidopsis* sextuple mutant impaired in 6 PYR/PYL receptors, namely PYR1, PYL1, PYL2, PYL4, PYL5 and PYL8, was able to germinate and grow even on 100  $\mu$ M ABA. Whole-rosette stomatal conductance (Gst) measurements revealed that leaf transpiration in the sextuple *pyr/pyl* mutant was higher than in the ABA-deficient *aba3-1* or ABA-insensitive *snrk2.6* mutants. The gradually increasing Gst values of plants lacking three, four, five and six PYR/PYLs indicate quantitative regulation of stomatal aperture by this family of receptors. The sextuple mutant lacked ABA-mediated activation of SnRK2s and ABA-responsive gene expression was dramatically impaired as was reported in *snrk2.2/2.3/2.6*. In summary, these results show that ABA perception by PYR/PYLs plays a major role to regulate seed germination and establishment, basal ABA signaling required for vegetative and reproductive growth, stomatal aperture and transcriptional response to the hormone.

## INTRODUCTION

The phytohormone abscisic acid (ABA) plays a key role to regulate different aspects of plant growth and development as well as plant response to both biotic and abiotic stress (Cutler et al., 2010). ABA elicits plant responses through binding to soluble PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) receptors, which constitute a 14-member family. All of them (except PYL13) are able to activate ABA-responsive gene expression using protoplast transfection assays (Fujii et al., 2009); however, according to their different expression patterns (Antoni et al., 2012; Kilian et al., 2007; Laubinger et al., 2008; Winter et al., 2007; Yang et al., 2008)(Supplemental Figure 1), substantial functional differences among them can be expected. For instance, expression of *PYL3* and *PYL10-13* is very low to undetectable in different whole-genome microarrays (Yamada et al., 2003; Chekanova et al., 2007; Laubinger et al., 2008), whereas significant expression levels are found for *PYR1* and *PYL1-9* in different tissues and in response to developmental and environmental cues (Kilian et al., 2007; Winter et al., 2007)(Supplemental Figure 1). From a biochemical point of view, recent studies reveal at least two families of PYR/PYL receptors, characterized by a different oligomeric state, some being dimeric (PYR1, PYL1 and PYL2), whereas others are monomeric (for instance PYL5, PYL6, PYL8) (Dupeux et al., 2011a; Hao et al., 2011). The dimeric receptors show a higher  $K_d$  for ABA ( $>50 \mu\text{M}$ , lower affinity) than monomeric ones ( $\sim 1 \mu\text{M}$ ), however, in the presence of certain clade A protein phosphatases 2C (PP2Cs), both groups of receptors form ternary complexes with high affinity for ABA ( $K_d$  30-60 nM) (Ma et al., 2009; Santiago et al., 2009a, b). The highest genetic impairment of PYR/PYL function is currently represented by the *pyr1pyl1pyl2pyl4* quadruple mutant, abbreviated as 1124, which shows strong ABA insensitivity, including reduced sensitivity to ABA-mediated inhibition of germination and root growth, impaired ABA-induced stomatal closure and ABA inhibition of stomatal opening as well as reduced expression of some ABA-responsive genes (Nishimura et al., 2010; Park et al., 2009).

PYR/PYL receptors perceive ABA intracellularly and as a result, form ternary complexes inhibiting clade A PP2Cs (Ma et al., 2009; Park et al., 2009). This allows the activation of downstream targets of the PP2Cs, such as the sucrose non-fermenting 1-related subfamily 2 (SnRK2s) protein kinases, i.e. SnRK2.2/D, 2.3/I and 2.6/OST1/E, which are key players to regulate ABA signaling, including regulation of transcriptional

response to ABA and stomatal aperture (Fujii and Zhu, 2009; Fujita et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). Indeed, a *snrk2.2/2.3/2.6* triple mutant shows a dramatic ABA-insensitive phenotype in different responses to the hormone, being able to germinate and establish in the range 50-300  $\mu$ M ABA (Fujii and Zhu, 2009; Fujita et al., 2009). The 1124 quadruple mutant shows impaired ABA-mediated-activation of the three SnRK2s because of reduced inhibition of clade A PP2Cs and, conversely, a *hab1-1 abil-2 pp2ca-1* triple pp2c knockout shows partial constitutive activation of SnRK2s (Fujii et al., 2009; Park et al., 2009; Rubio et al., 2009). Even though the 1124 quadruple mutant shows strong ABA-insensitivity, it was not able to establish and develop the first pair of true leaves in medium supplemented with 5  $\mu$ M ABA at 7 days after sowing (see below). Although ABA-induced activation of SnRK2s was notably impaired in 1124, some activation of SnRK2s in response to ABA was observed (Park et al., 2009). This result suggests that additional members of the PYR/PYL family are still able to inhibit clade A PP2Cs to a certain extent in 1124, leading to some activation of both SnRK2s and other PP2C targets. Additionally, other types of ABA receptors might contribute to ABA signaling in 1124 (Pandey et al., 2009; Shen et al., 2006).

Five different types of ABA receptors have been reported in the literature. The original article describing the first one, the RNA binding protein FCA involved in regulation of flowering time, was later on retracted (Razem et al., 2008). A second ABA binding protein, ABAR/CHLH, has been isolated from *Vicia faba* and *Arabidopsis* using an ABA-affinity chromatography technique that relies on the linkage of the carboxylic group of ABA to a functionalized Sepharose resin (Shen et al., 2006; Wu et al., 2009). ABAR/CHLH is a chloroplastic protein involved in both chlorophyll biosynthesis, acting as protoporphyrin IX-magnesium chelatase, and plastid-to-nucleus signaling, and according to recent results it also antagonizes a group of WRKY transcription factors to relieve inhibition of ABA-responsive genes (Shang et al., 2010). However, structural compelling evidence supporting ABA binding by ABAR/CHLH is still lacking (reviewed by Antoni et al., 2011). The third ABA receptor to be described was GCR2, which according to Liu et al., (2007) is a G protein-coupled protein that works as a plasma membrane receptor for ABA. However, there is controversy regarding its definition as a G-protein coupled receptor and its role in ABA signaling during germination and seedling establishment (reviewed by Cutler et al., 2010). Following pharmacological and genetic evidence suggesting the involvement of G-

protein coupled signaling in the ABA pathway, Pandey et al., (2009) reported a family of two G-protein coupled receptors, GTG1 and GTG2, which work as plasma membrane ABA receptors. Finally, Ma et al., (2009) and Park et al., (2009) reported the PYR/PYL/RCAR family of ABA receptors, which form a core hormone signaling pathway with clade A PP2Cs and SnRK2.2/2.3/2.6. Potential perception of ABA through different types of receptors or by different members of the PYR/PYL/RCAR family raises several questions that have not been addressed yet, such as what is the relative contribution of each type of receptor and how are multiple inputs of perception integrated into ABA signaling. In order to evaluate the relative contribution to ABA signaling of ABA perception mediated by the PYR/PYL/RCAR family, we aimed to generate a *pyr/pyl* mutant lacking ABA-mediated activation of SnRK2s. To this end, we knocked out six *PYR/PYL* genes that showed high expression level in different tissues (Supplemental Figure 1). Thus, we were able to generate a *pyr1pyl1pyl2pyl4pyl5pyl8* sextuple mutant that is extremely insensitive to ABA even though other branches of ABA perception remain functional.

## RESULTS

### Reporter gene analysis of *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* promoters

Data from whole-genome arrays (Yamada et al., 2003; Chekanova et al., 2007; Laubinger et al., 2008) found in the *Arabidopsis* transcriptome database indicate that *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* genes are expressed in different tissues, as we confirmed through a detailed reporter gene analysis (Figure 1; Supplemental Figure 1, 2 and 3). For instance, *PYR1*, *PYL1*, *PYL4* and *PYL8* genes rank among the top-four most expressed receptors of the *PYR/PYL* family in root, seedling, leaf young, stem, vegetative apex, fruit and whole inflorescence (Supplemental Figure 1D). In order to visualize the expression of *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* genes through histochemical staining, sequences comprising between 1.5 to 2 kb upstream of the ATG start codon and the first 30 bp of the ORF were fused to a reporter gene encoding  $\beta$ -glucuronidase (GUS). Independent transgenic lines were generated and the GUS expression pattern of at least three lines was analyzed by histochemical GUS staining (Figure 1). Germinating embryos were dissected from the seed coat and endosperm at 24 or 48 h after imbibition and imaging of GUS within germinating embryos was performed as previously described (Truernit et al., 2008). Interestingly, at 24 h, the

expression of *PYR1*, *PYL8* and to lower extent *PYL1* was detected in the endosperm (Figure 1B), whereas expression of *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* was detected in the peripheral layer of the embryo (embryo epidermal layer) as well as in the provascular cells within the cotyledons and hypocotyls, but not in the radicle (Figure 1A and C). However, at 48 h after imbibition and following the completion of germination, expression of *PYR1*, *PYL1*, *PYL2*, *PYL4* and *PYL8* could be detected in the vascular tissue of the root (Figure 1C).

In 5-d-old seedlings, expression of *PYR1*, *PYL1*, *PYL2*, *PYL4* and *PYL8* was detected in the vascular bundle of the primary root, whereas *PYR1* and *PYL5* were expressed in the cortex of the upper part of the root (Figure 1D; Supplemental Figure 2). Interestingly, *PYL1*, *PYL4* and *PYL8* were also expressed in the columella cells (Figure 1D). In 15-d-old seedlings, expression of *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* was detected in guard cells, and also in vascular tissue of the leaves with the exception of *PYL5* (Figure 1E-F). The predominant expression of *PYR/PYL* genes in vascular bundles of root and leaves is particularly interesting since the vascular system is a node of systemic stress responses and immunological studies have localized the NCED3, ABA2 and AAO3 ABA-biosynthetic enzymes in vascular parenchyma cells (Endo et al., 2008). Finally, ABA-treatment inhibited or strongly attenuated GUS expression driven by these promoters (Supplemental Figure 3). This result provides *in situ* evidence for the down-regulation of gene expression of members of the *PYR/PYL* family by ABA (Santiago et al., 2009a; Szostkiewicz et al., 2010).

### **Generation of *pyr1pyl1pyl2pyl4pyl5pyl8* sextuple mutant**

Different combinations of multiple mutants containing lesions in *PYR/PYL* genes were generated, namely *pyr1pyl4pyl5* (145), *pyl4pyl5pyl8* (458), *pyr1pyl4pyl8* (148) triple and *pyr1pyl4pyl5pyl8* (1458) quadruple mutants. Seed germination and seedling establishment analyses showed that these genotypes were less sensitive to ABA than wt (Figure 2). All of them, as well as the previously described *pyr1pyl1pyl4* (114) and 1124 mutants (Park et al., 2009), were able to establish in 1  $\mu$ M ABA; however, only 1458 was able to establish in 5  $\mu$ M ABA at 7-d after sowing, whereas 1124 established in 3  $\mu$ M ABA. We crossed the 1124 and 1458 quadruple mutants and selected F2 individuals able to germinate and establish in MS medium supplemented with 10  $\mu$ M ABA. PCR-based genotyping and gene sequencing of the *pyr1-1* allele identified

*pyr1pyl2pyl4pyl5pyl8* pentuple, abbreviated as 12458, and *pyr1pyl1pyl2pyl4pyl5pyl8* sextuple, abbreviated as 112458, mutants (Figure 3A). The 12458 and particularly 112458 mutants showed impaired growth, which was reminiscent of growth inhibition previously reported in the *snrk2.2/2.3/2.6* triple mutant (Figure 3B-C). Although lower growth and seed yield was observed in the sextuple mutant compared to wt, it could bolt, flower and produced viable seeds under greenhouse conditions (40-50% relative humidity) (Figure 3C-E). Increasing humidity (70-80%) improved growth and seed yield of 112458, however it also caused fungal contamination of the seeds.

### **Extreme ABA-insensitive phenotype of *pyr1pyl1pyl2pyl4pyl5pyl8* sextuple mutant**

We analyzed the effect of ABA to inhibit seed germination and seedling establishment of the pentuple and sextuple mutants in comparison to wt and the extremely ABA-insensitive *snrk2.2/2.3/2.6*. Radicle emergence of 12458, 112458 and *snrk2.2/2.3/2.6* was resistant even to 50-100  $\mu$ M ABA, however only the 112458 and *snrk2.2/2.3/2.6* mutants were able to develop expanded green cotyledons and the first pair of true leaves at such high ABA concentrations (Figure 4A, C and D). Root length in MS medium of 12458 and 112458 mutants was lower than wt, but it improved by the presence of 3-20  $\mu$ M ABA in the germination plate, which indicates that these mutants require ABA supplementation for optimal *in vitro* root growth (Figure 4A-B). The *snrk2.2/2.3/2.6* triple mutant also showed a reduced root growth in MS medium compared to wt, however, in contrast to pentuple and sextuple *pyr/pyl* mutants, ABA supplementation did not improve root growth (Figure 4B).

High concentrations of ABA inhibit seedling growth of wt, whereas certain ABA-insensitive mutants are resistant to inhibition of vegetative growth. We transferred 4-d-old seedlings from different genotypes to MS medium plates lacking or supplemented with 20 or 50  $\mu$ M ABA. Root growth was measured 10 days after transfer and as a result, 12458, 112458 and *snrk2.2/2.3/2.6* were resistant to ABA-mediated inhibition of root growth compared to wt (Figure 5A-B). Moreover, ABA supplementation improved slightly root growth of 12458 and 112458. Shoot growth was evaluated by either measuring the maximum rosette radius or fresh weight of plants grown for 11-d or 21-d, respectively, in MS medium either lacking or supplemented with ABA (Figure 5C-F). As a result, shoot growth of 12458 was found to be inhibited

by ABA, whereas both 112458 and *snrk2.2/2.3/2.6* were notably resistant to ABA-mediated inhibition of growth.

Previous microarray analyses (Yang et al., 2008) showed that the six *PYR/PYL* genes studied here were all expressed in guard cells (Supplemental Figure 4). Indeed, GUS expression driven by *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* promoters was detected in guard cells (Figure 1F). Therefore, to study the contribution of these genes to the regulation of stomatal aperture, we performed water-loss and stomatal assays in different genotypes. Water-loss assays were done using 15-d-old seedlings grown in a controlled environment growth chamber to reduce developmental differences among the different genotypes. As a result, enhanced shrinking and higher fresh weight-loss was found in the excised 12458, 112458 and *snrk2.2/2.3/2.6* plants compared to wt (Figure 6A and B). For instance, both 112458 and *snrk2.2/2.3/2.6* lost approximately 40% of fresh weight in 30 minutes whereas wt only 20%. Direct measurements of stomatal aperture using whole leaf imaging (Chitrakar and Melotto, 2010) revealed that stomata of both 112458 and *snrk2.2/2.3/2.6* were more open than in wt (Figure 6C) and 112458 was insensitive to ABA-induced stomatal closing (Figure 6D).

We also used a gas exchange system that monitors steady state stomatal conductance (Gst) of whole *Arabidopsis* rosette, enabling to analyze Gst in intact whole plants under basal conditions (Kollist et al., 2007; Vahisalu et al., 2008). Plants carrying different combinations of *pyr/pyl* mutations showed higher steady state Gst than wt, which indicates that stomata of different *pyr/pyl* mutants have higher aperture than wt (Figure 6E). Interestingly, both 12458 and 112458 showed more 2-fold higher Gst than well known wilted mutants such as *snrk2.6* or *aba3-1*. The *snrk2.6/ost1* mutant showed 1.7-fold higher Gst value than wt, whereas *snrk2.2/2.3* double mutant was similar to wt, which is in agreement with water-loss assays reported previously (Fujii and Zhu, 2009). We tried to perform Gst measurements with *snrk2.2/2.3/2.6*, but this mutant is severely impaired in growth and we could not obtain enough foliar surface to perform the experiments. A transgenic line harbouring the *hab1*<sup>G246D</sup> hypermorphic mutation (Robert et al., 2006), which represents a PP2C version refractory to PYR/PYL-mediated inhibition (Dupeux et al., 2011b), showed a dramatic increase in Gst compared to wt. This result is in agreement with the more open stomata phenotype of *pyr/pyl* mutants, since these mutants must contain higher PP2C activity because of reduced inhibition by PYR/PYL receptors and this in turn suppresses the activation of positive regulators of

stomatal closure, such as SnRK2.6. Taken together these results suggest that ABA and PYR/PYL receptors are required for adjustment of stomatal aperture in steady-state resting conditions.

### **Transcriptional response to ABA is severely impaired in *pyr1pyl1pyl2pyl4pyl5pyl8* sextuple mutant**

The phenotypes described above indicate that PYR/PYL receptors are major players for ABA perception and signaling. To examine the effect of impaired ABA perception via the PYR/PYL pathway on transcriptional response to ABA, we compared transcriptomic profiles of wt, 112458 and *snrk2.2/2.3/2.6* in response to ABA using Agilent's *Arabidopsis* 44k oligonucleotide microarrays (Figure 7A; Supplemental Figure 5). Large-scale transcriptome analysis has previously showed that ABA-dependent gene expression was globally and drastically impaired in *snrk2.2/2.3/2.6* (Fujita et al., 2009). We confirmed these results under our experimental conditions and found that 112458 also showed a globally impaired transcriptional response to ABA (Figure 7A). After 10  $\mu$ M ABA treatment for 3h, 2432 and 2283 genes showed reduced expression ( $\geq 2$ -fold, false discovery rate  $P < 0.05$ ) in the *snrk2.2/2.3/2.6* and 112458 mutants compared to the wt, respectively. Among them, 1974 genes overlapped, which indicates that more than 85% of the genes whose expression was impaired in 112458 upon ABA-treatment were regulated in the wt through the activity of SnRK2.2/2.3/2.6 kinases. Among the different groups of ABA-responsive genes that showed diminished expression in *snrk2.2/2.3/2.6* and 112458 mutants, we found for instance the clade A *PP2Cs*, *ABI5/ABFs/AREBs* bZIP family, the proline biosynthetic gene  $\Delta^1$ -pyrroline-5-carboxylate synthetase 1 (*P5CS1*), a high number of late embryogenesis-abundant (*LEA*) genes or different ABA- and osmotic stress marker genes that belong to responsive to ABA (*RAB*), desiccation/dehydration (*RD/ERD*) and cold-inducible/cold-responsive/low temperature inducible gene families (*KIN/COR/LTI*) (Figure 7B). A complete list is provided in Supplemental Table 1. We confirmed the data described above using RT-qPCR analysis. For instance, ABA-mediated induction of the genes *RAB18*, *RD29B* and *KIN1* was dramatically reduced both in *snrk2.2/2.3/2.6* and 112458 (Figure 7C). Other *pyr/pyl* mutants also showed a reduced expression of these genes; however triple *pyr/pyl* mutants such as 148 or 458 still retained between 25-40% of wt expression, whereas 112458 showed only residual 4 %, 0.3 % and 1.5 % of wt expression for *RAB18*, *RD29B* and *KIN1*, respectively. Interestingly, the *PYL1* gene

appears to provide an important contribution to the induction of these genes, since their induction by ABA in 1124 was more impaired than in 1458 or 12458 mutants (Figure 7C).

Finally, we monitored the *in vivo* activation status of SnRK2s by an in-gel kinase assay using protein extracts from Col wt, 112458 and *snrk2.2/2.3/2.6* (Figure 7D). The in-gel-kinase assay here reported uses a  $\Delta$ CABF2 fragment (amino acids 1-173) as substrate and the three ABA-activated SnRK2s were identified as a double band between 42-44 kDa that was present in ABA-treated Col wt but absent in *snrk2.2/2.3/2.6*. Likewise, in 112458, the in-gel-kinase assay did not detect activation of the SnRK2s by 100  $\mu$ M ABA treatment, which is in agreement with gene expression data shown above for 112458 and *snrk2.2/2.3/2.6*.

## DISCUSSION

ABA perception by different types of ABA receptors has been reported during the last years (Shen et al., 2006; Liu et al., 2007; Pandey et al., 2009; Ma et al., 2009; Park et al., 2009). Perception through PYR/PYL receptors is evolutionary conserved from bryophytes and presumably represents an essential mechanism to mediate, for instance, plant adaptive responses to drought in crops (Umezawa et al., 2010). In this work, we show that impairment of ABA perception mediated by key members of the PYR/PYL family leads to a global dramatic ABA-insensitive phenotype, impaired growth and seed production as well as constitutively more open stomata phenotype. Impaired growth and reproduction has been previously documented in ABA-deficient and ABA-insensitive mutants, and it could not be fully restored by growing plants in high humidity conditions (Barrero et al., 2005; Cheng et al., 2002; Fujii and Zhu, 2009). Indeed, even a mild reduction in basal ABA levels negatively affects vegetative growth (Frey et al., 2011). Therefore, our results show that ABA perception through the PYR/PYL receptors is required for the basal ABA signaling that promotes plant growth, normal seed production and regulates steady-state transpiration. Even under *in vitro* conditions of high humidity and sucrose supplementation of the medium, both the pentuple and sextuple mutants here described showed reduced root growth compared to wt, which was restored by ABA supplementation. These results suggest that the residual perception mediated by other PYR/PYLs or alternative receptors is required for optimal root growth, which is in agreement with the reported role of ABA to maintain primary

root growth during water deficits (Sharp et al., 2004) and low ABA concentrations (<1  $\mu$ M) to stimulate root growth under non-stress conditions (Zeevaart and Creelman, 1998). Several mechanisms have been proposed to explain root growth promotion by ABA, such as restriction of ethylene production, enhanced proline accumulation in the tip of water-stressed roots, induction of certain cell wall-loosening proteins and antioxidant enzymes as well as control of K<sup>+</sup> translocation from root to shoot (Gaymard et al., 1998; Sharp et al., 2004). Recently, it has been confirmed in 17 natural accessions of *Arabidopsis* that a key physiological response to soil drying is an increased root versus shoot biomass partitioning, and the key role played by the transcriptomic response to ABA for coping with drought stress (Des Marais et al., 2012). On the other hand, growth inhibition of *snrk2.2/2.3/2.6* could not be complemented by ABA, which likely reflects a bottleneck in ABA signaling downstream of ABA perception or additional functions of these kinases. Indeed, SnRK2.6 has been also shown to be involved in the regulation of sucrose metabolism and plant growth (Zheng et al., 2010).

To our knowledge, both 112458 and *snrk2.2/2.3/2.6* are the most ABA-insensitive mutants described so far. For instance, 112458 was able to germinate and establish in the presence of 100  $\mu$ M ABA, which was not possible for other ABA-insensitive mutants impaired in ABA receptors (Pandey et al., 2009; Shen et al., 2006). Likewise, both 112458 and *snrk2.2/2.3/2.6* were able to maintain sustained growth in 50  $\mu$ M ABA for 21 days, which was close to that of wt in MS medium. These results, together with the partial constitutive activation of ABA signaling found in triple *pp2c* knockouts (Fujii et al., 2009; Rubio et al., 2009), reinforce the importance of the core components, i.e. PYR/PYL receptors, PP2Cs and SnRK2s. Moreover, even though other branches of ABA perception such as ABAR/CHLH and GTG1/GTG2 remain presumably active in the sextuple *pyr/pyl* mutant, they do not show a major effect on ABA-mediated growth inhibition.

The sextuple *pyr/pyl* mutant, in addition to showing strongly reduced sensitivity to ABA-mediated inhibition of germination and growth, was also drastically impaired in the regulation of both stomatal aperture and ABA-responsive gene expression. The stomatal aperture in the sextuple *pyr/pyl* mutant under steady state conditions was 70% higher than in wt and stomata did not close in response to ABA. Stomatal conductance measurements of different triple *pyr/pyl* mutants rendered values similar to the *open*

*stomatal1* mutant, *ost1-3/snrk2.6*, which was originally identified because of a defective regulation of transpiration upon water stress (Mustilli et al., 2002; Yoshida et al., 2002). Moreover, the sextuple *pyr/pyl* mutant rendered record Gst values, more than 2-fold higher than in *ost1-3/snrk2.6* or the ABA-deficient *aba3-1* mutant. Therefore, our results highlight that PYR/PYL receptors play a major role in basal ABA signaling required for regulation of stomatal aperture even under non-stress conditions. The progressive inactivation of *PYR/PYL* genes generated a clear additive effect on stomatal conductance, which can be illustrated by the three successive steps of increasing Gst values represented by triple/quadruple, pentuple and sextuple *pyr/pyl* mutants (Figure 6E). Microarray data and gene-reporter analysis have showed that different PYR/PYL receptors co-exist in the same tissues and therefore can combine their different biochemical properties and preferential inhibition of certain clade A PP2Cs to quantitatively modulate ABA sensitivity (Antoni et al., 2012; Dupeux et al., 2011a; Hao et al., 2011). Moreover, since the six receptors here studied are expressed in guard cells at different levels (Yang et al., 2008), we might expect, for instance, different phenotypes in triple combinations of *pyr/pyl* mutant loci. Apparently this was not the case, since 114, 148, 145 and 458 triple mutants rendered similar Gst values. Thus, some functional redundancy for regulation of stomatal aperture occurs among these receptors and these results suggest that a similar degree of PP2C inhibition can be attained by combined action of different PYR/PYL receptors in guard cells. Further studies to address protein levels of PYR/PYL receptors in guard cells and additional combinations of *pyr/pyl* mutants might shed novel light on this subject.

On the other hand, different evidences indicate non-redundant functions for *PYR/PYL* genes. First, the histochemical analysis of *PYR/PYL* expression patterns points out to specific functions of certain members of the family in different tissues. For instance, expression of *PYR1*, *PYL8* and to lower extent *PYL1*, but not *PYL2*, *PYL4* and *PYL5*, could be detected in the endosperm at 24 h after imbibition. Imaging of GUS staining in the embryo at 24 or 48 h after imbibition suggests spatio-temporal regulation of ABA signaling by certain receptors. Likewise, root ABA signaling seems to use different types of receptors whether we consider expression of *PYR/PYLs* in root vascular bundle, cortex or columella cells. Second, some ABA responses of multiple *pyr/pyl* mutants were clearly different depending on the combination considered. For instance, the 1458 mutant was less sensitive to ABA-mediated inhibition of seedling

establishment than 1124. Induction of *RAB18*, *RD29B* and *KIN1* was more impaired in 1124 than 1458 or 12458, which suggests PYL1 might play a more relevant role to control transcriptional response to ABA of certain genes.

Finally, ABA-responsive gene expression was dramatically impaired in 112458 as it was in *snrk2.2/2.3/2.6* (Fujii and Zhu, 2009; Fujita et al., 2009)(Figure 7). Previously, expression of three ABA-responsive genes, *RD29A*, *NCED3* and *P5CS1*, was found to be diminished in 1124 compared to wt (Park et al., 2009), but no global analysis of ABA response in *pyr/pyl* mutants had been previously reported. Our results provide evidence that perception of ABA through the PYR/PYL receptors exerts a major control on the transcriptional response to ABA. Numerous osmotic stress-responsive genes were notably down-regulated in 112458, which together with the important role of PYR/PYL receptors to regulate stomatal aperture highlights the relevance of the PYR/PYL pathway to cope with drought stress. Additionally, the strong overlap between the impaired response to ABA of 112458 and *snrk2.2/2.3/2.6* mutants was biochemically corroborated by an in-gel kinase assay that shows lack of ABA-mediated activation of SnRK2s in 112458 (Figure 7D). In summary, using large-scale experiments and biochemical analysis, we show that PYR/PYL receptors exert a major control on ABA transcriptional response through PP2C-dependent regulation of SnRK2s. Future comparative studies using transcript profiling of mutants impaired in other types of receptors could shed additional light on the regulation of transcriptional response to ABA.

## METHODS

### **Plant material and growth conditions.**

*Arabidopsis thaliana* plants were routinely grown under greenhouse conditions (40-50% relative humidity) in pots containing a 1:3 vermiculite-soil mixture. For plants grown under growth chamber conditions, seeds were surface sterilized by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5 % sodium hypochlorite) containing 0.05 % Triton X-100 for 10 min, and finally, four washes with sterile distilled water. Stratification of the seeds was conducted in the dark at 4°C for 3 days. Then, seeds were sowed on Murashige-Skoog (MS) plates composed of MS basal salts,

0.1% 2-[N-morpholino]ethanesulfonic acid, 1% sucrose and 1% agar. The pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber at 22°C under a 16 h light, 8 h dark photoperiod at 80-100  $\mu\text{E m}^{-2} \text{sec}^{-1}$ . The *pyr1-1* allele and the T-DNA insertion lines for *pyl1*, *pyl2*, *pyl4* and *pyl5* have been described previously (Lackman et al., 2011; Park et al., 2009). Seeds of *snrk2.6/ost1-3* and *pyl8* insertion lines, SALK\_008068 and SAIL\_1269\_A02, respectively, were obtained from the Nottingham *Arabidopsis* Stock Centre.

### **ProPYR1, ProPYL1, ProPYL2, ProPYL4, ProPYL5 and ProPYL8:GUS fusions.**

To construct the *ProPYL8:GUS* gene, a fragment comprising 2 kb 5' upstream of the ATG start codon plus the first 30 bp of the PYL8 coding sequence was amplified by PCR and cloned into pCR8/GW/TOPO T/A. Next, it was recombined by Gateway LR reaction into pMDC163 destination vector (Curtis and Grossniklaus, 2003). To generate *ProPYR1*, *ProPYL1*, *ProPYL2*, *ProPYL4* and *ProPYL5:GUS* genes, the upstream sequence amplified was approximately of 1.5 kb to avoid overlapping with regulatory sequences of neighboring genes. The different pMDC163-based constructs carrying *ProPYR/PYL:GUS* genes were transferred to *Agrobacterium tumefaciens* pGV2260 (Deblaere et al., 1985) by electroporation and used to transform Col wt plants by the floral dipping method. Seeds of transformed plants were harvested and plated on hygromycin (20  $\mu\text{g/ml}$ ) selection medium to identify T1 transgenic plants and T3 progenies homozygous for the selection marker were used for further studies. Imaging of GUS within germinating embryos was performed as previously described (Truernit et al., 2008).

### **RNA analyses.**

ABA treatment, total RNA extraction and RT-quantitative PCR amplifications were performed as previously described (Saez et al., 2004). Briefly, about 10-12 seven-day-old seedlings were transferred from MS plates to 100-ml flasks containing 2.5 ml of MS solution and 1 % sucrose. Seedlings were grown in a controlled environment growth chamber at 22° under a 16 h light, 8 h dark photoperiod at 80-100  $\mu\text{E m}^{-2} \text{sec}^{-1}$ . After 10 days, seedlings were either mock- or 10  $\mu\text{M}$  ABA-treated for 3 h. Transcriptome analysis was done using the Agilent Arabidopsis (V4) Gene Expression 4x44,000

Microarray, which contained 43,803 probes (60-mer oligonucleotides) and was used in a two color experimental design according to MIAME guidelines (Brazma et al., 2001). Four biological replicas for each genotype, 112458, *snrk2.2/2.3/2.6* and Col wt plants, were analyzed and each mutant line was compared with the wt, with dye-swap. Total RNA integrity was assessed using the 2100 Bioanalyzer (Agilent). Sample RNA (0.5 µg) was amplified and labeled with the Agilent Low Input Quick Amp Labeling Kit. Agilent's Spike-In Kit was used to assess the labeling and hybridization efficiencies. Hybridization and slide washing were carried out with the Gene Expression Hybridization Kit and Gene Expression Wash Buffers, respectively. After washing and drying, slides were scanned in an Agilent G2565AA microarray scanner, at 5 µm resolution, and using the double scanning, as recommended. Image files were analyzed with the Feature Extraction software 9.5.1. Inter-array analyses were performed with the GeneSpring 11.5 software. To ensure high quality dataset, control features were removed, and only features for which the 'IsWellAboveBG' parameter was 1 at least in three out of four replicas were selected (31,912 and 31908 features, representing 21,392 and 21,438 genes for 112458 and *snrk2.2/2.3/2.6* mutant analysis, respectively). To identify significantly expressed genes in each comparison, a t-test analysis was carried out with FDR adjustment according to Benjamini and Hochberg's method. Features were selected only if p-value was below 0.05 after correction for multiple-testing and expression ratio was above 2-fold difference. Gene Ontology (GO) analysis of the Biological Process level, with corrected p-value of 0.05, was carried out with the GeneSpring software.

### **Seed germination and seedling establishment assays.**

After surface sterilization of the seeds, stratification was conducted in the dark at 4°C for 3 d. Next, approximately 100 seeds of each genotype were sowed on MS plates supplemented with different ABA concentrations per experiment. To score seed germination, radical emergence was analyzed at 72 h after sowing. Seedling establishment was scored as the percentage of seeds that developed green expanded cotyledons and the first pair of true leaves at 7-d. Additionally, root length of seedlings germinated and grown on different ABA concentrations was measured at 7-d.

### **Root and shoot growth assays.**

Seedlings were grown on vertically oriented MS plates for 4 to 5 days. Afterwards, 20 plants were transferred to new MS plates lacking or supplemented with the indicated concentrations of ABA. The plates were scanned on a flatbed scanner after 10-d to produce image files suitable for quantitative analysis of root growth using the NIH Image software ImageJ v1.37. As an indicator of shoot growth, either the maximum rosette radius or fresh weight was measured after 11 or 21-d, respectively.

### **Water-loss and stomatal aperture assays.**

2-3 weeks-old seedlings grown in MS plates were used for water-loss assays. Four seedlings per genotype with similar growth, three independent experiments, were submitted to the drying atmosphere of a flow laminar hood. Kinetic analysis of water-loss was performed and represented as the percentage of initial fresh weight loss at each scored time point. Stomatal aperture measurements were done in leaves of 5-week-old plants using whole leaf imaging (Chitrakar and Melotto, 2010). To score ABA-induced stomatal closing, leaves were first incubated for 2 h in stomatal opening buffer, 10 mM KCl and 10 mM MES-KOH pH 6.2, at 20°C. Then, they were incubated for 2 h in the same buffer supplemented or not with 1  $\mu$ M ABA. Next, staining of whole leaves with propidium iodide was conducted and the aperture of 30-40 stomata (ratio width/length, two independent experiments) was measured using a Leica TCS-SL confocal microscope.

### **Whole-rosette stomatal conductance measurements.**

The *Arabidopsis* whole-rosette gas exchange measurement device, plant growth practice and custom written program to calculate G<sub>st</sub> for water vapour have been described previously (Vahisalu et al., 2008). For gas-exchange experiments, 21-26-d-old plants (rosette area 5-15 cm<sup>2</sup>) were used. Until measurements, plants were grown in growth chambers (AR-66LX and AR-22L, Percival Scientific, IA, USA) at 12/12 photoperiod, 23/18°C temperature, air relative humidity of 70-80% and 150  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> light.

### **In-gel kinase assay.**

It was performed as described previously (Fujii et al., 2007). Proteins were extracted

from 12-d-old seedlings that were either mock- or 100  $\mu$ M ABA-treated for 30 min. As kinase substrate we used His<sub>6</sub>- $\Delta$ CABF2 (amino acids 1-173) (Antoni et al., 2012).

### Accession numbers

The Arabidopsis Genome Initiative locus identifiers for *PYR1*, *PYL1*, *PYL2*, *PYL3*, *PYL4*, *PYL5*, *PYL6*, *PYL7*, *PYL8*, *PYL9*, *PYL10*, *PYL11*, *PYL12* and *PYL13* are, *At4g17870*, *At5g46790*, *At2g26040*, *At1g73000*, *At2g38310*, *At5g05440*, *At2g40330*, *At4g01026*, *At5g53160*, *At1g01360*, *At4g27920*, *At5g45860*, *At5g45870* and *At4g18620*, respectively.

Raw microarray data have been deposited in the Gene Expression Omnibus: [www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=vbahzkiuseisszw&acc=GSE36692](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=vbahzkiuseisszw&acc=GSE36692)

### Supplemental data

The following materials are available in the online version of this article

**Supplemental Figure 1.** Gene expression levels of the PYR/PYL/RCAR ABA receptors in the *Arabidopsis* transcriptome genomic express database and *Arabidopsis* whole-genome tiling array (At-TAX).

**Supplemental Figure 2.** Photographs showing GUS expression driven by *ProPYL1*, *ProPYR1*, *ProPYL2*, *ProPYL4*, *ProPYL5* and *ProPYL8:GUS* genes in roots of 5-d-old seedlings.

**Supplemental Figure 3.** ABA treatment inhibits or attenuates GUS expression driven by *ProPYL1*, *ProPYR1*, *ProPYL2*, *ProPYL4*, *ProPYL5* and *ProPYL8:GUS* genes.

**Supplemental Figure 4.** Expression of PYR/PYL genes in guard cells mock or 100  $\mu$ M ABA-treated.

**Supplemental Figure 5.** Scheme of the transcriptomic experiment. ABA-response of wt, 112458 and *snrk2.2/2.3/2.6* mutants was compared using Agilent's *Arabidopsis* 44k oligonucleotide microarrays.

### ACKNOWLEDGEMENTS

We thank Joseph Ecker and the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed *Arabidopsis* T-DNA insertion mutants, the *Arabidopsis* Biological Resource Center/Nottingham *Arabidopsis* Stock Centre for distributing these

seeds and Sean Cutler for the *pyrI-1*, 114 and 1124 mutants. This work was supported by the Ministerio de Ciencia e Innovacion, Fondo Europeo de Desarrollo Regional and Consejo Superior de Investigaciones Cientificas (grant BIO2011-23446 to P.L.R; fellowship to R.A.; Juan de la Cierva contract to MGG), by Estonian Ministry of Science and Education (ETF7763 and ETF9208, SF0180071s07), by European Regional Fund (Center of Excellence in Environmental Adaptation) and a Marie Curie I.I.F. to G.W.B.

### Figure legends.

**Figure 1.** Photographs showing GUS expression driven by *ProPYL1*, *ProPYR1*, *ProPYL2*, *ProPYL4*, *ProPYL5* and *ProPYL8:GUS* genes in different tissues. **(A, C)** Embryos dissected from mature seeds imbibed for 24 h or 48 h, respectively. **(B)** Seed coat and endosperm imbibed for 24 h. **(D)** Primary root from 5-d-old seedlings. **(E, F)** Vascular tissue and guard cells in leaves of 15-d-old seedlings, respectively. The bar corresponds to 100  $\mu$ m.

**Figure 2.** Quantification of ABA-mediated inhibition of germination and seedling establishment of Col wt compared to different genotypes. Approximately 100 seeds of each genotype (three independent experiments) were sowed on each plate and scored for radicle emergence 3-d-later **(A)** or for the presence of both green cotyledons and the first pair of true leaves 7-d-later **(B)**. Values are averages  $\pm$ SE.

**Figure 3.** Combined loss of function of *PYR/PYL* genes impairs plant growth and seed yield. **(A)** Schematic diagram of the *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* genes showing the position of the T-DNA insertion or the nonsense mutation in the *pyr1-1* allele. RT-PCR analyses of mRNAs from wt, 12458 and 112458. The position of the primers used for genotyping and RT-PCR is indicated by arrows. **(B, C, D)** Photographs show the impairment of growth and reproduction in extreme ABA-insensitive mutants. Photographs of 24-d-old plants **(B)**, 50-d-old plants **(C)** and siliques **(D)** grown under greenhouse conditions of Col wt, 12458, 112458 and *snrk2.2/2.3/2.6*. **(E)** Quantification of maximum rosette radius, plant height, seed production and silique length of the different genotypes. Data are average values obtained for 20 plants.

**Figure 4.** Extreme ABA-insensitive phenotype of 112458 for germination and seedling establishment assays. **(A)** Photographs of Col wt, 12458, 112458 and *snrk2.2/2.3/2.6* grown for 7-d on MS medium either lacking or supplemented with different concentrations of ABA. **(B)** Quantification of root length in 7-d-old seedlings of panel A. Data are averages  $\pm$ SE from three independent experiments (n =15 each). The asterisk indicates  $P < 0.01$  (Student's t test) with respect to medium lacking ABA. **(C, D)** Quantification of ABA-mediated inhibition of germination and seedling establishment

of Col wt compared to 12458, 112458 and *snrk2.2/2.3/2.6*. Approximately 100 seeds of each genotype were sowed on each plate and scored for radicle emergence 3-d-later (C) or for the presence of both green cotyledons and the first pair of true leaves 7-d-later (D). SE values were lower than 7% and are not indicated.

**Figure 5.** ABA-insensitive phenotype of 112458 for ABA-mediated inhibition of growth assays. (A) Photograph of representative seedlings 10 d after the transfer of 4-d-old seedlings to MS plates lacking or supplemented with 50  $\mu$ M ABA. (B) Quantification of ABA-mediated root growth inhibition of Col wt compared to 12458, 112458 and *snrk2.2/2.3/2.6*. Data are averages  $\pm$ SE from three independent experiments (n =15 each). The asterisk indicates  $P < 0.01$  (Student's t test) with respect to medium lacking ABA. (C, E) Photograph of representative seedlings 11-d or 21-d after the transfer of 4-d-old seedlings from MS medium to plates lacking or supplemented with ABA. (D, F) Quantification of ABA-mediated shoot growth inhibition of Col wt compared to 12458, 112458 and *snrk2.2/2.3/2.6*. Data are averages  $\pm$ SE from three independent experiments (n =15 each).

**Figure 6.** Water-loss and stomatal conductance assays in *pyr/pyl* mutants. (A) Photograph of representative excised plants submitted for 90 minutes to the drying atmosphere of a flow laminar hood. (B) Loss-of-fresh weight of 18-d-old excised plants that were submitted to the drying atmosphere of a flow laminar hood. (C) Increased stomatal aperture of 21-d-old plants of 112458 and *snrk2.2/2.3/2.6* compared to wt. (D) ABA-insensitive stomatal closing of 21-d old plants of 112458 compared to wt. (E) Leaf gas exchange measurements reveal increased stomatal conductance (Gst) in different *pyr/pyl* mutants and additive effects upon progressive inactivation of *PYR/PYL* genes. The different letters denote significant differences between mutants ( $P < 0.05$ , n=5-17, Tukey's post-hoc comparison, Student's t and Fisher LSD tests for comparison between *snrk2.6* and Col wt).).

**Figure 7.** ABA-responsive gene expression is drastically impaired in 112458. (A) Global impairment of transcriptional response to ABA in 112458 and *snrk2.2/2.3/2.6* compared to Col wt. Genes showing  $\geq 2$ -fold higher expression upon ABA-treatment in Col than in the mutants (false discovery rate  $p < 0.05$ ) are represented using Venn diagram. Analyses were made in quadruplicate on independent RNA samples of 2-week-old seedlings that were treated with 10  $\mu$ M ABA for 3 h. The transcriptome

profile was obtained using the Agilent's gene expression 4x44000 microarray. **(B)** Relative induction level of selected genes after ABA treatment in each mutant compared to wt (value 1). **(C)** Relative expression of three ABA-responsive genes in the indicated genotypes after ABA treatment compared to wt (value 1) as determined by RT-qPCR. Expression of *RAB18*, *RD29B* and *KIN1* was up-regulated 86-, 634- and 312-fold by ABA in the wt, respectively. **(D)** SnRK2s are not activated by ABA in 112458. Proteins extracted from wt, 112458 and *snrk2.2/2.3/2.6* seedlings that were either mock (-) or 100  $\mu$ M ABA-treated (+) for 30 min were analysed by an in-gel-kinase assay.

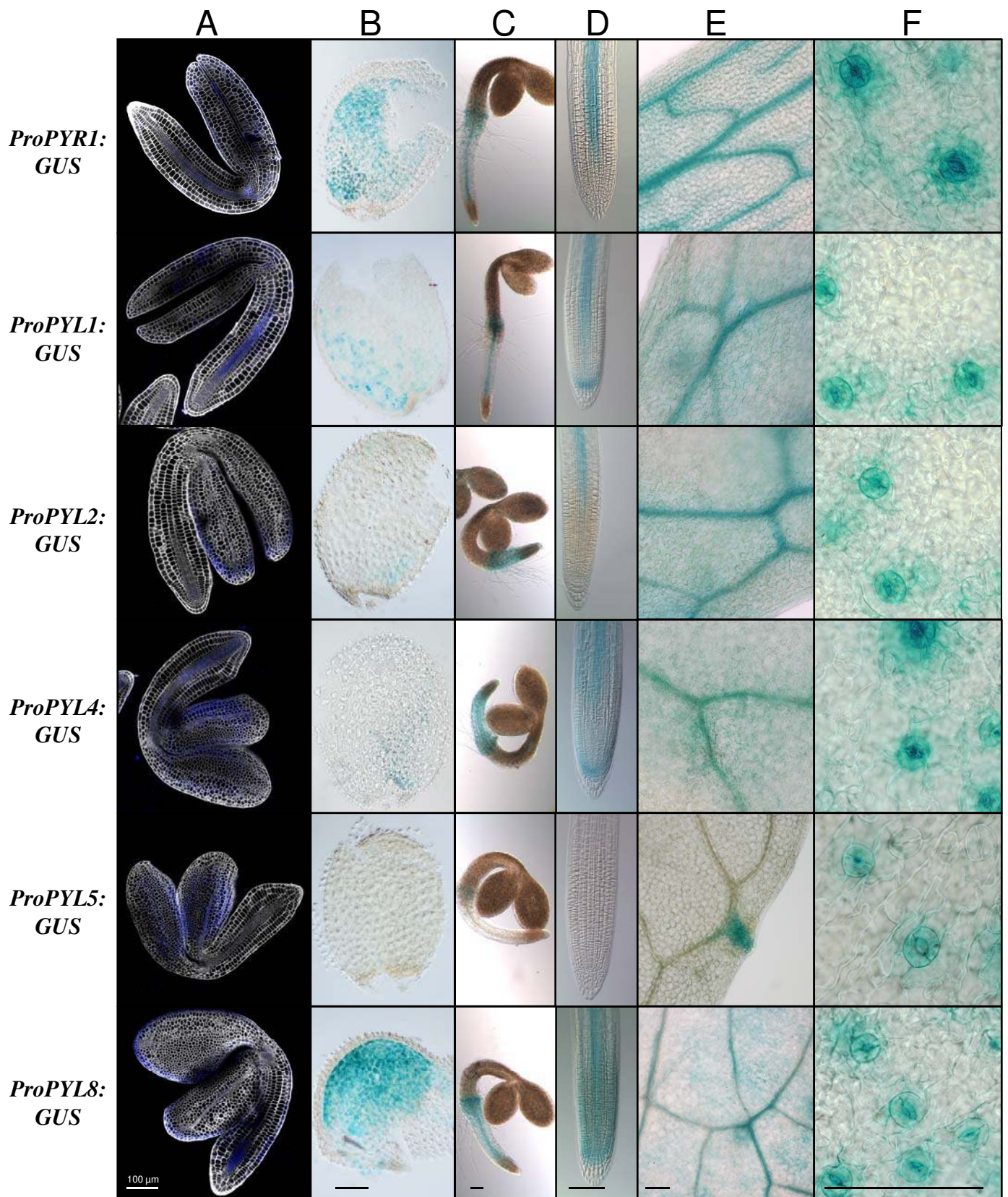
## References

- Antoni, R., Rodriguez, L., Gonzalez-Guzman, M., Pizzio, G. A., and Rodriguez, P. L.** (2011). News on ABA transport, protein degradation, and ABFs/WRKYs in ABA signaling. *Curr.Opin.Plant Biol.* **14**, 547-553.
- Antoni, R., Gonzalez-Guzman, M., Rodriguez, L., Rodrigues, A., Pizzio, G. A., and Rodriguez, P. L.** (2012). Selective Inhibition of Clade A Phosphatases Type 2C by PYR/PYL/RCAR Absciscic Acid Receptors. *Plant Physiol* **158**, 970-980.
- Barrero, J. M., Piqueras, P., Gonzalez-Guzman, M., Serrano, R., Rodriguez, P. L., Ponce, M. R., and Micol, J. L.** (2005). A mutational analysis of the ABA1 gene of *Arabidopsis thaliana* highlights the involvement of ABA in vegetative development. *J.Exp.Bot.* **56**, 2071-2083.
- Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C. A., Causton, H. C., Gaasterland, T., Glenisson, P., Holstege, F. C., Kim, I. F., Markowitz, V., Matese, J. C., Parkinson, H., Robinson, A., Sarkans, U., Schulze-Kremer, S., Stewart, J., Taylor, R., Vilo, J., and Vingron, M.** (2001). Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nat.Genet.* **29**, 365-371.
- Chekanova, J. A., Gregory, B. D., Reverdatto, S. V., Chen, H., Kumar, R., Hooker, T., Yazaki, J., Li, P., Skiba, N., Peng, Q., Alonso, J., Brukhin, V., Grossniklaus, U., Ecker, J. R., and Belostotsky, D. A.** (2007). Genome-wide high-resolution mapping of exosome substrates reveals hidden features in the *Arabidopsis* transcriptome. *Cell* **131**, 1340-1353.
- Cheng, W. H., Endo, A., Zhou, L., Penney, J., Chen, H. C., Arroyo, A., Leon, P., Nambara, E., Asami, T., Seo, M., Koshiba, T., and Sheen, J.** (2002). A unique short-chain dehydrogenase/reductase in *Arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. *Plant Cell* **14**, 2723-2743.
- Chitrakar, R. and Melotto, M.** (2010). Assessing stomatal response to live bacterial cells using whole leaf imaging. *J.Vis.Exp.*
- Curtis, M. D. and Grossniklaus, U.** (2003). A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol* **133**, 462-469.
- Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R., and Abrams, S. R.** (2010). Absciscic acid: emergence of a core signaling network. *Annu.Rev.Plant Biol.* **61**, 651-679.
- Deblaire, R., Bytebier, B., De Greve, H., Deboeck, F., Schell, J., Van Montagu, M., and Leemans, J.** (1985). Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. *Nucleic Acids Res.* **13**, 4777-4788.
- Des Marais, D. L., McKay, J. K., Richards, J. H., Sen, S., Wayne, T., and Juenger, T. E.** (2012). Physiological genomics of response to soil drying in diverse *Arabidopsis* accessions. *Plant Cell* **24**, 893-914.
- Dupeux, F., Santiago, J., Betz, K., Twycross, J., Park, S. Y., Rodriguez, L., Gonzalez-Guzman, M., Jensen, M. R., Krasnogor, N., Blackledge, M., Holdsworth, M., Cutler, S. R., Rodriguez, P. L., and Marquez, J. A.** (2011a). A thermodynamic switch modulates abscisic acid receptor sensitivity. *EMBO J.* **30**, 4171-4184.
- Dupeux, F., Antoni, R., Betz, K., Santiago, J., Gonzalez-Guzman, M., Rodriguez, L., Rubio, S., Park, S. Y., Cutler, S. R., Rodriguez, P. L., and Marquez, J. A.** (2011b). Modulation of Absciscic Acid Signaling in Vivo by an Engineered Receptor-Insensitive Protein Phosphatase Type 2C Allele. *Plant Physiol* **156**, 106-116.
- Endo, A., Sawada, Y., Takahashi, H., Okamoto, M., Ikegami, K., Koiwai, H., Seo, M., Toyomasu, T., Mitsuhashi, W., Shinozaki, K., Nakazono, M., Kamiya, Y., Koshiba, T., and Nambara, E.** (2008). Drought induction of *Arabidopsis* 9-cis-epoxycarotenoid dioxygenase occurs in vascular parenchyma cells. *Plant Physiol* **147**, 1984-1993.
- Frey, A., Effroy, D., Lefebvre, V., Seo, M., Perreau, F., Berger, A., Sechet, J., To, A., North, H. M., and Marion-Poll, A.** (2011). Epoxycarotenoid cleavage by NCED5 fine-tunes ABA accumulation and affects seed dormancy and drought tolerance with other NCED family members. *Plant J.*
- Fujii, H., Verslues, P. E., and Zhu, J. K.** (2007). Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in *Arabidopsis*. *Plant Cell* **19**, 485-494.
- Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S. Y., Cutler, S. R., Sheen, J., Rodriguez, P. L., and Zhu, J. K.** (2009). In vitro reconstitution of an abscisic acid signalling pathway. *Nature* **462**, 660-664.

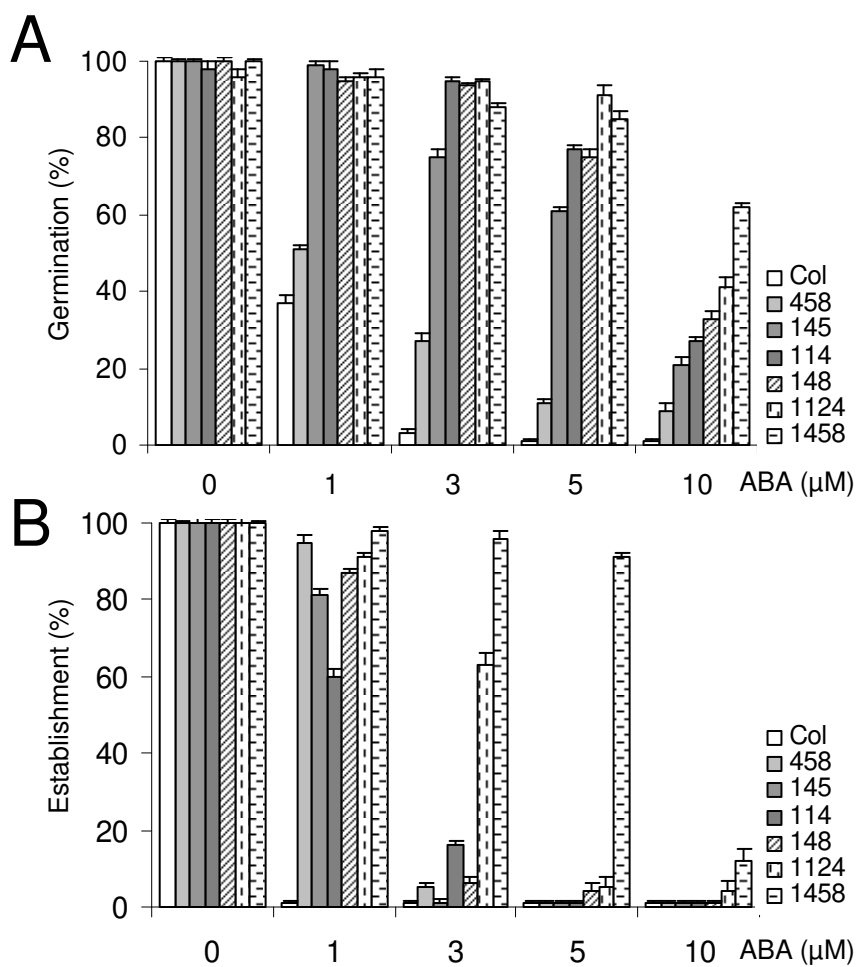
- Fujii, H. and Zhu, J. K.** (2009). Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. *Proc.Natl.Acad.Sci.U.S.A* **106**, 8380-8385.
- Fujita, Y., Nakashima, K., Yoshida, T., Katagiri, T., Kidokoro, S., Kanamori, N., Umezawa, T., Fujita, M., Maruyama, K., Ishiyama, K., Kobayashi, M., Nakasone, S., Yamada, K., Ito, T., Shinozaki, K., and Yamaguchi-Shinozaki, K.** (2009). Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in Arabidopsis. *Plant Cell Physiol* **50**, 2123-2132.
- Gaymard, F., Pilot, G., Lacombe, B., Bouchez, D., Bruneau, D., Boucherez, J., Michaux-Ferriere, N., Thibaud, J. B., and Sentenac, H.** (1998). Identification and disruption of a plant shaker-like outward channel involved in K<sup>+</sup> release into the xylem sap. *Cell* **94**, 647-655.
- Hao, Q., Yin, P., Li, W., Wang, L., Yan, C., Lin, Z., Wu, J. Z., Wang, J., Yan, S. F., and Yan, N.** (2011). The Molecular Basis of ABA-Independent Inhibition of PP2Cs by a Subclass of PYL Proteins. *Mol.Cell* **42**, 662-672.
- Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., D'Angelo, C., Bornberg-Bauer, E., Kudla, J., and Harter, K.** (2007). The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant J.* **50**, 347-363.
- Kollist, T., Moldau, H., Rasulov, B., Oja, V., Ramma, H., Huve, K., Jaspers, P., Kangasjarvi, J., and Kollist, H.** (2007). A novel device detects a rapid ozone-induce transient stomatal closure in intact Arabidopsis and its absence in *abi2* mutant. *Physiol Plant* **129**, 796-803.
- Lackman, P., Gonzalez-Guzman, M., Tilleman, S., Carqueijeiro, I., Perez, A. C., Moses, T., Seo, M., Kanno, Y., Hakkinen, S. T., Van Montagu, M. C., Thevelein, J. M., Maaheimo, H., Oksman-Caldentey, K. M., Rodriguez, P. L., Rischer, H., and Goossens, A.** (2011). Jasmonate signaling involves the abscisic acid receptor PYL4 to regulate metabolic reprogramming in Arabidopsis and tobacco. *Proc.Natl.Acad.Sci.U.S.A* **108**, 5891-5896.
- Laubinger, S., Zeller, G., Henz, S. R., Sachsenberg, T., Widmer, C. K., Naouar, N., Vuylsteke, M., Scholkopf, B., Ratsch, G., and Weigel, D.** (2008). At-TAX: a whole genome tiling array resource for developmental expression analysis and transcript identification in Arabidopsis thaliana. *Genome Biol.* **9**, R112.
- Liu, X., Yue, Y., Li, B., Nie, Y., Li, W., Wu, W. H., and Ma, L.** (2007). A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. *Science* **315**, 1712-1716.
- Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A., and Grill, E.** (2009). Regulators of PP2C Phosphatase Activity Function as Abscisic Acid Sensors. *Science* **324**, 1064-1068.
- Mustilli, A. C., Merlot, S., Vavasseur, A., Fenzi, F., and Giraudat, J.** (2002). Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* **14**, 3089-3099.
- Nishimura, N., Sarkeshik, A., Nito, K., Park, S. Y., Wang, A., Carvalho, P. C., Lee, S., Caddell, D. F., Cutler, S. R., Chory, J., Yates, J. R., and Schroeder, J. I.** (2010). PYR/PYL/RCAR family members are major in-vivo ABI1 protein phosphatase 2C-interacting proteins in Arabidopsis. *Plant J.* **61**, 290-299.
- Pandey, S., Nelson, D. C., and Assmann, S. M.** (2009). Two novel GPCR-type G proteins are abscisic acid receptors in Arabidopsis. *Cell* **136**, 136-148.
- Park, S. Y., Fung, P., Nishimura, N., Jensen, D. R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Chow, T. F. F., Alfred, S. E., Bonetta, D., Finkelstein, R., Provart, N. J., Desveaux, D., Rodriguez, P. L., McCourt, P., Zhu, J. K., Schroeder, J. I., Volkman, B. F., and Cutler, S. R.** (2009). Abscisic Acid Inhibits Type 2C Protein Phosphatases via the PYR/PYL Family of START Proteins. *Science* **324**, 1068-1071.
- Razem, F. A., El Kereamy, A., Abrams, S. R., and Hill, R. D.** (2008). Retraction. The RNA-binding protein FCA is an abscisic acid receptor. *Nature* **456**, 824.
- Robert, N., Merlot, S., N'guyen, V., Boisson-Dernier, A., and Schroeder, J. I.** (2006). A hypermorphic mutation in the protein phosphatase 2C HAB1 strongly affects ABA signaling in Arabidopsis. *FEBS Lett.* **580**, 4691-4696.
- Rubio, S., Rodrigues, A., Saez, A., Dizon, M. B., Galle, A., Kim, T. H., Santiago, J., Flexas, J., Schroeder, J. I., and Rodriguez, P. L.** (2009). Triple loss of function of protein phosphatases type 2C leads to partial constitutive response to endogenous abscisic acid. *Plant Physiol* **150**, 1345-1355.
- Saez, A., Apostolova, N., Gonzalez-Guzman, M., Gonzalez-Garcia, M. P., Nicolas, C., Lorenzo, O., and Rodriguez, P. L.** (2004). Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C HAB1 reveal its role as a negative regulator of abscisic acid signalling. *Plant J.* **37**, 354-369.

- Santiago, J., Rodrigues, A., Saez, A., Rubio, S., Antoni, R., Dupeux, F., Park, S. Y., Marquez, J. A., Cutler, S. R., and Rodriguez, P. L. (2009a). Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs. *Plant J.* **60**, 575-588.
- Santiago, J., Dupeux, F., Round, A., Antoni, R., Park, S. Y., Jamin, M., Cutler, S. R., Rodriguez, P. L., and Marquez, J. A. (2009b). The abscisic acid receptor PYR1 in complex with abscisic acid. *Nature* **462**, 665-668.
- Shang, Y., Yan, L., Liu, Z. Q., Cao, Z., Mei, C., Xin, Q., Wu, F. Q., Wang, X. F., Du, S. Y., Jiang, T., Zhang, X. F., Zhao, R., Sun, H. L., Liu, R., Yu, Y. T., and Zhang, D. P. (2010). The Mg-chelatase H subunit of Arabidopsis antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition. *Plant Cell* **22**, 1909-1935.
- Sharp, R. E., Poroyko, V., Hejlek, L. G., Spollen, W. G., Springer, G. K., Bohnert, H. J., and Nguyen, H. T. (2004). Root growth maintenance during water deficits: physiology to functional genomics. *J. Exp. Bot.* **55**, 2343-2351.
- Shen, Y. Y., Wang, X. F., Wu, F. Q., Du, S. Y., Cao, Z., Shang, Y., Wang, X. L., Peng, C. C., Yu, X. C., Zhu, S. Y., Fan, R. C., Xu, Y. H., and Zhang, D. P. (2006). The Mg-chelatase H subunit is an abscisic acid receptor. *Nature* **443**, 823-826.
- Szostkiewicz, I., Richter, K., Kepka, M., Demmel, S., Ma, Y., Korte, A., Assaad, F. F., Christmann, A., and Grill, E. (2010). Closely related receptor complexes differ in their ABA selectivity and sensitivity. *Plant J.* **61**, 25-35.
- Truernit, E., Bauby, H., Dubreucq, B., Grandjean, O., Runions, J., Barthelemy, J., and Palauqui, J. C. (2008). High-resolution whole-mount imaging of three-dimensional tissue organization and gene expression enables the study of Phloem development and structure in Arabidopsis. *Plant Cell* **20**, 1494-1503.
- Umezawa, T., Sugiyama, N., Mizoguchi, M., Hayashi, S., Myouga, F., Yamaguchi-Shinozaki, K., Ishihama, Y., Hirayama, T., and Shinozaki, K. (2009). Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 17588-17593.
- Umezawa, T., Nakashima, K., Miyakawa, T., Kuromori, T., Tanokura, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2010). Molecular basis of the core regulatory network in aba responses: sensing, signaling and transport. *Plant Cell Physiol* **51**, 1821-1839.
- Vahisalu, T., Kollist, H., Wang, Y. F., Nishimura, N., Chan, W. Y., Valerio, G., Lamminmaki, A., Brosche, M., Moldau, H., Desikan, R., Schroeder, J. I., and Kangasjarvi, J. (2008). SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. *Nature* **452**, 487-491.
- Vlad, F., Rubio, S., Rodrigues, A., Sirichandra, C., Belin, C., Robert, N., Leung, J., Rodriguez, P. L., Lauriere, C., and Merlot, S. (2009). Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in Arabidopsis. *Plant Cell* **21**, 3170-3184.
- Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G. V., and Provart, N. J. (2007). An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. *PLoS One* **2**, e718.
- Wu, F. Q., Xin, Q., Cao, Z., Liu, Z. Q., Du, S. Y., Mei, C., Zhao, C. X., Wang, X. F., Shang, Y., Jiang, T., Zhang, X. F., Yan, L., Zhao, R., Cui, Z. N., Liu, R., Sun, H. L., Yang, X. L., Su, Z., and Zhang, D. P. (2009). The magnesium-chelatase H subunit binds abscisic acid and functions in abscisic acid signaling: new evidence in Arabidopsis. *Plant Physiol* **150**, 1940-1954.
- Yamada, K., et al., (more than 20 authors) (2003). Empirical analysis of transcriptional activity in the Arabidopsis genome. *Science* **302**, 842-846.
- Yang, Y., Costa, A., Leonhardt, N., Siegel, R. S., and Schroeder, J. I. (2008). Isolation of a strong Arabidopsis guard cell promoter and its potential as a research tool. *Plant Methods* **4**, 6.
- Yoshida, R., Hobo, T., Ichimura, K., Mizoguchi, T., Takahashi, F., Aronso, J., Ecker, J. R., and Shinozaki, K. (2002). ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in Arabidopsis. *Plant Cell Physiol* **43**, 1473-1483.
- Zeevaart, J. A. and Creelman, R. A. (1988). Metabolism and physiology of abscisic acid. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **39**, 439-473.
- Zheng, Z., Xu, X., Crosley, R. A., Greenwalt, S. A., Sun, Y., Blakeslee, B., Wang, L., Ni, W., Sopko, M. S., Yao, C., Yau, K., Burton, S., Zhuang, M., McCaskill, D. G., Gachotte, D., Thompson, M., and Greene, T. W. (2010). The protein kinase SnRK2.6 mediates the regulation of sucrose metabolism and plant growth in Arabidopsis. *Plant Physiol* **153**, 99-113.

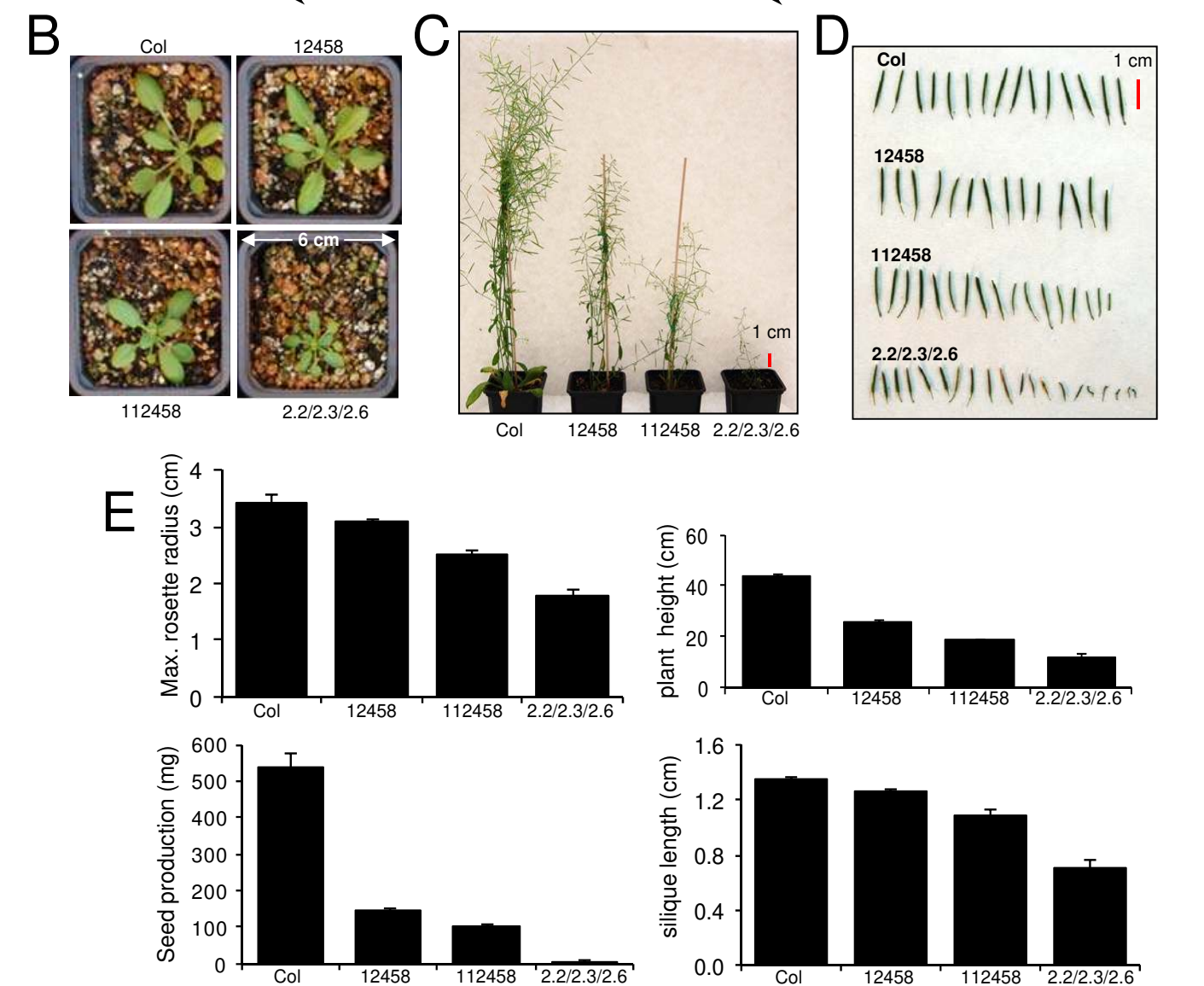
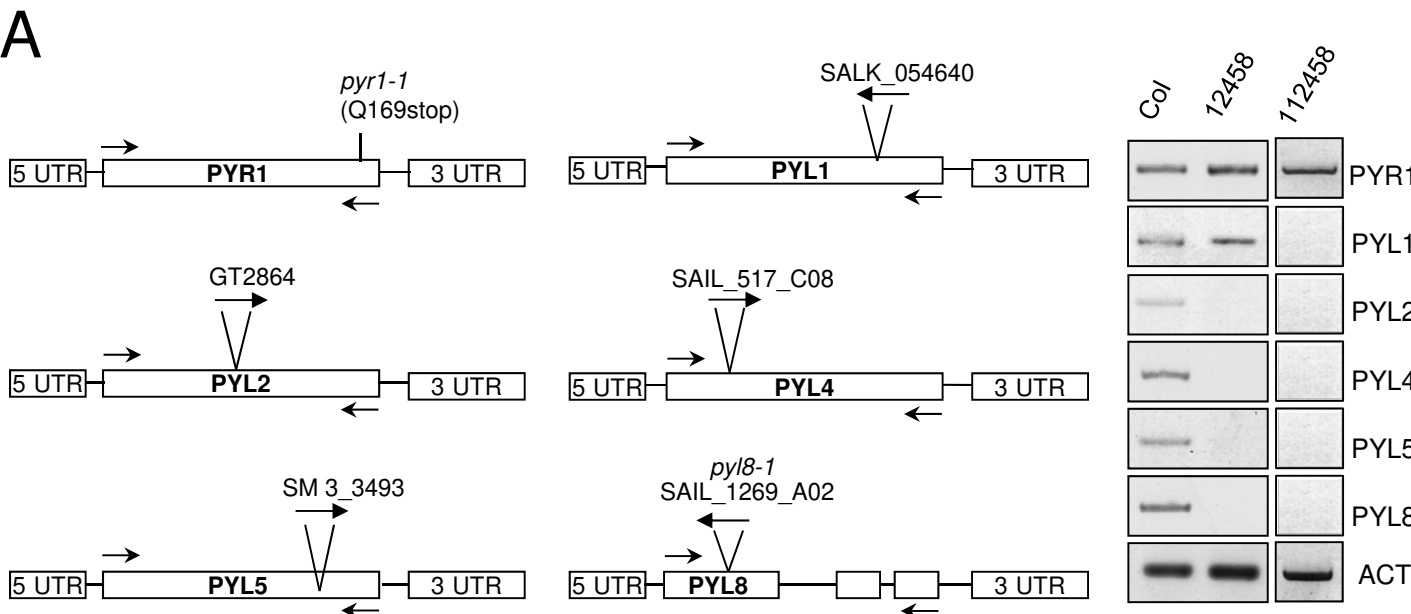
## \References



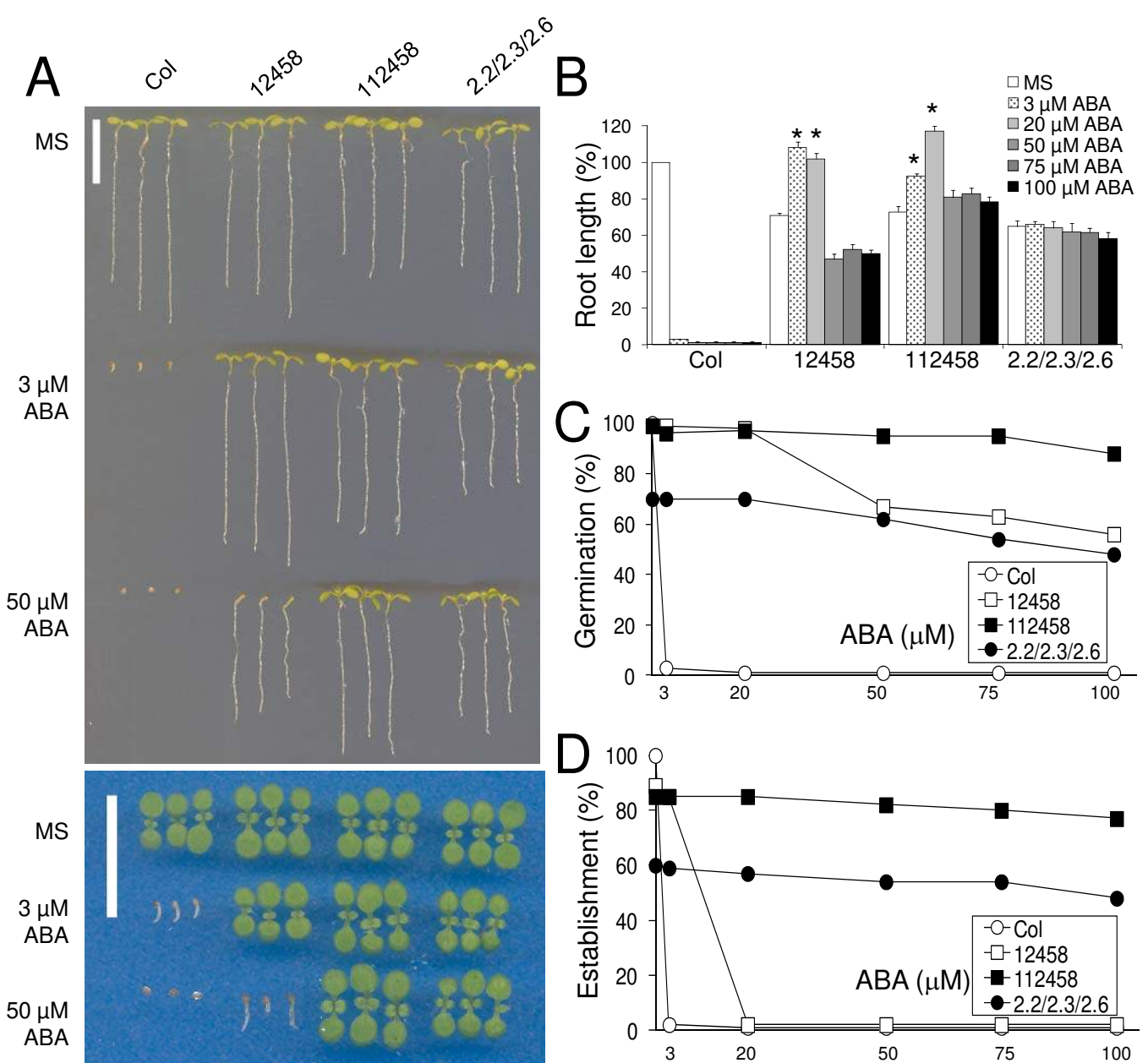
**Figure 1.** Photographs showing GUS expression driven by *ProPYL1*, *ProPYR1*, *ProPYL2*, *ProPYL4*, *ProPYL5* and *ProPYL8*:*GUS* genes in different tissues. (A, C) Embryos dissected from mature seeds imbibed for 24 h or 48 h, respectively. (B) Seed coat and endosperm imbibed for 24 h. (D) Primary root from 5-d-old seedlings. (E, F) Vascular tissue and guard cells in leaves of 15-d-old seedlings, respectively. The bar corresponds to 100  $\mu$ m.



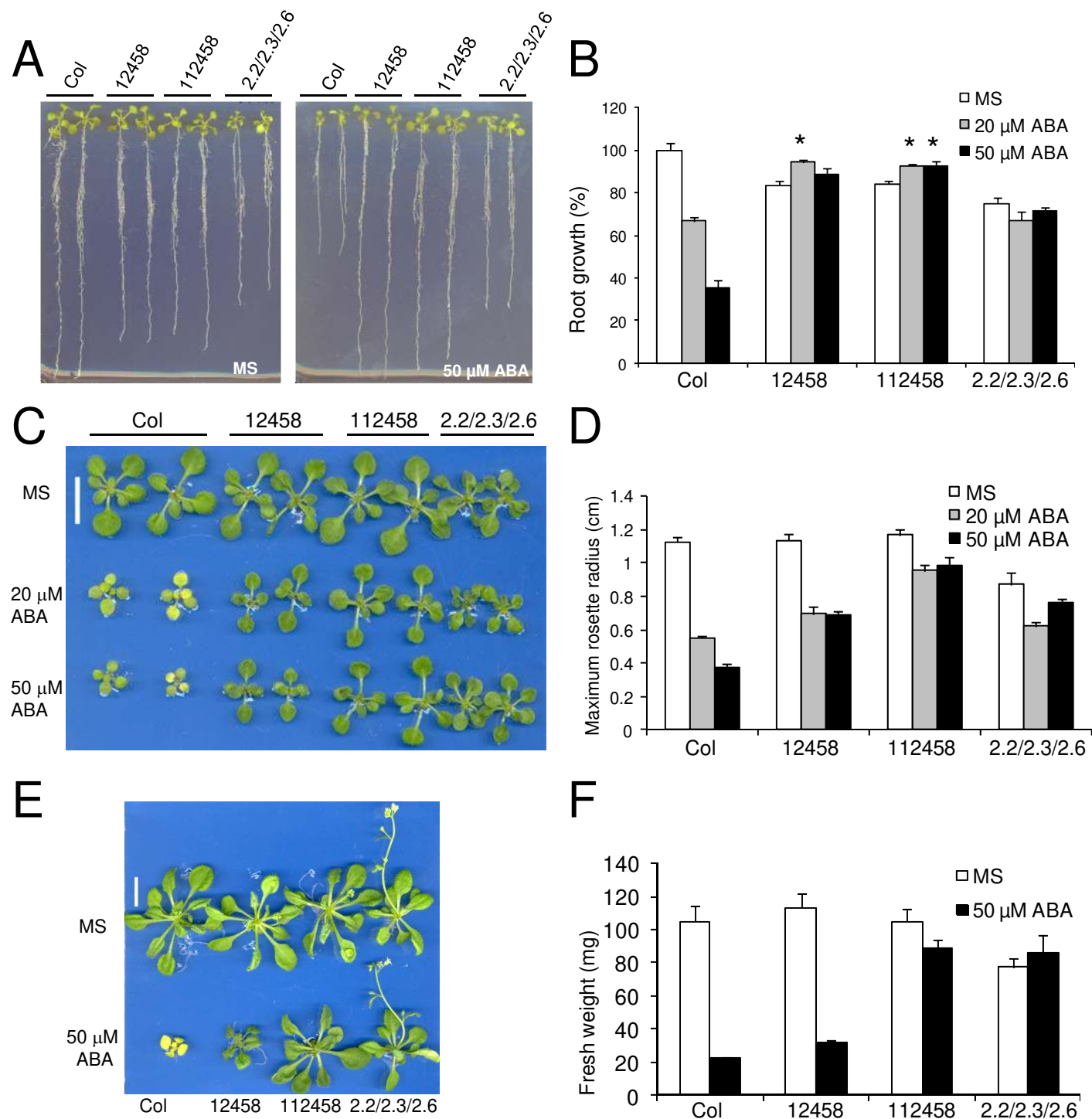
**Figure 2.** Quantification of ABA-mediated inhibition of germination and seedling establishment of Col wt compared to different genotypes. Approximately 100 seeds of each genotype (three independent experiments) were sowed on each plate and scored for radicle emergence 3-d-later (**A**) or for the presence of both green cotyledons and the first pair of true leaves 7-d-later (**B**). Values are averages  $\pm$ SE.



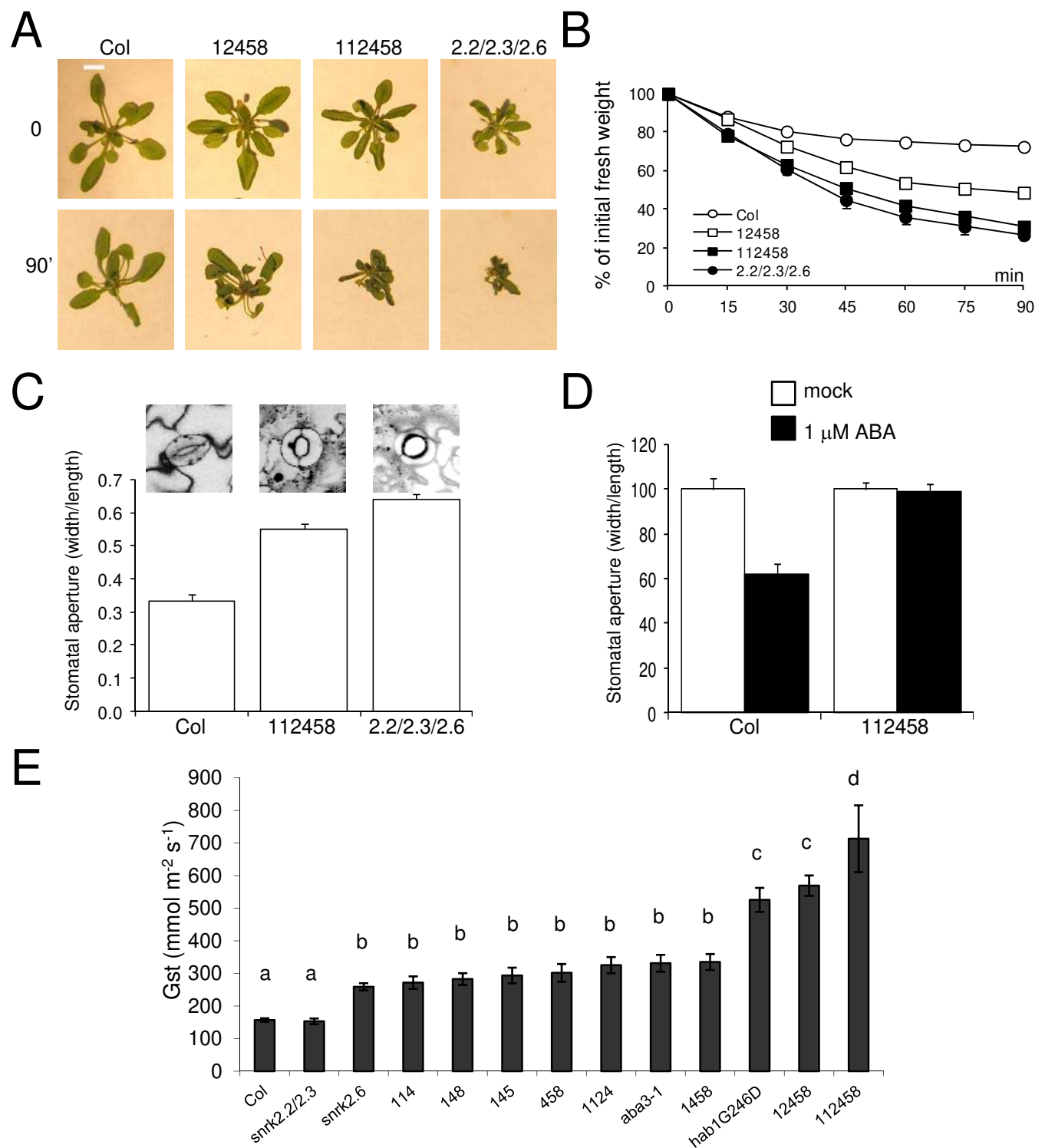
**Figure 3.** Combined loss of function of *PYR/PYL* genes impairs plant growth and seed yield. **(A)** Schematic diagram of the *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* genes showing the position of the T-DNA insertion or the nonsense mutation in the *pyr1-1* allele. RT-PCR analyses of mRNAs from wt, 12458 and 112458. The position of the primers used for genotyping and RT-PCR is indicated by arrows. **(B, C, D)** Photographs show the impairment of growth and reproduction in extreme ABA-insensitive mutants. Photographs of 24-d-old plants **(B)**, 50-d-old plants **(C)** and siliques **(D)** grown under greenhouse conditions of Col wt, 12458, 112458 and *snrk2.2/2.3/2.6*. **(E)** Quantification of maximum rosette radius, plant height, seed production and silique length of the different genotypes. Data are average values obtained for 20 plants.



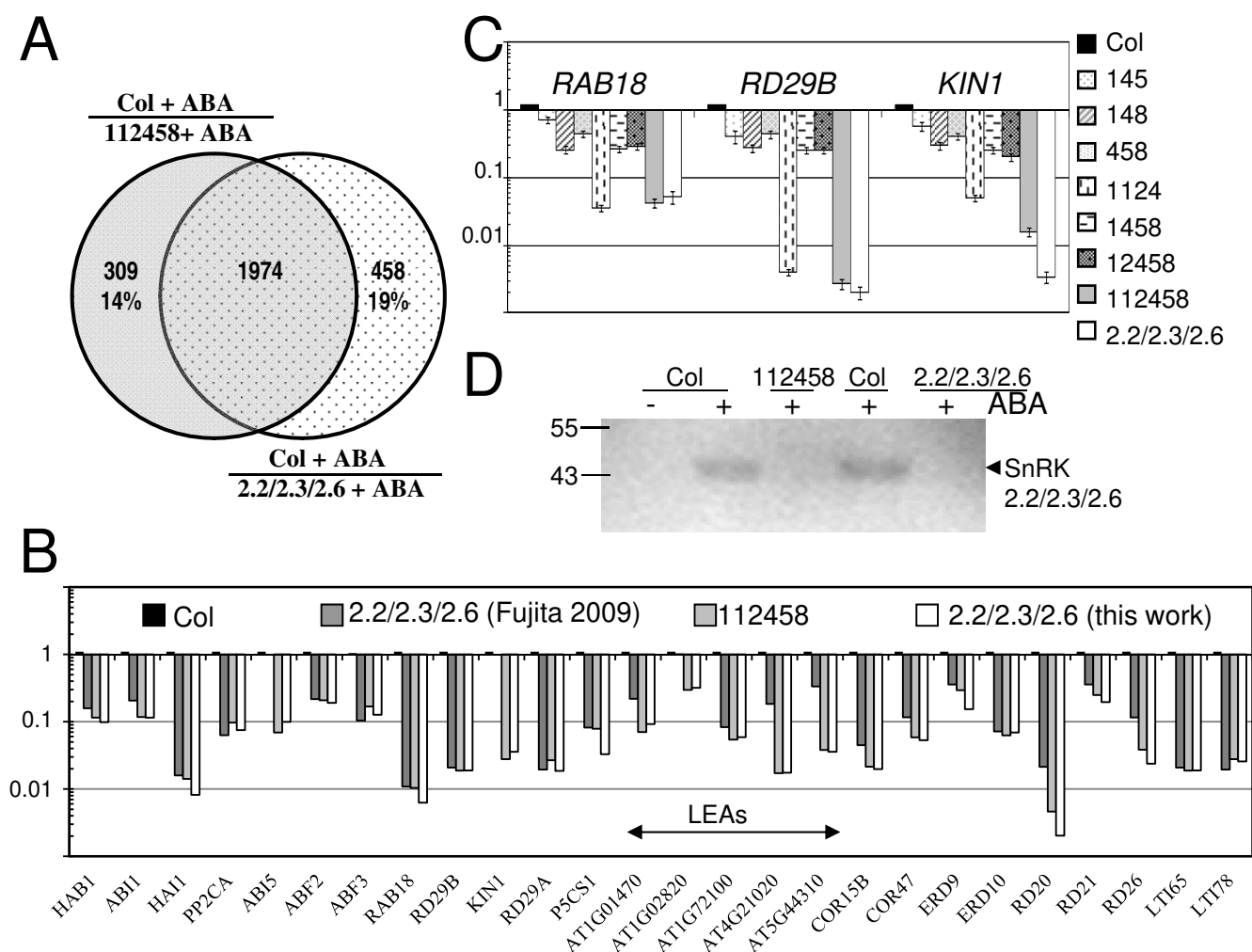
**Figure 4.** Extreme ABA-insensitive phenotype of 112458 for germination and seedling establishment assays. (A) Photographs of Col wt, 12458, 112458 and *snrk2.2/2.3/2.6* grown for 7-d on MS medium either lacking or supplemented with different concentrations of ABA. (B) Quantification of root length in 7-d-old seedlings of panel A. Data are averages  $\pm$ SE from three independent experiments (n =15 each). The asterisk indicates  $P < 0.01$  (Student's t test) with respect to medium lacking ABA. (C, D) Quantification of ABA-mediated inhibition of germination and seedling establishment of Col wt compared to 12458, 112458 and *snrk2.2/2.3/2.6*. Approximately 100 seeds of each genotype were sowed on each plate and scored for radicle emergence 3-d-later (C) or for the presence of both green cotyledons and the first pair of true leaves 7-d-later (D). SE values were lower than 7% and are not indicated.



**Figure 5.** ABA-insensitive phenotype of 112458 for ABA-mediated inhibition of growth assays. **(A)** Photograph of representative seedlings 10 d after the transfer of 4-d-old seedlings to MS plates lacking or supplemented with 50  $\mu$ M ABA. **(B)** Quantification of ABA-mediated root growth inhibition of Col wt compared to 12458, 112458 and *snrk2.2/2.3/2.6*. Data are averages  $\pm$ SE from three independent experiments (n =15 each). The asterisk indicates P<0.01 (Student's t test) with respect to medium lacking ABA. **(C, E)** Photograph of representative seedlings 11-d or 21-d after the transfer of 4-d-old seedlings from MS medium to plates lacking or supplemented with ABA. **(D, F)** Quantification of ABA-mediated shoot growth inhibition of Col wt compared to 12458, 112458 and *snrk2.2/2.3/2.6*. Data are averages  $\pm$ SE from three independent experiments (n =15 each).



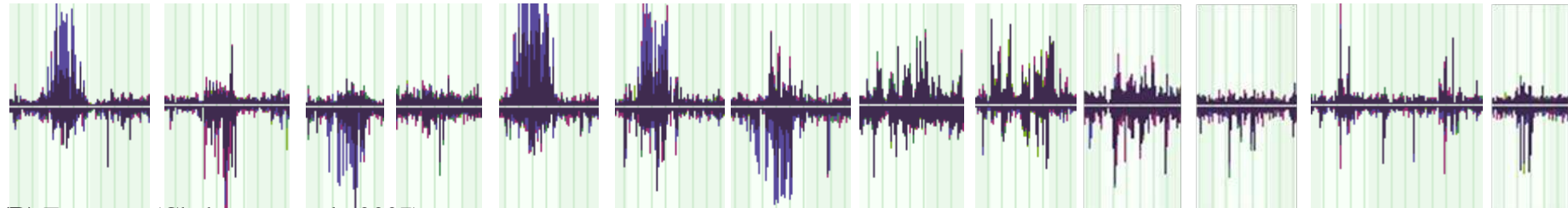
**Figure 6.** Water-loss and stomatal conductance assays in *pyr/pyl* mutants. **(A)** Photograph of representative excised plants submitted for 90 minutes to the drying atmosphere of a flow laminar hood. **(B)** Loss-of-fresh weight of 18-d-old excised plants that were submitted to the drying atmosphere of a flow laminar hood. **(C)** Increased stomatal aperture of 21-d-old plants of 112458 and *snrk2.2/2.3/2.6* compared to wt. **(D)** ABA-insensitive stomatal closing of 21-d old plants of 112458 compared to wt. **(E)** Leaf gas exchange measurements reveal increased stomatal conductance (Gst) in different *pyr/pyl* mutants and additive effects upon progressive inactivation of *PYR/PYL* genes. The different letters denote significant differences between mutants ( $P < 0.05$ ,  $n = 5-17$ , Tukey's post-hoc comparison, Student's *t* and Fisher LSD tests for comparison between *snrk2.6* and Col wt).



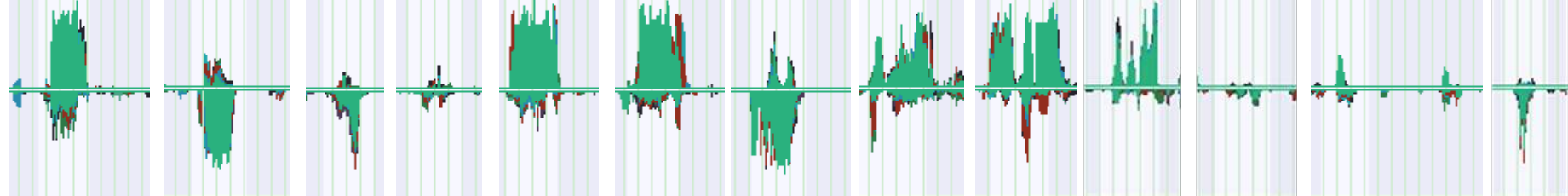
**Figure 7.** ABA-responsive gene expression is drastically impaired in 112458. **(A)** Global impairment of transcriptional response to ABA in 112458 and *snrk2.2/2.3/2.6* compared to Col wt. Genes showing  $\geq 2$ -fold higher expression upon ABA-treatment in Col than in the mutants (false discovery rate  $p < 0.05$ ) are represented using Venn diagram. Analyses were made in quadruplicate on independent RNA samples of 2-week-old seedlings that were treated with 10  $\mu$ M ABA for 3 h. The transcriptome profile was obtained using the Agilent's gene expression 4x44000 microarray. **(B)** Relative induction level of selected genes after ABA treatment in each mutant compared to wt (value 1). **(C)** Relative expression of three ABA-responsive genes in the indicated genotypes after ABA treatment compared to wt (value 1) as determined by RT-qPCR. Expression of *RAB18*, *RD29B* and *KIN1* was up-regulated 86-, 634- and 312-fold by ABA in the wt, respectively. **(D)** SnRK2s are not activated by ABA in 112458. Proteins extracted from wt, 112458 and *snrk2.2/2.3/2.6* seedlings that were either mock (-) or 100  $\mu$ M ABA-treated (+) for 30 min were analysed by an in-gel-kinase assay.

**Supplemental Figure 1. Gene expression levels of the *PYR/PYL/RCAR* ABA receptors in the *Arabidopsis* transcriptome genomic express database and *Arabidopsis* whole-genome tiling array (At-TAX).** (A-C) Gene expression data were obtained from the *Arabidopsis* transcriptome genomic express database (<http://signal.salk.edu>). The figure shows the gene expression picture for each *PYR/PYL* gene using whole-genome microarrays (A) Salk transcriptome (Yamada et al., 2003), (B) Exosome (Chekanova et al., 2007) and (C) At-TAX (Laubinger et al., 2008), *Arabidopsis* whole-genome tiling array (<http://www.weigelworld.org/resources/microarray/at-tax>). (D) *Arabidopsis thaliana* Tiling Array Express (At-TAX) expression data of the *PYR/PYL/RCAR* gene family. Gene expression data were obtained from the Weigel World webpage (<http://www.weigelworld.org/resources/microarray/at-tax>) using the TileViz web resource (<http://jsp.weigelworld.org/tileviz/tileviz.jsp>). Data correspond to 9 different tissues at various stages of plant development from Col-0 wild-type. Root, seedling and vegetative apex materials were obtained from 7-d-old plants; young and old leaf materials were obtained from 17 and 35 day-old plants respectively. Remaining plant material was obtained from plants grown for 21 or more days. Y axis represents the intensity of the gene expression in arbitrary units.

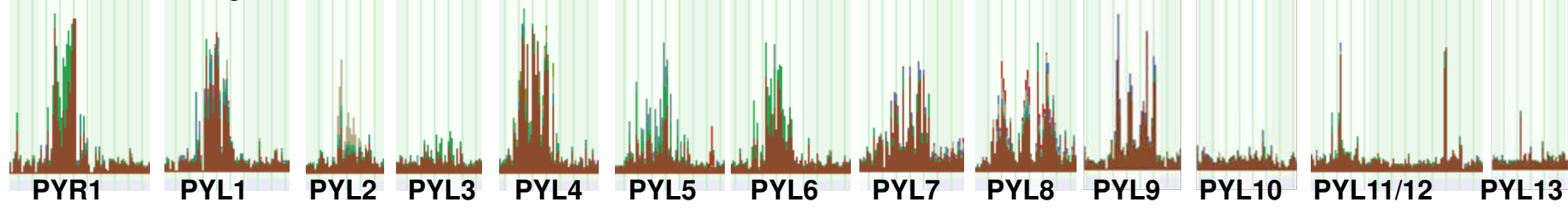
(A) Salk transcriptome (Yamada et al., 2003)



(B) Exosome (Chekanova et al., 2007)

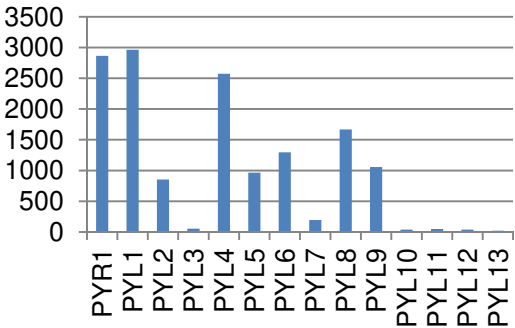


(C) At-TAX (Laubinger et al., 2008)

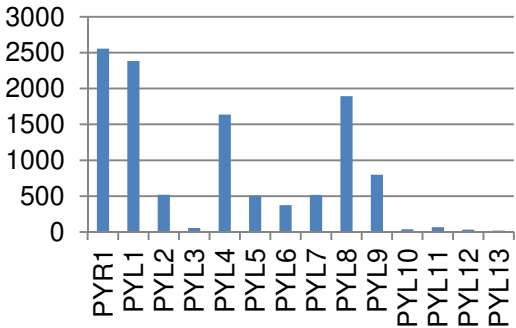


(D)Arabidopsis thaliana Tiling Array Express (At-TAX) expression data of the PYR/PYL/RCAR gene family.

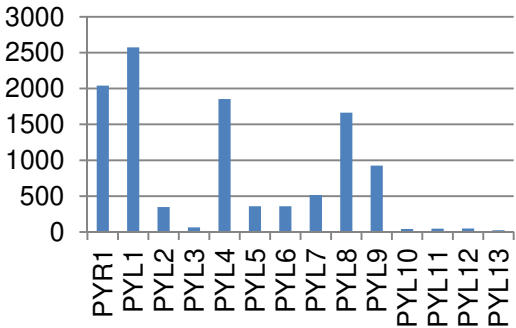
Root



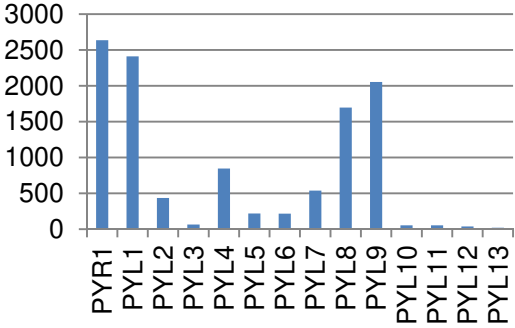
Seedling



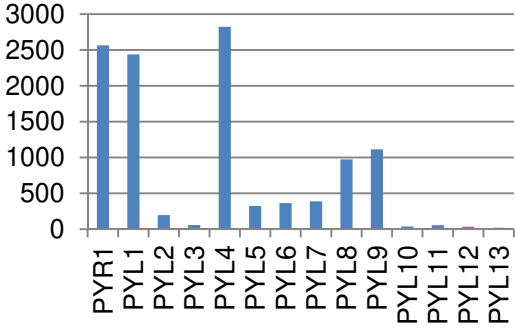
Leaf young



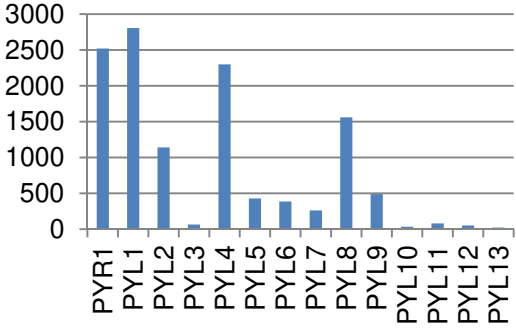
Leaf old



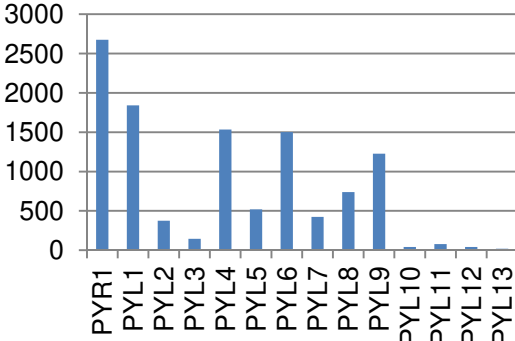
Stem



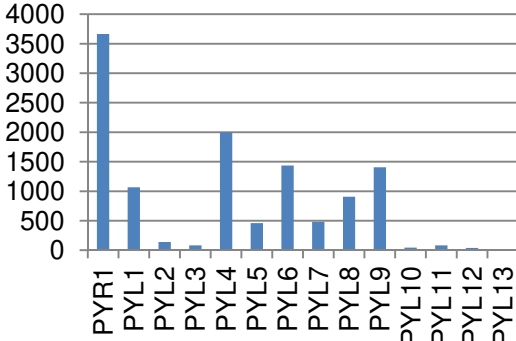
Veg. apex



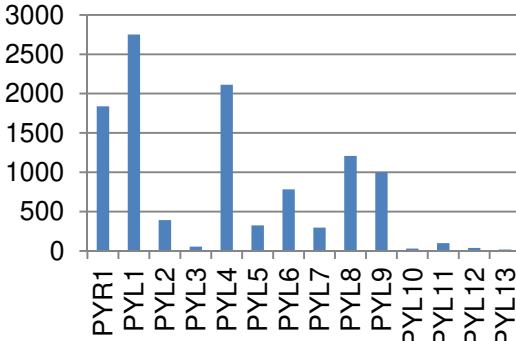
Flower

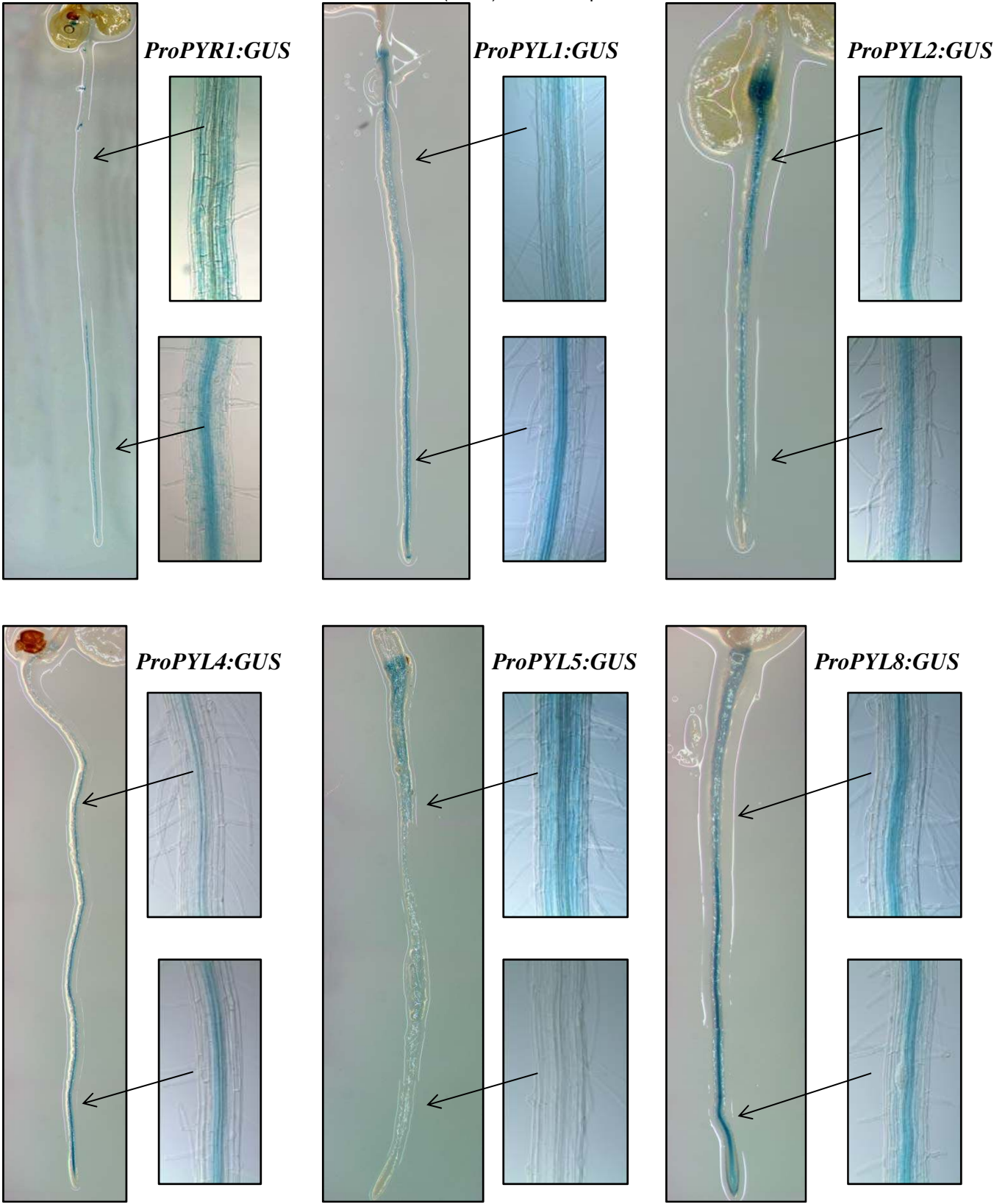


Fruit

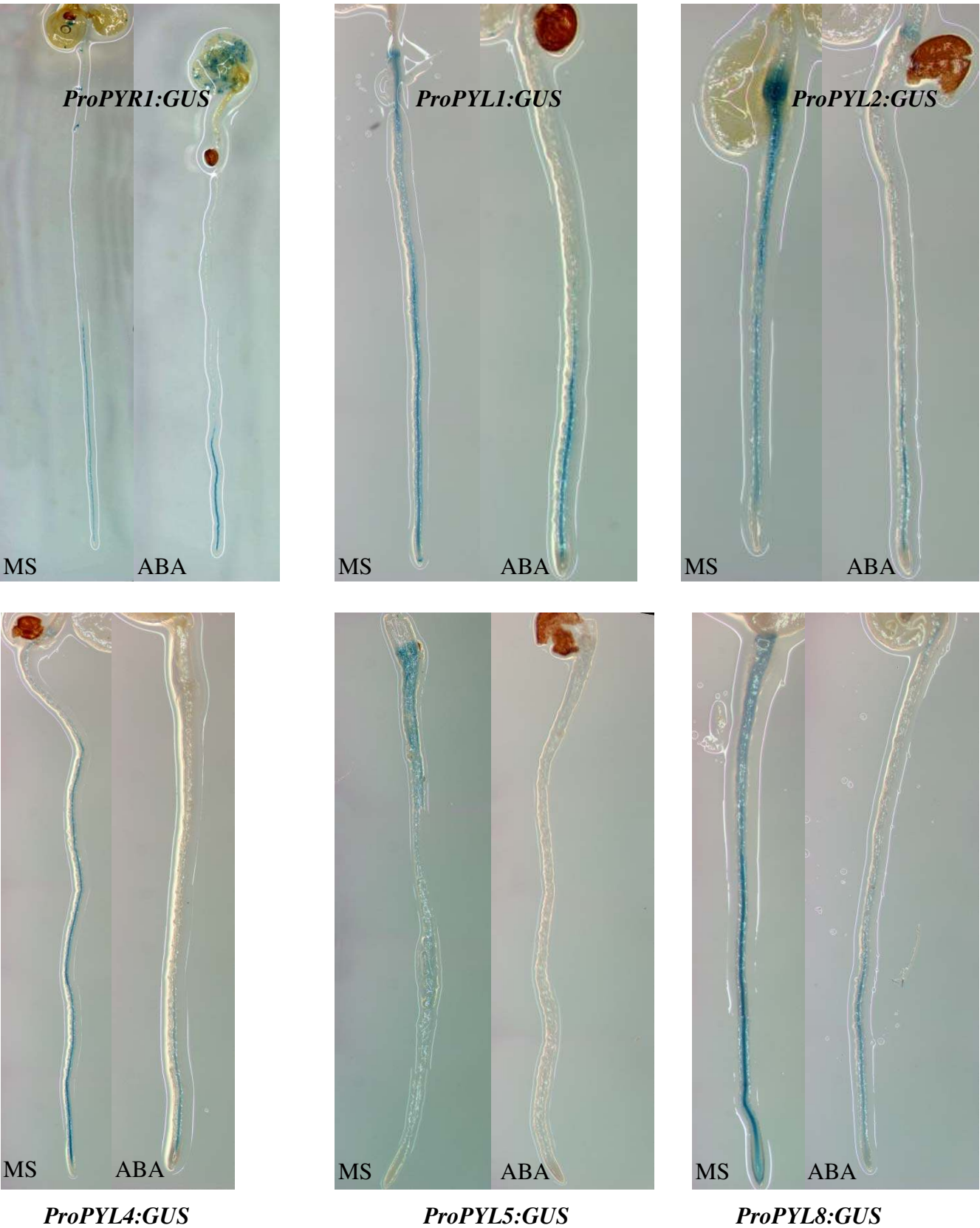


Whole Inf.

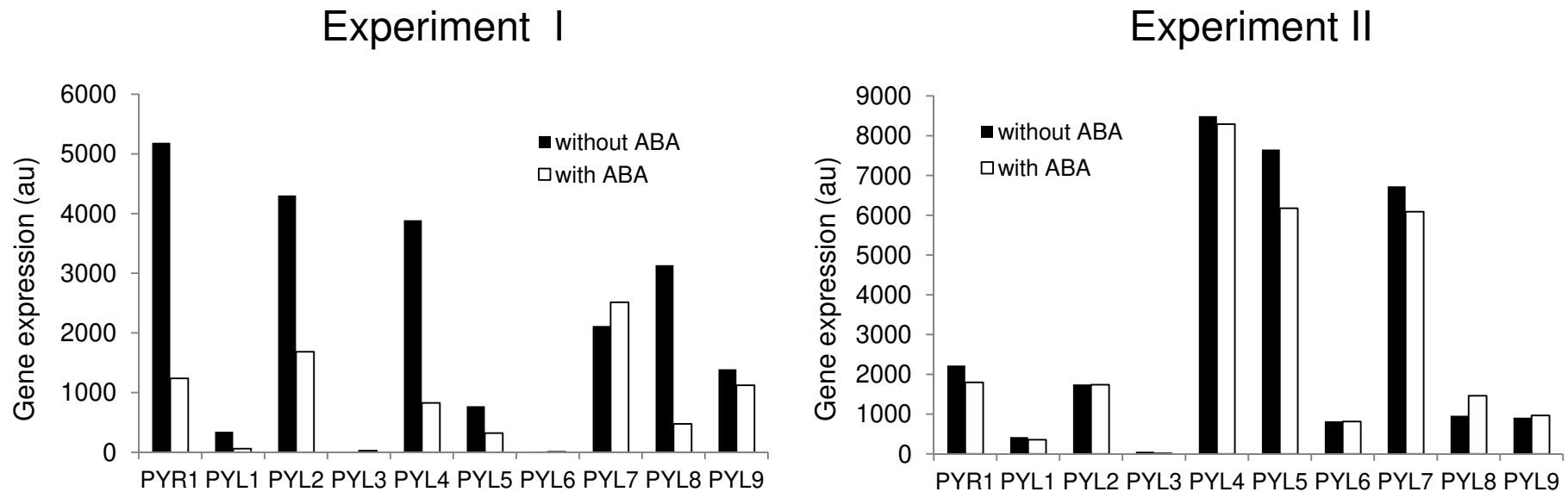




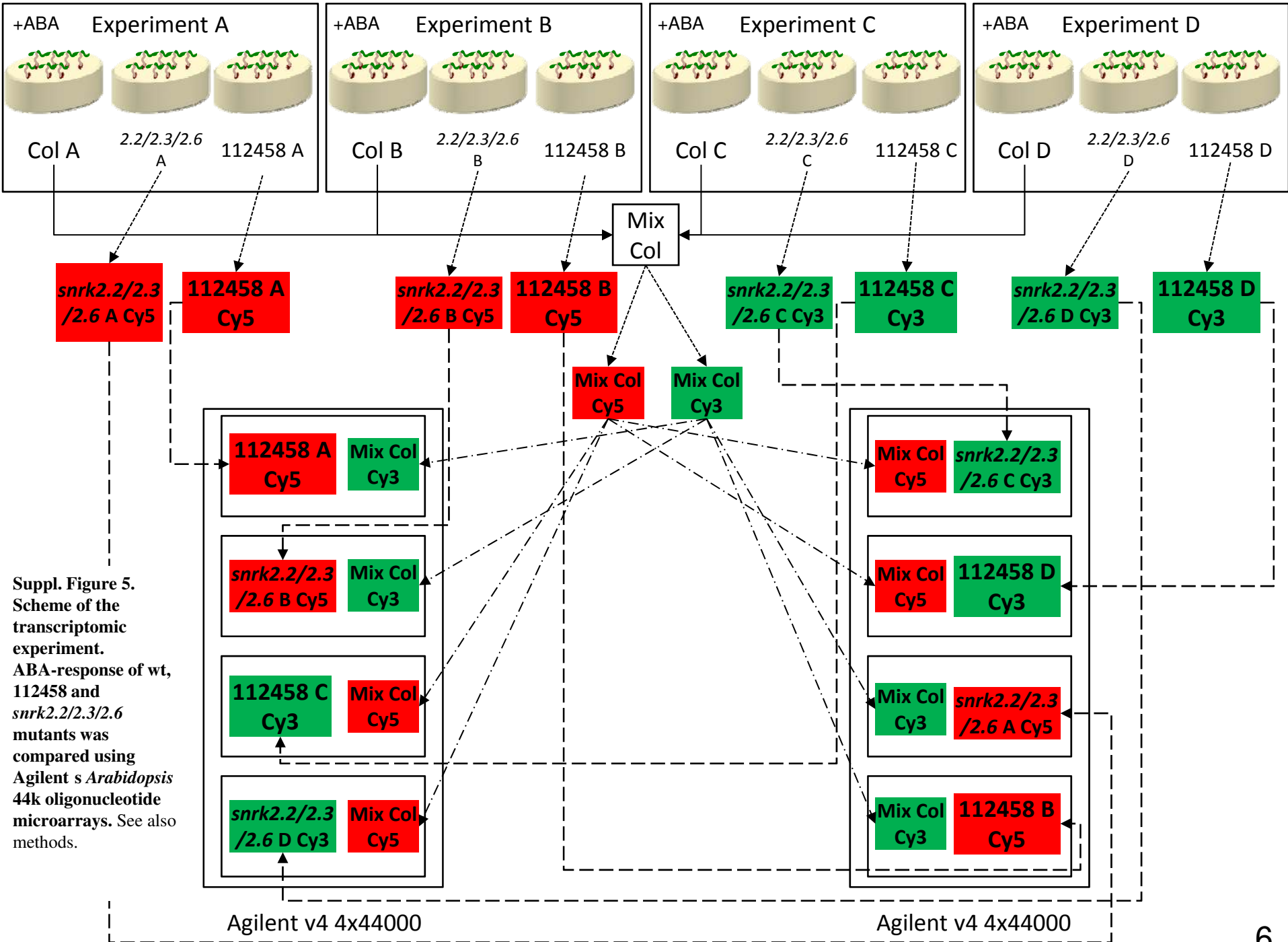
**Supplemental Figure 2. Photographs showing GUS expression driven by *ProPYL1*, *ProPYR1*, *ProPYL2*, *ProPYL4*, *ProPYL5* and *ProPYL8:GUS* genes in roots of 5-d-old seedlings.** Upper arrows point to mature root, while lower arrows point the root differentiation zone. Mature roots from *ProPYR1:GUS* and *ProPYL5:GUS* lines show cortex expression while expression of the other receptors is located only in vascular tissue.



**Supplemental Figure 3. ABA treatment inhibits or attenuates GUS expression driven by *ProPYL1*, *ProPYR1*, *ProPYL2*, *ProPYL4*, *ProPYL5* and *ProPYL8:GUS* genes.** Photographs show 5-d-old seedlings that were either mock or 10  $\mu$ M ABA -treated for 10 h.



**Supplemental Figure 4. Expression of *PYR/PYL* genes in guard cells mock or 100  $\mu$ M ABA-treated.** Data were obtained from experiments reported by Yang et al., (2008). Five-six weeks old plants were sprayed with 100  $\mu$ M ABA or water for 4h before cell isolation. Next, guard cell protoplast isolation and RNA extraction were performed as previously described by Leonhardt et al., (2004). Experiment I included the addition of 33 mg/L actinomycin D and 100 mg/L cordycepin during protoplast isolation to inhibit modulation of gene expression in response to stress during guard cell protoplast preparation. Experiment II lacked these compounds.



## Supplemental references

- Chekanova, J. A., Gregory, B. D., Reverdatto, S. V., Chen, H., Kumar, R., Hooker, T., Yazaki, J., Li, P., Skiba, N., Peng, Q., Alonso, J., Brukhin, V., Grossniklaus, U., Ecker, J. R., and Belostotsky, D. A.** (2007). Genome-wide high-resolution mapping of exosome substrates reveals hidden features in the Arabidopsis transcriptome. *Cell* **131**, 1340-1353.
- Laubinger, S., Zeller, G., Henz, S. R., Sachsenberg, T., Widmer, C. K., Naouar, N., Vuylsteke, M., Scholkopf, B., Ratsch, G., and Weigel, D.** (2008). At-TAX: a whole genome tiling array resource for developmental expression analysis and transcript identification in *Arabidopsis thaliana*. *Genome Biol.* **9**, R112.
- Leonhardt, N., Kwak, J. M., Robert, N., Waner, D., Leonhardt, G., and Schroeder, J. I.** (2004). Microarray expression analyses of Arabidopsis guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. *Plant Cell* **16**, 596-615.
- Yamada, K., Lim, J., Dale, J. M., Chen, H., Shinn, P., Palm, C. J., Southwick, A. M., Wu, H. C., Kim, C., Nguyen, M., Pham, P., Cheuk, R., Karlin-Newmann, G., Liu, S. X., Lam, B., Sakano, H., Wu, T., Yu, G., Miranda, M., Quach, H. L., Tripp, M., Chang, C. H., Lee, J. M., Toriumi, M., Chan, M. M., Tang, C. C., Onodera, C. S., Deng, J. M., Akiyama, K., Ansari, Y., Arakawa, T., Banh, J., Banno, F., Bowser, L., Brooks, S., Carninci, P., Chao, Q., Choy, N., Enju, A., Goldsmith, A. D., Gurjal, M., Hansen, N. F., Hayashizaki, Y., Johnson-Hopson, C., Hsuan, V. W., Iida, K., Karnes, M., Khan, S., Koesema, E., Ishida, J., Jiang, P. X., Jones, T., Kawai, J., Kamiya, A., Meyers, C., Nakajima, M., Narusaka, M., Seki, M., Sakurai, T., Satou, M., Tamse, R., Vaysberg, M., Wallender, E. K., Wong, C., Yamamura, Y., Yuan, S., Shinozaki, K., Davis, R. W., Theologis, A., and Ecker, J. R.** (2003). Empirical analysis of transcriptional activity in the Arabidopsis genome. *Science* **302**, 842-846.
- Yang, Y., Costa, A., Leonhardt, N., Siegel, R. S., and Schroeder, J. I.** (2008). Isolation of a strong Arabidopsis guard cell promoter and its potential as a research tool. *Plant Methods* **4**, 6.