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Additional Information

PYL8 plays an important role for regulation of abscisic acid signaling in root

Antoni, R.², Gonzalez-Guzman, M.², Rodriguez, L., Peirats-Llobet, M., Pizzio, G.A., Fernandez, M.A., De Winne N., De Jaeger G., Dietrich, D., Bennett M.J., Rodriguez P.L.*

Instituto de Biologia Molecular y Celular de Plantas, Consejo Superior de Investigaciones Cientificas-Universidad Politecnica de Valencia, ES-46022 Valencia, Spain (R.A., M. G.-G., L.R., M.P., G.A.P., M.A.F., P.L.R.)

VIB, Department of Plant Systems Biology and Department of Plant Biotechnology and Bioinformatics, Ghent University, B-9052 Ghent, Belgium (D.E., G.J.)

Centre for Plant Integrative Biology, University of Nottingham, LE12 5RD, UK (D.D., M.J.B.)

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²These authors contributed equally to the article

*Corresponding author; e-mail prodriguez@ibmcp.upv.es

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the Journal policy described in the Instructions for Authors (<http://www.plantphysiol.org>) is: Pedro L. Rodriguez (prodriguez@ibmcp.upv.es)

ABSTRACT

Abscisic acid (ABA) signaling plays a critical role to regulate root growth and root system architecture. ABA-mediated growth promotion and root tropic response under water stress are key responses for plant survival under limiting water conditions. In this work we have explored the role of *Arabidopsis thaliana* PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) ABA receptors for root ABA signaling. As a result, we discovered that PYL8 plays a non-redundant role for regulation of root ABA sensitivity. Unexpectedly, given the multigenic nature and partial functional redundancy observed in the PYR/PYL family, the single *pyl8* mutant showed reduced sensitivity to ABA-mediated root growth inhibition. This effect was due to lack of PYL8-mediated inhibition of several clade A PP2Cs since PYL8 interacted *in vivo* with at least five PP2Cs, namely HAB1, HAB2, ABI1, ABI2 and PP2CA/AHG3, as revealed by tandem affinity purification and mass spectrometry proteomic approaches. We also discovered that PYR/PYL receptors and clade A PP2Cs are crucial for the hydrotropic response that takes place to guide root growth far from regions with low water potential. Thus, an ABA-hypersensitive *pp2c* quadruple mutant showed enhanced hydrotropism, whereas an ABA-insensitive sextuple *pyr/pyl* mutant showed reduced hydrotropic response, indicating that ABA-dependent inhibition of PP2Cs by PYR/PYLS is required for proper perception of a moisture gradient.

Introduction

Control of ABA signaling by PYR/PYL/RCAR ABA-receptors involves direct ABA-dependent inhibition of clade A phosphatases type-2C (PP2Cs), such as ABI1, HAB1, PP2CA, which are key negative regulators of the pathway (Saez et al., 2004, 2006; Rubio et al., 2009). Under resting conditions clade A PP2Cs can interact with and dephosphorylate SnRK2.2, 2.3 and 2.6/OST1, reducing their catalytic activity (Umezawa *et al.*, 2009; Vlad *et al.*, 2009; Fujii et al., 2009). The increase of ABA levels in the plant cell leads to PYR/PYL receptor-mediated inhibition of PP2C activity, which results in the activation of the three SnRK2s, and ultimately of the ABA signaling pathway (Ma *et al.*, 2009; Park *et al.*, 2009; Umezawa *et al.*, 2009; Vlad *et al.*, 2009; Gonzalez-Guzman et al., 2012). Biochemically, SnRK2s are activated through phosphorylation of certain Ser residues of their activation loop, including Ser175, either by autophosphorylation or by yet unidentified upstream activating kinases that are staurosporine-resistant (Boudsocq et al., 2007; Fujii et al., 2009; Umezawa *et al.*, 2009; Vlad *et al.*, 2009, 2010). Next, the SnRK2s directly phosphorylate transcription factors that bind to ABA-responsive promoter elements (ABREs), named ABFs/AREBs for ABRE-binding factors, and components of the machinery regulating stomatal aperture like the slow anion channel SLAC1, K⁺ inward channel KAT1 or ROS-biosynthetic enzymes as the NADPH oxidase AtrbohF (Kobayashi et al., 2005; Fujii *et al.*, 2009; Geiger *et al.*, 2009; Lee *et al.*, 2009; Sato et al., 2009; Sirichandra et al., 2009).

PYR/PYL ABA receptors constitute a 14-member family and all of them (except PYL13) are able to activate ABA-responsive gene expression using protoplast transfection assays (Fujii *et al.*, 2009). However, gene expression patterns obtained from public databases and GUS-reporter gene

analyses have revealed substantial differences among them (Gonzalez-Guzman et al., 2012). Thus, expression of PYL3 and PYL10-13 is very low to undetectable in different whole-genome microarrays, whereas expression of PYR1 and the rest of PYL1-9 could be detected in both vegetative and reproductive tissues, although at different levels (Gonzalez-Guzman et al., 2012). 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 y PYL2), whereas others are monomeric (for instance PYL5, PYL6, PYL8) (Dupeux et al., 2011). The dimeric receptors show a higher K_d for ABA (>50 μM, lower affinity) than monomeric ones (~1 μM); however, in the presence of the PP2C, 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) and physiological characterization of some ABA responses in different multiple *pyr/pyl* mutants did not reveal a clear difference between dimeric and monomeric receptors (Gonzalez-Guzman et al., 2012). Finally, both the biochemical properties of the PYR/PYL receptors and in silico modeling of the ABA activation pathway reveal adequate coverage of the full spectrum of physiological ABA concentrations, ranging from basal ABA levels (nM range) to high levels induced by water stress (μM range) (Priest et al., 2006).

Gene expression patterns, biochemical analysis of different PP2C-PYL receptor complexes and genetic analysis of different *pyr/pyl* mutants suggest the function of PYR/PYL proteins is not completely redundant (Santiago et al., 2009; Szostkiewicz et al., 2010; Antoni et al., 2012; Gonzalez-Guzman et al., 2012). However, some functional redundancy exists since the generation of a *pyr1pyl1pyl2pyl4* quadruple mutant, 1124, was required to obtain robust ABA-insensitive phenotypes (Park et al.,

2009), and a *pyr1pyl1pyl2pyl4pyl5pyl8* sextuple mutant, 112458, is at least one order of magnitude more ABA-insensitive than 1124 (Gonzalez-Guzman et al., 2012). Recently, analysis of mutants lacking three, four, five or six PYR/PYLs has revealed quantitative regulation of ABA signaling by this family of receptors (Gonzalez-Guzman et al., 2012). Finally, GUS-reporter analyses of *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* promoters has showed both overlapping and differential expression in different tissues (Gonzalez-Guzman et al., 2012). For instance, in 5-d-old seedlings, only *PYR1* and *PYL5* were expressed in the cortex of the upper part of the root, whereas *PYL1*, *PYL4* and *PYL8* were expressed in the columella cells. On the other side, overlapping expression of *PYR1*, *PYL1*, *PYL2*, *PYL4* and *PYL8* in root vascular tissue was observed (Gonzalez-Guzman et al., 2012)

ABA regulates root growth and root architecture, likely interacting with other hormones in these processes, such as auxins, gibberellins or brassinosteroids (Swarup et al., 2005; Deak and Malamy 2005; Rodrigues et al., 2009; Peret et al. 2009; Ubeda-Tomas et al., 2009 and 2012; Hacham et al., 2011). ABA signaling in the root is required for different processes, such as maintenance of primary root elongation and repression of lateral root formation when water availability is reduced (Sharp et al., 2004; Deak and Malamy 2005). Recent results in 17 natural accessions of *Arabidopsis* reveal increased root versus shoot biomass partitioning as a crucial plant response to cope with water stress (Des Marais et al., 2012). Several mechanisms dependent on ABA signaling have been proposed to maintain root elongation at low water potentials, such as osmotic adjustment in the root tip, restriction of ethylene production and control of K⁺ translocation from root to shoot (Gaymard et al., 1998; Sharp et al., 2004). Enhanced cell wall loosening is required to maintain root elongation at low water potential, and indeed ABA induces xyloglucan endotransglycosylase,

which is a cell wall degrading enzyme (Wu et al., 1994). Thus, the role of ABA in maintaining root growth under water deficits has been well established (Sharp et al., 2004); however, high concentrations of ABA inhibit root growth. Another important function of ABA is the regulation of the hydrotropic response, i.e. a genuine response of roots to a moisture gradient. Results from Takahashi et al., (2002) indicate that ABA constitutes an intrinsic signal in hydrotropism since both *aba1-1* and *abi2-1* mutants were less sensitive to hydrotropic stimulation, whereas addition of ABA to *aba1-1* restored its capacity to perceive the moisture gradient. Additionally, the no hydrotropic response (*nhr1*) mutant of *Arabidopsis* showed reduced ABA sensitivity in root (Eapen et al., 2003) and ABA induces the expression of MIZ1, a gene essential for hydrotropism (Kobayashi et al., 2007). Regulation of root growth by ABA must be closely connected with hydrotropism since the hydrotropic response likely involves asymmetric transmission of ABA signaling to the root sides that are in contact with different water potentials.

To further understand ABA perception and signaling in the root, we have analyzed root ABA sensitivity of 9 *pyr/pyl* mutants, i.e. *pyr1*, *pyl1*, *pyl2*, *pyl4*, *pyl5*, *pyl6*, *pyl7*, *pyl8* and *pyl9*, as well as different combinations among them. Unexpectedly, given the partial functional redundancy among PYR/PYL genes, we found that *pyl8* single knockout showed reduced sensitivity to ABA-mediated inhibition of root growth. Combination of different *pyr/pyl* mutations enhanced this phenotype. This genetic evidence together with root expression analyses served to pinpoint relevant ABA receptors in the root, particularly PYL8. Moreover, using tandem affinity purification (TAP) technology and mass-spectrometrical (MS) analyses, we were able to identify clade A PP2Cs that interacted *in vivo* with PYL8, providing new *in vivo* evidence for the mechanism of action of PYL8 to

regulate ABA signaling. Finally, we found that PYR/PYLs and clade A PP2Cs play an important role for the ABA-mediated root hydrotropic response.

Results

Regulation of ABA signaling in root by PYR/PYL receptors

To further understand ABA perception in the root, we have analyzed root ABA sensitivity of different *pyr/pyl* single mutants, namely *pyr1-1*, *pyl1*, *pyl2*, *pyl4*, *pyl5*, *pyl6*, *pyl7*, *pyl8-1* and *pyl9* (Figure 1A; Supplemental Figure S1). All the mutants were generated in Col background, with the exception of *pyl2*, which is in *La-er* background (Park et al., 2009). We found that *pyl8-1* showed reduced inhibition of root growth compared to wt, whereas the rest of single mutants did not show significant differences to wt in that response (Figure 1A). ABA-mediated inhibition of seedling establishment in *pyr/pyl* single mutants was quite similar to wt, although slight differences could be noticed in some mutants (Figure 1B). Root growth of *pyl8-1* was even resistant to 20 μ M ABA compared to wt, whereas the other single mutants did not show significant differences ($P < 0.01$) compared to wt at 10 and 20 μ M ABA (Figure 1A). To further verify that the phenotype observed in *pyl8-1* was a consequence of impaired *PYL8* expression, we analyzed the phenotype of a second *pyl8* allele, *pyl8-2*, and we found it also showed a reduced sensitivity to ABA-mediated inhibition of root growth (Figure 1, A, B and C). Finally, the phenotype of *pyl8-1* was complemented by introduction of a *35S:PYL8* transgene (Figure 1D).

During the course of these experiments, we analyzed *HAB1*-overexpressing lines (*HAB1* OE), where additional copies of *HAB1* have been introduced under control of its own promoter, *ProHAB1:HAB1-dHA*,

and we found they showed reduced sensitivity to ABA-mediated inhibition of root growth, which mimicked the *pyl8* phenotype (Saez et al., 2004; Figure 1, A and C). Interestingly, these lines showed lower root growth than wt in medium lacking exogenous ABA and ABA supplementation improved root growth of HAB1 OE plants between 20-30% compared to growth in medium lacking ABA (Figure 1, A and C). Recently, improved root growth by ABA supplementation has also been reported for strong ABA insensitive mutants lacking five or six PYR/PYL receptors (Gonzalez-Guzman et al., 2012).

GUS-reporter analysis of *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* promoters has been recently reported (Gonzalez-Guzman et al., 2012). We have completed this analysis by generating additional GUS-reporter lines for *PYL6*, *PYL7* and *PYL9* promoters (Supplemental Figure S2). Root expression of GUS driven by *PYL6* promoter was almost undetectable, expression driven by *PYL7* promoter was weak and could only be detected after 6h incubation with the GUS substrate, whereas *ProPYL9:GUS* lines showed GUS staining after 3h (Supplemental Figure S2). Additionally, we have used a modified pseudo-Schiff propidium iodide (PS-PI) staining method to get a detailed GUS staining of the apical root (Figure 2A). After 3 h incubation with the GUS substrate, we could detect GUS staining in stele cells of the *ProPYR1*, *ProPYL1*, *ProPYL2*, *ProPYL4*, *ProPYL8* and *ProPYL9:GUS* lines, as well as root epidermis and lateral root cap for *PYL8* (Figure 2A and 2D). PS-PI staining combined with confocal laser scanning microscopy produced high-resolution images; however it eliminated GUS staining of columella cells in *ProPYL1*, *ProPYL4* and *ProPYL8:GUS* lines, which was previously detected (Gonzalez-Guzman et al., 2012; Figure 2E). In order to get an estimation of GUS expression in the whole root, we performed a quantitative GUS activity assay in extracts

of root tissue prepared from 15-d-old seedlings by using 4-methyl umbelliferyl b-D-glucuronide (MUG) as a substrate (Figure 2B). GUS activity was particularly high for *ProPYL8*, *ProPYL1*, *ProPYR1*, *ProPYL9* and *ProPYL2:GUS* genes, whereas expression of *ProPYL4*, *ProPYL5* and *ProPYL7:GUS* genes was lower and *ProPYL6:GUS* was almost undetectable (Figure 2B). These results were in good agreement with immunoblot analysis of the corresponding protein extracts using anti-GUS antibody (Figure 2C) and they provide a quantitative estimation on the expression of the different PYR/PYL receptors in root.

Since different PYR/PYL receptors are expressed at high levels in the root, we performed root growth assays of different triple, quadruple, pentuple and sextuple mutants (Figure 3). To this end, we transferred 4-d-old seedlings to MS medium plates lacking or supplemented with 10, 20 or 50 μ M ABA. Some mutants were more resistant to ABA-mediated root-growth inhibition than *pyl8* and combination of *pyl8* with other mutant loci enhanced the *pyl8* phenotype, particularly evident at 50 μ M ABA when compared to pentuple and sextuple mutants (Figure 3). Triple mutants lacking the *pyl8* locus such as 114 or 145, also showed an ABA-insensitive phenotype, which indicates that in addition to PYL8, other loci contribute to root ABA sensitivity and additional *pyr/pyl* combinations will be required for a more comprehensive analysis. In spite of this limitation, in different combined mutants we observed additive effects by progressive inactivation of *PYR/PYL* genes (Figure 3). For instance, the ABA insensitive phenotype was clearly increased when pentuple and sextuple mutants were compared to *pyl8* in 50 μ M ABA, indicating a quantitative regulation of root ABA sensitivity by *PYR/PYL* genes (Figure 3). Finally, as previously described for 12458 and 112458 mutants (Gonzalez-Guzman et al., 2012), ABA supplementation (10-20 μ M) slightly improved root

growth of 1458 and 11458 mutants compared to growth in the absence of supplemented ABA (Figure 3).

PYL8 regulates root ABA signaling through interaction with clade A PP2Cs

Given the important role of PYL8 to control root sensitivity to ABA, we decided to further pursue the study of its mechanism of action. To this end, we followed both a genetic and a biochemical approach. Firstly, since PYR/PYL ABA receptors inhibit PP2Cs in an ABA-dependent manner and PYL8 inhibited *in vitro* several PP2Cs (Santiago et al., 2009, Antoni et al., 2012), we reasoned that *pyl8* phenotype might be due to enhanced *in vivo* activity of PP2Cs. To test this hypothesis, we crossed *pyl8-1* with the *hab1-1abi1-2pp2ca-1* triple mutant to generate different combinations of *pyl8-1* with *pp2c* mutants. The reduced sensitivity of *pyl8* to ABA-mediated inhibition of root growth was notably diminished when *pyl8* was combined either with *abi1-2*, *hab1-1* or *pp2ca-1* (Figure 4A). Therefore, these results are in agreement with the *in vitro* inhibition of clade A PP2Cs by PYL8 and reveal genetic interaction of PYL8 with different clade A PP2Cs.

Secondly, we followed a biochemical approach to identify PYL8 interacting proteins *in vivo* using *Arabidopsis* suspension cells that express a protein G-streptavidin (GS) tagged PYL8 as a bait for tandem affinity purification (TAP) (Van Leene et al., 2008). The GS tag combines two IgG-binding domains of protein G with a streptavidin-binding peptide, separated by two tobacco etch virus (TEV) cleavage sites, and it has proved to be a good tag for TAP approaches (Van Leene et al., 2008). Previously, PYL8 and other PYR/PYL ABA-receptors have been recovered *in vivo* as ABI1-interacting proteins using affinity protein complex purifications and subsequent identification by MS analyses (Nishimura et al., 2010). Therefore, it has been demonstrated that a single PP2C interacts *in vivo*

with different ABA receptors. We wondered whether a single ABA-receptor might be able to interact *in vivo* with different PP2Cs. To this end, we performed TAP of protein complexes in *Arabidopsis* suspension cells that express PYL8 fused to the N- or C-terminal GS tag (GS-PYL8 and PYL8-GS, respectively) as bait, and subsequently, we proceeded to the identification of interacting partners by MS analyses (Supplemental Table S1). Recombinant GS-PYL8 or PYL8-GS protein production was confirmed by immunoblot analysis (Figure 4B). PYL8-bound complexes were recovered from both cultures (two technical repeats per culture) treated with 50 μ M ABA and finally they were eluted from streptavidin beads and analyzed by SDS-PAGE (Figure 4B). MALDI-TOF/TOF-MS analysis of these complexes revealed that five clade A PP2Cs, i.e. HAB1, HAB2, ABI1, ABI2 and PP2CA/AHG3, were able to interact with PYL8 in samples supplemented with 50 μ M ABA (Figure 4B and 4C; Supplemental Table S1). Therefore, these results provide evidence that a PYR/PYL ABA receptor can form complexes with several clade A PP2Cs *in vivo* in *Arabidopsis*.

We did not recover any ABA-activated SnRK2 in these complexes, which suggests that the ABA-dependent interaction of PYL8 with the PP2Cs effectively competed *in planta* with the interaction of PP2Cs and SnRK2s or alternatively, that the residual interaction of the SnRK2s (via the ABA box) and PP2Cs was not enough to withstand the double purification of the TAP procedure. When samples were not supplemented with exogenous ABA, only one peptide that significantly matched both to HAB1 and HAB2 (VIQWQGAR, identical sequence in both PP2Cs) could be recovered from PYL8-interacting proteins in one experiment (Figure 4C; Supplemental Table S1). Thus, exogenous ABA supplementation

dramatically increased the recovery of PP2Cs as PYL8-interacting proteins in TAP complexes.

PYR/PYL receptors and clade A PP2Cs mediate the root hydrotropic response

PYR/PYL receptors and clade A PP2Cs are key players for ABA signaling in root, and taking into account the important role of ABA for hydrotropism, we decided to investigate their role in the root hydrotropic response. Since PYL8 plays an important role for ABA signaling in root and it interacts at least with 5 clade A PP2Cs, we generated an *abi1-2 abi2-2 hab1-1 pp2ca-1* quadruple mutant, abbreviated as *Qabi2-2* (Figure 5A and 5B). The *Qabi2-2* mutant is impaired in four PYL8-interacting PP2Cs and it turned out to be very hypersensitive to ABA-mediated inhibition of root and shoot growth (Figure 5A and 5B). Using the experimental system developed by Takahashi et al., (2002), i.e. split agar plates containing sorbitol in the region with low water potential, we measured the hydrotropic response of mutants showing enhanced or impaired ABA signaling (Figure 5C and 5D). In this assay, MS medium containing 1% agar and agar containing 400 mM sorbitol are placed side-by-side, which generates a water potential gradient at the border between the two media (see Figure 5E and 5F). Thus, we analysed the hydrotropic response of the strongly ABA-hypersensitive *Qabi2-2* mutant and the ABA-insensitive 112458 sextuple mutant, which is strongly impaired in ABA perception through PYR/PYL receptors (Gonzalez-Guzman et al., 2012). As a result, we found that *Qabi2-2* mutant showed enhanced root curvature compared to wt when faced with a medium containing -1 MPa sorbitol (Figure 5C). Conversely, the 112458 mutant showed reduced root curvature compared to wt (Figure 5D). This response had important consequences, since seedlings of the *Qabi2-2* mutant avoided better than wt the entrance in

medium with low water potential, whereas seedlings of 112458 mutant were impaired in that response (Figure 5E and 5F).

DISCUSSION

Previous analyses of loss-of-function mutants indicated that combination of several *pyr/pyl* loci was required to impair ABA signaling (Park et al., 2009; Gonzalez-Guzman et al., 2012). Thus, the generation of *pyr1pyl1pyl4* triple or *pyr1pyl1pyl2pyl4* quadruple mutants (Park et al., 2009) or different combinations of triple mutants (Gonzalez-Guzman et al., 2012) was at least required to obtain a robust ABA-insensitive phenotype, which suggested certain functional redundancy among *PYR/PYL* genes. Only the *pyr1* mutant was reported to show a phenotype by inactivation of a single *PYR/PYL* gene, i.e. pyrabactin resistance (Park et al., 2009). Given these precedents, we were surprised to find that the single *pyl8* mutant showed reduced ABA-mediated inhibition of root growth compared to wt.

Several reasons might explain the non-redundant role of *PYL8* to regulate root sensitivity to ABA. First, the root expression pattern of *PYL8* shows some specificity with respect to other *PYR/PYL* receptors (discussed below). Second, biochemically *PYL8* is a monomeric receptor (higher affinity for ABA than dimeric receptors) that shows higher capacity (lower inhibitory concentration 50) to inhibit *in vitro* certain PP2Cs (*ABI1*, *PP2CA*, *HAI1*) than other *PYR/PYLs* (Santiago et al., 2009; Antoni et al., 2012). For instance, the ABA-dependent inhibition of *PP2CA* and *HAI1* by *PYL8* was almost one order of magnitude higher than by other *PYR/PYLs* (Antoni et al., 2012). Third, we have shown that at least five clade A PP2Cs interacted *in vivo* with *PYL8* in an ABA-dependent manner. We can't exclude that other clade A PP2Cs that are strongly induced by ABA (*HAI1*, *HAI2*, *HAI3*) but otherwise expressed at low levels could also interact with *PYL8*. The *Arabidopsis* suspension cells used in TAP

experiments were supplemented with ABA to prepare protein extracts but they were not grown in the presence of ABA to avoid growth impairment and overexpression of the other PP2Cs. Therefore, lack of PYL8 function likely leads to a globally enhanced activity of PP2Cs or diminished capacity to inhibit them. Indeed, both the analysis of root ABA sensitivity of *pyl8 pp2c* mutants (Figure 4A) and HAB1 OE plants (Figure 1, A and C) support this idea. Thus, root ABA sensitivity of HAB1 OE plants mimicked *pyl8* or *pyr/pyl* combined mutants, which suggests that these phenotypes are due to enhanced clade A PP2C activity. Interestingly, HAB1 OE plants as well as severe ABA-insensitive *pyr/pyl* mutants (1458, 11458, 12458 and 112458) showed improved root growth upon supplementation of the growth media with ABA (Figure 1 and 3; Gonzalez-Guzman et al., 2012). Therefore, although ABA has an inhibitory role on root growth depending on the concentration and plant growth conditions, it is clear that ABA contributes to root growth stimulation under certain conditions. Low concentrations of ABA (<1 μ M) are known to stimulate root growth in *Arabidopsis* wt (Ephritikhine et al., 1999; Fuji et al., 2007). ABA is also required to maintain primary root growth at low water potentials in maize seedlings, and one of the factors that explain it is the restriction of ethylene production by ABA (reviewed by Sharp et al., 2004). Finally, mutants whose threshold for ABA perception and signaling has been dramatically altered can reveal the ABA stimulatory effect on root growth at concentrations that would be inhibitory for wt. Different physiological mechanisms have been pointed out to explain this effect at low water potentials (Sharp et al., 2004). The interaction of ABA with other hormones that regulate root growth, although not well known yet, will be another possible explanation.

Our TAP experiments, together with those of Nishimura et al., (2010) using YFP-ABI1 as a bait, provide evidence that multiple interactions among PP2Cs and PYR/PYLs occur *in vivo*, generating a regulatory network that offers a wide sensitivity and combinatorial possibilities to modulate ABA signaling. Mathematical modeling will be required to provide quantitative insights on the complex combinatorial of the PP2C-ABA-PYL interactions. However, our results differed from Nishimura et al., (2010) because we did not recover any ABA-activated SnRK2 in our complexes. Although *in vitro* experiments have suggested the possible existence of quaternary PYL-ABA-PP2C-SnRK2 complexes (Soon et al., 2012), the actual fact is that such complexes have not been recovered from plant extracts yet. The ABI1-interacting proteins identified by MS analyses by Nishimura et al., (2010) might simply reflect the recovery of independent PYL-ABA-ABI1 and ABI1-SnRK2 complexes. Alternatively, TAP is a more stringent technique than single GFP affinity purification, which might result in loss of the SnRK2 ABA box-PP2C interaction or weaker interactions than ternary ABA complexes. This could explain another divergence with respect to Nishimura et al., (2010), i.e. the dramatic increase in the recovery of PP2C peptides by exogenous addition of ABA. Thus, without ABA supplementation, GS-PYL8 only recovered one PP2C peptide in one experiment (none with PYL8 tagged at the C-terminus), compared to 28 peptides when 50 μ M ABA was supplemented (Figure 4C; Supplemental Table S1). These results suggest that *in vivo* PYL8 only shows a weak/transient interaction with clade A PP2Cs when ABA levels are low, further supporting the current model for PYR/PYL-mediated signaling able to perceive changes in ABA levels (Cutler et al., 2010).

The expression in the root of PYR1, PYL1, PYL2, PYL4, PYL8 and PYL9 was predominant in the vascular tissue (Figure 2; Gonzalez-Guzman et al., 2012), where ABA-biosynthetic enzymes are localized too (Cheng et al., 2002; Tan et al., 2003). Active ABA signaling in the root vascular tissue that carries out ABA biosynthesis might act as a positive feedback for ABA production or play a regulatory role for different transport processes (Barrero et al., 2005; Gaymard et al., 1998). Additionally, expression in columella cells could also be detected for PYL1, PYL4 and PYL8. Active pools of ABA have been detected in the columella cells by a *ProRD29B:GUS* reporter system, which suggests that even in the absence of stress ABA signaling occurs in these cells (Christmann et al., 2005). Root columella cells play a key role for sensing gravity in a process governed by auxins, and the presence of ABA receptors in this region suggests that ABA signaling might somehow affect auxin signaling in this area. For instance, it has been proposed that degradation of starch grains in amyloplasts in columella cells is required to have a hydrotropic response, since gravitropism would be inhibitory to hydrotropism (Takahashi et al., 2003). It has been also suggested that starch degradation in the columella cells of roots subjected to osmotic stress might be an osmoregulatory mechanism to increase osmolite concentration and to sustain glucose supply under water stress (Ponce et al., 2008). Since ABA signaling is required both for hydrotropism and osmoregulation of water-stressed roots (Takahashi et al., 2002; Sharp et al., 2004; this work), the presence of PYR/PYL receptors in columella cells might contribute to regulate both processes.

Finally, expression of PYL8 was also documented in the root epidermis and lateral root cap. The localization of the moisture-gradient sensing apparatus has not been precisely defined, likely because different

root tissues might be required for proper hydrotropic perception and response. However, root cap has been suggested to play a role for moisture-gradient perception (Eapen et al., 2003; Kobayashi et al., 2007). The localization of at least three ABA receptors in this area and the presence of PYL8 in root epidermis and lateral root cap fit well with the requirement of ABA signaling for root hydrotropism. Moreover, we provide evidence that *pyr/pyl* mutants are impaired in hydrotropism, indicating that ABA perception by these receptors is required for a proper response. Interestingly, this response can be enhanced by multiple knocking out of the PP2Cs that represses ABA signaling under basal conditions. Thus, the enhanced hydrotropic response of *pp2c* knockouts together with their reduced water loss and enhanced transcriptional response to ABA constitute a powerful mechanism to cope with water stress (Saez et al., 2006; Rubio et al., 2009). Future studies on the role played by ABA signaling for hydrotropism should answer important questions, such as how ABA generates the asymmetric growth required to escape from low water potential regions of the soil or whether ABA gradients are generated in the root in an analogous manner to auxins.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana plants were routinely grown under greenhouse conditions 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 (1962) (MS) plates composed of MS basal salts, 0.1% 2-[N-morpholino]ethanesulfonic acid 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*, *pyl5* and *pyl8-1* have been described previously (Lackman et al., 2011; Park et al., 2009; Gonzalez-Guzman et al., 2012). Seeds of *pyl6*, *pyl7* and *pyl8-2* insertion lines, SAIL_1179_D01, SALK_012096 and SALK_033867, respectively, were obtained from the Nottingham *Arabidopsis* Stock Centre. The *abi1-2 abi2-2 hab1-1 pp2ca-1* quadruple mutant was generated by crossing two triple *pp2c* mutants described in Rubio et al., (2009). The *pyl8-1* allele was crossed with the *abi1-2 hab1-1 pp2ca-1* triple mutant to generate different combinations of *pyl8* and *pp2c* knockout alleles.

Generation of transgenic lines and GUS analyses.

To construct the *ProPYL6* and *ProPYL8:GUS* genes, a fragment comprising 1.5 kb 5' upstream of the ATG start codon plus the first 30 bp of the PYL6 or PYL8 coding sequences, respectively, 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 the *ProPYL7:GUS* gene, the upstream sequence amplified was of 0.5 kb to avoid overlapping with regulatory sequences of the At4g01023 neighboring gene. 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 and GUS quantitative assays were performed as described by Jefferson et al., (1987). Root GUS staining was also visualized using modified pseudo-Schiff propidium iodide staining and confocal laser scanning microscopy as previously described (Truernit et al., 2008)

The coding sequence of PYL8 was amplified by PCR and cloned into pCR8/GW/TOPO (Santiago et al., 2009a). Next, it was recombined by LR reaction into the ALLIGATOR2 destination vector (Bensmihen *et al.*, 2004). The ALLIGATOR2-35S:3HA-PYL8 construct was transferred to *Agrobacterium tumefaciens C58C1* (pGV2260) (Deblaere *et al.*, 1985) by electroporation and used to transform *pyl8-1* plants by the floral dip method. T1 transgenic seeds were selected based on GFP

visualization and T3 progenies homozygous for the selection marker were used for further studies. The generation of *ProHAB1:HAB1-dHA* lines was described previously (Saez *et al.*, 2004).

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 lacking or supplemented with 0.5 μ M ABA. 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.

Root 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.

Tandem Affinity Purification (TAP)

Cloning of transgenes encoding GS-tagged fusions under control of the constitutive cauliflower tobacco mosaic virus 35S promoter and transformation of *Arabidopsis* cell suspension cultures were carried out as previously described (Van Leene *et al.*, 2007). Briefly, the GS tag combines two IgG-binding domains of protein G with a streptavidin-binding peptide, separated by two tobacco etch virus (TEV) cleavage sites. In GS-PYL8, the two IgG-binding domains of protein G are placed in front of the streptavidin-binding peptide; in PYL8-GS, the order of these domains is opposite. Tandem affinity purification of protein complexes was done using the GS tag (Bürckstümmer *et al.*, 2006) followed by protein precipitation and separation, according to Van Leene *et al.* (2008). For the protocols of proteolysis and peptide isolation, acquisition of mass spectra by a 4800 MALDI TOF/TOF Proteomics Analyzer (AB SCIEX), and MS-based protein homology identification based on the TAIR genomic database, we refer to Van Leene *et al.* (2010). Experimental background proteins were subtracted based on approximately 40 TAP experiments on wild type cultures and cultures expressing TAP-tagged mock proteins GUS, RFP and GFP (Van Leene *et al.*, 2010).

Hydrotropism assay

The hydrotropic response was analyzed in 7-d-old *Arabidopsis* seedlings as described by Takahashi et al., (2002). Briefly, plastic square plates were filled with 1% agar containing MS medium. After the solidification of the agar, half of the medium was removed by cutting with a scalpel in an angle of 36° and replaced with 1% agar containing MS medium supplemented with 400 mM sorbitol. Root tips were placed in the border between these two media, where a water potential gradient was generated, and plates were positioned vertically. After 14 hours the hydrotropic response was calculated by measuring root curvature angle.

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.

Supplemental material

The following supplemental material is available for this article online:

Supplemental Figure S1. Schematic diagram of the *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5*, *PYL6*, *PYL7*, *PYL8* and *PYL9* genes showing the position of the T-DNA insertion or the nonsense mutation in the *pyr1-1* allele.

Supplemental Figure S2. GUS expression driven by ProPYL6, ProPYL7 and ProPYL9:GUS genes in roots of 5-d-old seedlings

Supplemental Table S1. Summary of results obtained in TAP experiments and peptide identification using the 4800 MALDI TOF/TOFTM Proteomics analyzer (AB SCIEX) and the GPS explorer v3.6 (AB SCIEX) software package.

Supplemental Table S2. List of oligonucleotides used in this work

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We thank J. Ecker and the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed *Arabidopsis* T-DNA insertion mutants, and the SYNGENTA and SAIL *Arabidopsis* Biological Resource Center/ Nottingham *Arabidopsis* Stock Centre for distributing these seeds. We thank Ebe Merilo for help with statistical analysis.

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Figure legends

Figure 1. PYL8 plays a non-redundant role for root sensitivity to ABA. A, Quantification of ABA-mediated root growth inhibition of *pyr/pyl* mutants compared with the wild type. Data are averages \pm SE from three independent experiments (n = 15 each). * P < 0.01 (Student's t test) with respect to the wild type in the same experimental condition. B, Seedling establishment of *pyr/pyl* mutants compared with Columbia (Col) and *Lar* wild types in medium supplemented with 0.5 μ M ABA at 3, 5, 7 and 10 days after sowing. Data show the percentage of seeds that germinated and developed green cotyledons. Values are averages \pm SE for three independent experiments (100 seeds each). * P<0.01 (Student's t test) with respect to the wild type in the same experimental condition. C, ABA insensitive phenotype of *pyl8-1* and *pyl8-2* alleles compared to Col wt. Photographs show representative seedlings 10-d after the transfer of 4-d-old seedlings from MS medium to plates lacking or supplemented with 10 μ M ABA. D, Complementation of the *pyl8-1* allele by introduction of a *35S:3HA-PYL8* transgene (*pyl8-1* complemented). Photograph shows representative seedlings 12-d after the transfer of 4-d-old seedlings from MS medium to plates supplemented with 20 μ M ABA.

Figure 2. GUS expression driven by *ProPYR1*, *ProPYL1*, *ProPYL2*, *ProPYL4*, *ProPYL5*, *ProPYL6*, *ProPYL7*, *ProPYL8* and *ProPYL9:GUS* genes in the apical root. A, GUS expression visualized using modified pseudo-Schiff propidium iodide staining and confocal laser scanning microscopy. B, Quantification of GUS activity in 15-d-old roots using MUG as a substrate. C, Immunoblot analysis of protein extracts from 15-d-old roots using anti-GUS antibody. Ponceau staining from a 43 kDa protein is shown as loading control. D, Magnification of the apical root from *ProPYL8:GUS* lines that were stained as described in A. E, GUS

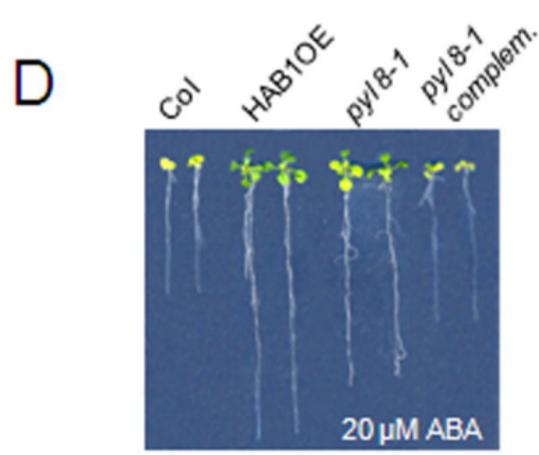
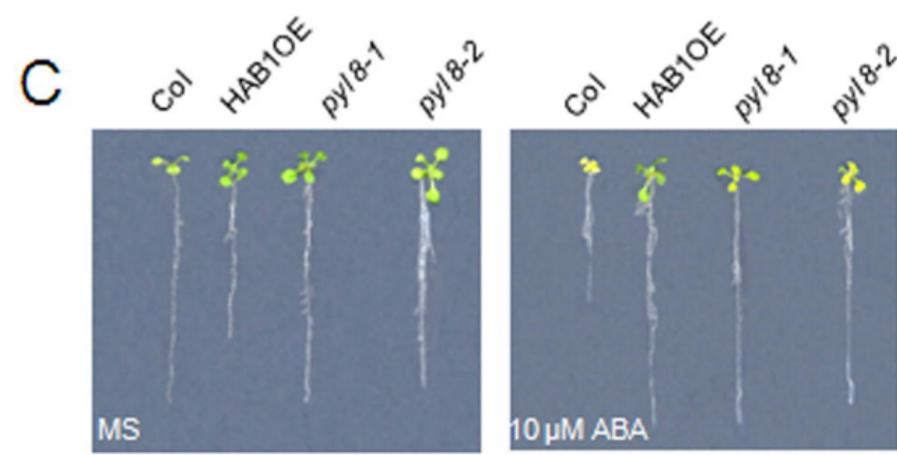
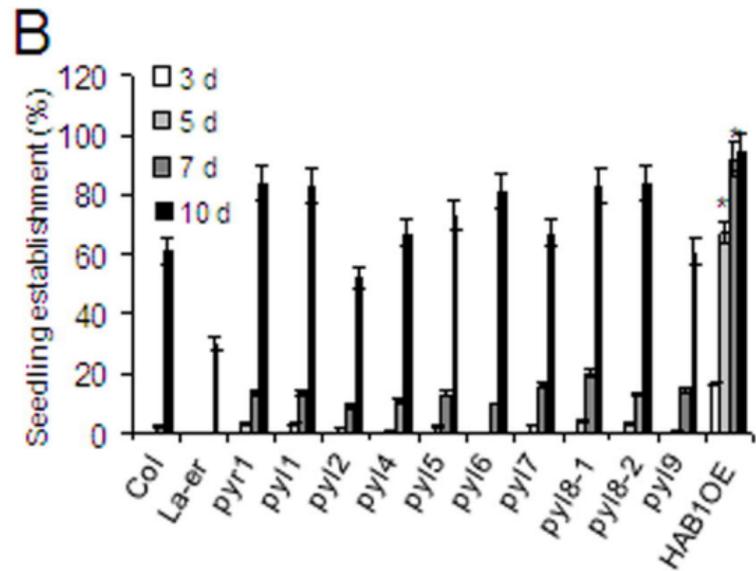
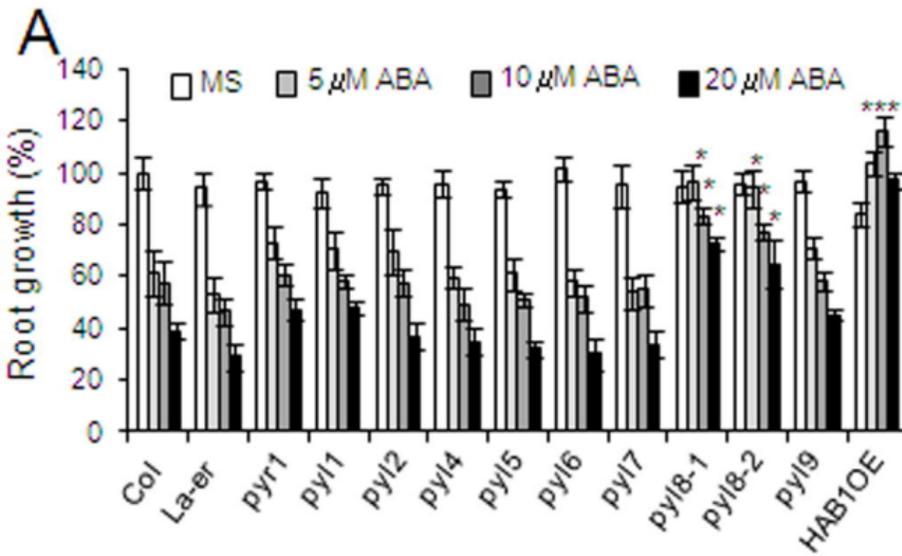
expression driven by *ProPYL1*, *ProPYL4* and *ProPYL8:GUS* genes in columella cells. GUS staining observed in the absence of subsequent PS-PI staining.

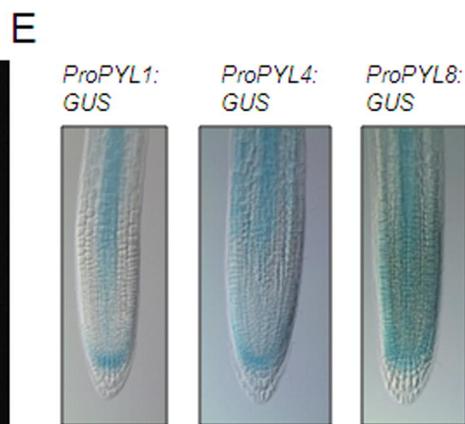
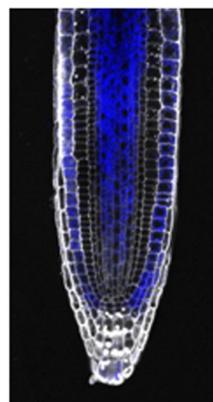
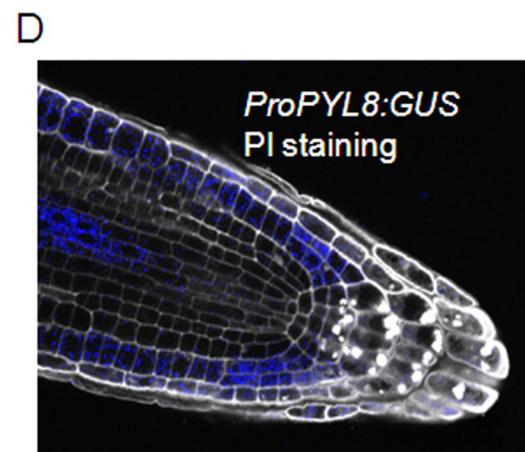
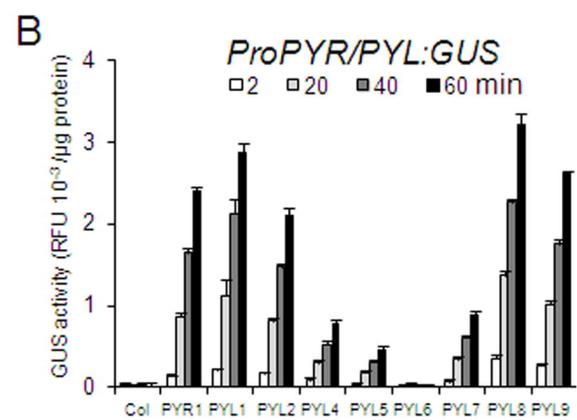
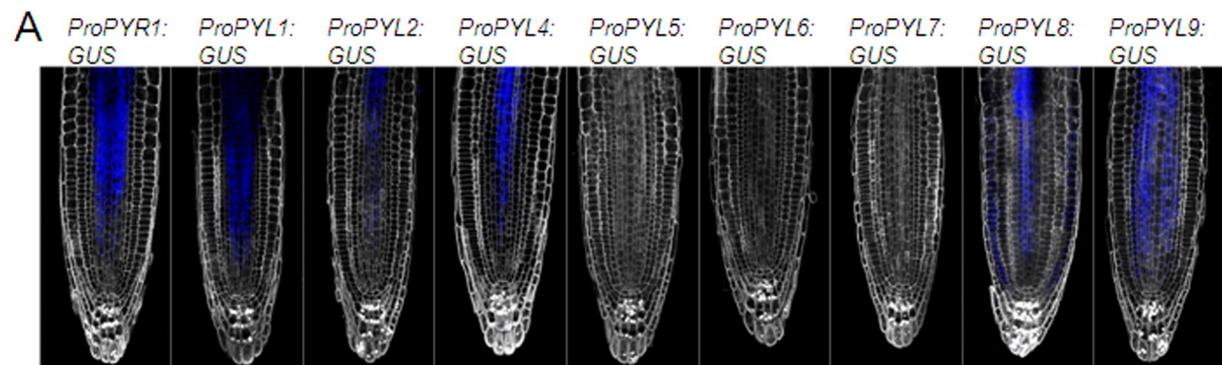
Figure 3. Quantification of ABA-mediated inhibition of root growth of *pyr/pyl* mutants compared with the wild type. Data are averages \pm SE from three independent experiments (n = 15 each). The letters denote significant differences among the different genetic backgrounds (P<0.05, Fisher LSD tests). Primary root length of 15 plants per genotype (three independent experiments) was measured after 8 days in medium lacking or supplemented with 10, 20 or 50 μ M ABA. The 145, 148, 1458 and 12458 mutants contain the *pyr1-1* allele; the 114, 1124, 11458 and 112458 mutants contain both *pyr1-1* and *pyl1* alleles. The rest of abbreviations reflect the corresponding *pyl* number.

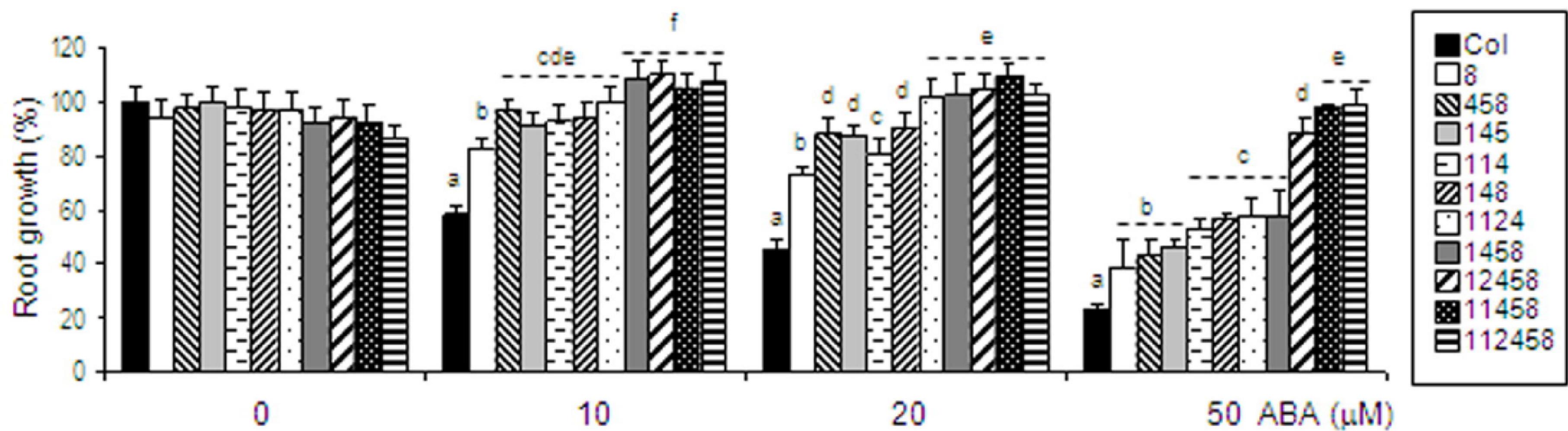
Figure 4. Genetic and biochemical interaction of PYL8 with clade A PP2Cs. A, The reduced sensitivity of *pyl8* to ABA-mediated inhibition of root growth is abrogated by knocking out clade A PP2Cs. Quantification of ABA-mediated root growth inhibition of the indicated genotypes compared with the wild type. Data are averages \pm SE from three independent experiments (n = 15 each). * P < 0.01 (Student's t test) with respect to the wild type in the same growth conditions. Photographs show representative seedlings 10-d after the transfer of 4-d-old seedlings to MS plates lacking or supplemented with 5 μ M ABA. Bars = 1 cm. B, Clade A PP2Cs interact *in vivo* with PYL8 in the presence of ABA. GS-PYL8 and PYL8-GS interact with clade A PP2Cs expressed in *Arabidopsis* cell suspension cultures. The SDS-PAGE analysis shows the zone were HAB1/HAB2 (two upper bands) and ABI1/ABI2/PP2CA (lower bands) were recovered as interacting partners of PYL8 when extracts and TAP purification buffers were supplemented with 50 μ M ABA. C, Quantification of significantly

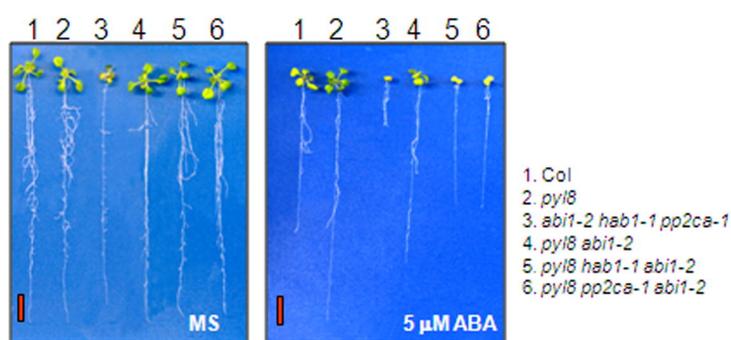
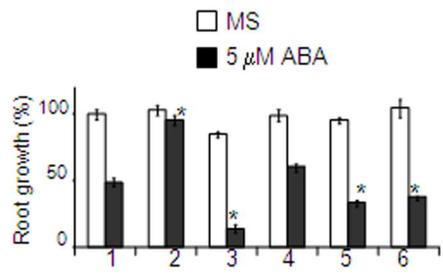
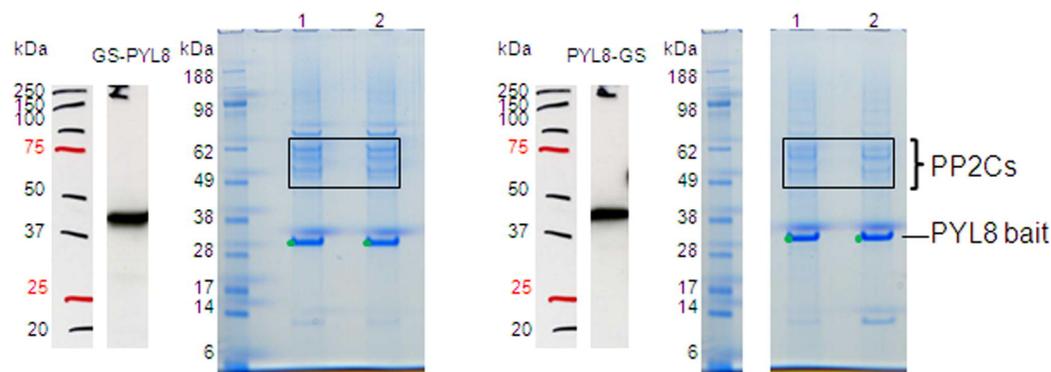
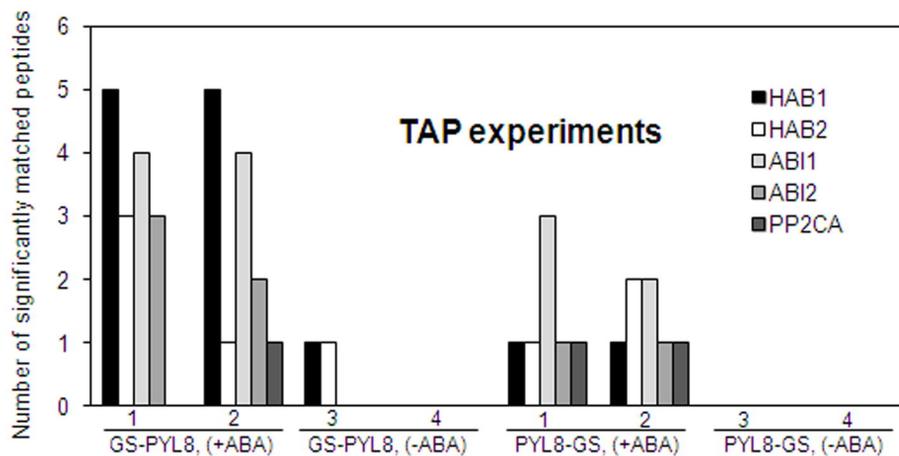
($p > 95\%$) matched peptides of clade A PP2Cs recovered in independent TAP experiments using either GS-PYL8 or PYL8-GS as baits. ABA supplementation (50 μM , +ABA) dramatically increased the recovery of clade A PP2Cs compared to samples lacking ABA supplementation (-ABA). Detailed results of the peptides identified by MS analyses are provided as supplemental table S1.

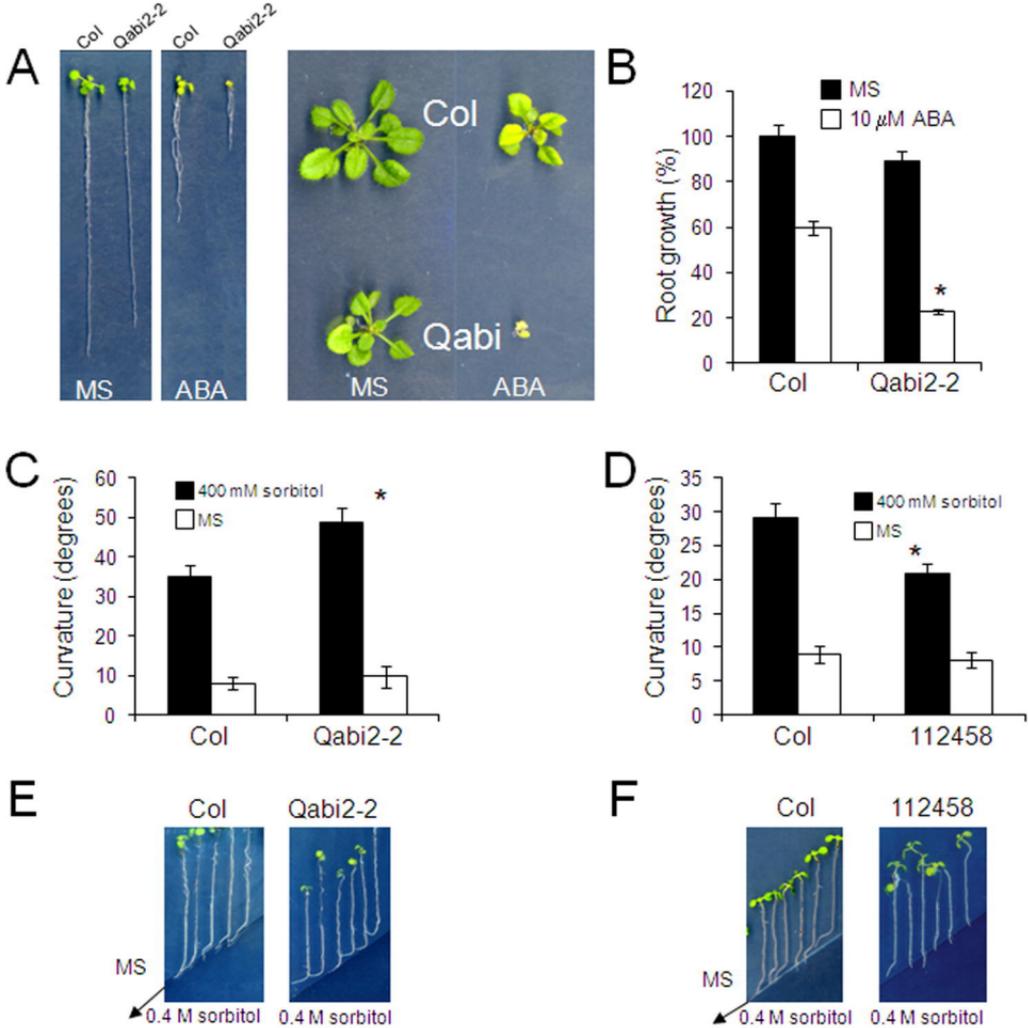
Figure 5. Enhanced hydrotropic response of *pp2c* quadruple mutant and reduced hydrotropic response of *pyr/pyl* sextuple mutant. A, ABA-hypersensitive phenotype of *hab1-labi1-2pp2ca-labi2-2* quadruple mutant, abbreviated as *Qabi2-2*, compared to Col wild-type. Photograph of representative seedlings 10 d (left) or 20 d (right) after the transfer of 4-d-old seedlings to MS plates lacking or supplemented with 10 μM ABA. B, ABA-hypersensitive root growth inhibition of *Qabi2-2* mutant compared to Col wild-type. C, Enhanced hydrotropic response of *Qabi2-2* mutant compared to wt. D, Reduced hydrotropic response of *pyr/pyl* sextuple mutant compared to wt. C, D, Hydrotropism assays with 7-d-old *Arabidopsis* seedlings. Data represent measures of the root curvature angle taken 14 h after transfer of 7-d-old seedlings to split agar plates containing 0.4 M sorbitol in the region with low water potential. Values are averages from three independent experiments \pm SE ($n = 42$ each). * indicates $P < 0.05$ (Student's *t* test) when comparing data from each genotype and wild type in the same assay conditions. E, F, Photographs show the experiments described in C and D, respectively, after 3 days of the transfer of 7-d-old seedlings to split agar plates containing 0.4 M sorbitol. The arrow marks the limit between MS medium and medium supplemented with 0.4 M sorbitol.

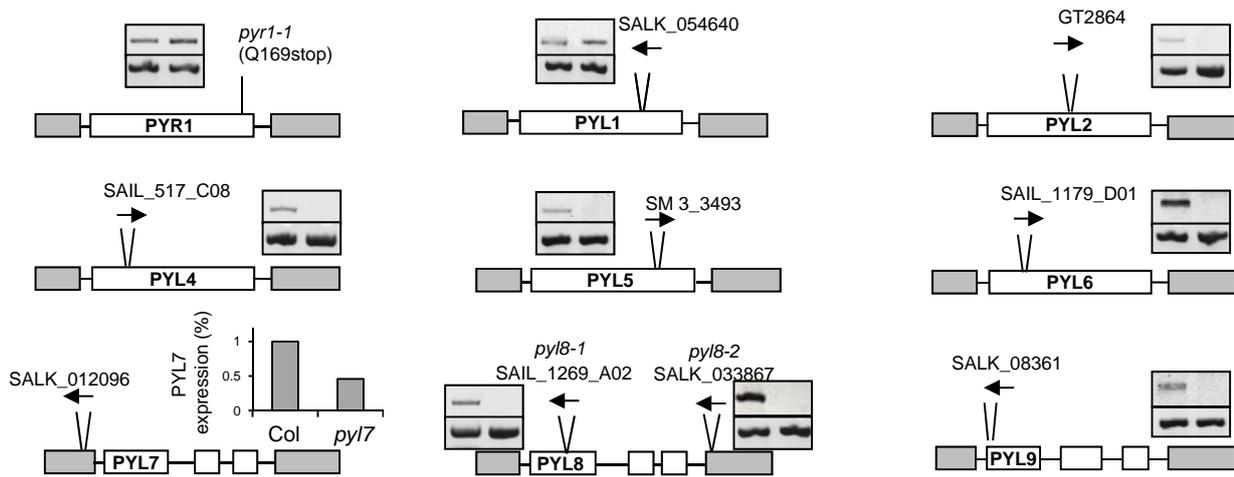






A**B****C**





Supplemental Figure S1. Schematic diagram of the *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5*, *PYL6*, *PYL7*, *PYL8* and *PYL9* genes showing the position of the T-DNA insertion in different *pyl* mutants or the nonsense mutation in the *pyr1-1* allele. RT-PCR analyses of mRNAs from wt and the corresponding *pyr/pyl* mutant are shown over each gene (wt, left; mutant, right); the upper box corresponds to the analysis of each *PYR/PYL* gene and the lower box to the *ACTIN* control of cDNA. In the case of *pyl7*, where the T-DNA insertion into the *PYL7* promoter leads to a knock-down mutation, quantitative RT-PCR analysis is shown.

Supplemental Figure S2. GUS expression driven by *ProPYL6*, *ProPYL7* and *ProPYL9:GUS* genes in roots of 5-d-old seedlings. Photographs show 5-d-old seedlings that were either mock or 10 μ M ABA-treated for 10 h.

ProPYL6:GUS

ProPYL7:GUS

ProPYL9:GUS



Supplemental Table S1. Summary of results obtained in TAP experiments and peptide identification using the 4800 MALDI TOF/TOFTM Proteomics analyzer (AB SCIEX) and the GPS explorer v3.6 (AB SCIEX) software package with search engine Mascot version 2.2 (Matrix Science) and database TAIR10. Four TAP experiments (two samples each) were performed using N-terminal (Nm) GS-tagged PYL8, two in the presence of 50 μ M supplemented ABA and two in the absence of exogenous ABA. Additionally, four TAP experiments were performed using C-terminal (Cm) SG-tagged PYL8, in the same conditions as above. The table shows the significantly ($p > 95\%$) matched peptides recovered in the experiments. Column headers for Protein and Peptide data are explained below. **Protein score:** The score calculated by the Mascot search engine for each protein. This score is based on the probability that peptide mass matches are non-random events. If the Protein Score is equal to or greater than the Mascot@ Significance Level calculated for the database search, the protein match is considered to be statistically non-random at the 95% confidence interval. Protein score = $-10 * \text{Log}(P)$, where P is the probability that the observed match is a random event. **Expect:** Protein score expectation value. **RMS error (ppm):** RMS error of the set of matched mass values, in ppm. **Sequence coverage %:** Percentage of protein sequence covered by assigned peptide matches. **Unique peptides:** The number of peptides with unique sequences matching the selected protein. **Total Ion Score:** A score calculated by weighting Ion Scores for all individual peptides matched to a given protein. **Peptide Number:** Peptide index number within the list of peptides associated with a given protein. **Start:** The starting position of the peptide in the protein. **End:** The ending position of the peptide in the protein. **Observed:** The observed monoisotopic mass of the peptide in the spectrum (m/z). **Mr (Exp):** The experimental mass of the peptide calculated from the observed m/z value. **Mr (Calc):** The theoretical mass of the peptide based on its sequence. **Delta (Da):** The difference between the theoretical (Mr (Calc)) and experimental (Mr (Exp)) masses, in daltons. **Miss:** Number of missed Trypsin cleavage sites. **Ions score:** The Ions Score is calculated by the Mascot search engine for each peptide matched from MS/MS peak lists. This score is based on the probability that ion fragmentation matches are non-random events. If the Ion Score is equal to or greater than the Mascot@ Significance Level calculated for the database search, the peptide match is considered to be statistically non-random at the 95% confidence interval. Ions score = $-10 * \text{Log}(P)$, where P is the probability that the observed match is a random event. **Best Ions score:** The highest individual Ion Score for a given protein identification. **Expect:** Ions score expectation value. **Peptide:** The amino acid sequence of the selected peptide. **Variable Modification:** Variable modification type on the peptide.

Supplemental Table S2. List of oligonucleotides used in this work.

For amplification of the promoters:

FpromPYR1: ACT AAA CTA ATC ATT CTC TAC
RpromPYR1: TCGTTCTTCTGGTGTAACTCCG
FpromPYL1: AGCAATATAATTTTACACTCTGGA
RprmPYL1: GGAGGAGGACTCTGAATTCGCCAT
FpromPYL2: AAACCTATCGAACTGGAATTG
RprmPYL2: TAGGCCTTTTACGGCCGGGGATG
FpromPYL4: TGTGTCCATGTCCGGAGTTGT
RpromPYL4: GGCGGAAGAAGGACGGTGAACG
FpromPYL5: GGTGTGATGGTCAATATTT
RpromPYL5: GCCGTGTTGGAGTTGCACCGGT
FpromPYL8: ACTGTGAAGCAAACCCTATATAT
RpromPYL8: CGTCAAGTTCTCAATCCCGTTAG
FpromPYL6: ATTATGTCACCTAACCCGAAAGC
RpromPYL6: GGATCTCTGAAACTGTATCGACG
FpromPYL7: TCTCATGCTGACATCAGCTAC
RpromPYL7: ATCTGTATCGTCTCCTCCGATC
FpromPYL9: TTTTATCTGATAAGGTTACTGT
RpromPYL9: CGCCGTGCCGCCTTCAACGCCG

For genotyping of the mutants:

FNc4g17870: ACCATGGCTTCGGAGTTAACACCA
R4g17870: TCACGTAC CTGAGAACCACT
FPYL1: ATGGCGAATTCAGAGTCCTCC
RPYL1: TTACCTAACCTGAGAAGAGTT
FwNcoPYL2: ACCATGGGCTCATCCCCGGCCGTGA
RvStopPYL2: TTATTCATCATCATGCATAGGTG
FPYL4: ACCATGGTTGCCGTTACCGTCCTT
RPYL4: TCACAGAGACATCTTCTTCTTGC
FPYL5: ATGAGGTCACCGGTGCAACT
RPYL5: TTATTGCCGGTTGGTACTTCGA
FwMEANpyl8: ATGGAAGCTAACGGGATTGAG
RvESRVpyl8 : TTAGACTCTCGATTCTGTCTG
pROK2: GCCGATTTTCGGAACCACCATC
Spm3: ACCGTCGACTACCTTTTTTCTTGTAGTG
LB3SAIL: TAGCATCTGAATTCATAACCAATCTCGATACAC
DS5: GTTCGAAATCGATCGGGATAAAAC
Fpyl6.1: GCCTCGAGACAGTAGAAGATTG
Rpyl6.1: CGTATGACTCAACGACACGTG
FpromPYL7: TCTCATGCTGACATCAGCTAC
RpromPYL7: ATCTGTATCGTCTCCTCCGATC
Fpyl9.1: GGTTTAAAGGCAACGAGAGC
Rpyl9.1: TCACTGAGTAATGTCCTGAGA
Fp1470PYL9 (to identify T-DNA insertion): CCT GAT ACA TGG AGA ATC ACT CA

TAP constructs

FattBPYL8: AAAAAGCAGGCTCCACCATGGAAGCTAACGGGATTGAG
RPYL8StopattB: AGAAAGCTGGGTCTTAGACTCTCGATTCTGTCTG
RPYL8attB: AGAAAGCTGGGTCTGAGACTCTCGATTCTGTCTG