

# Interactions between soybean ABA receptors and type 2C protein phosphatases

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**Abstract** The plant hormone abscisic acid (ABA) plays important roles in regulating plant growth, development, and responses to environmental stresses. Proteins in the PYR/PYL/RCAR family (hereafter referred to as PYLs) are known as ABA receptors. Since most studies thus far have focused on Arabidopsis PYLs, little is known about PYL homologs in crop plants. We report here the characterization of 21 PYL homologs (GmPYLs) in soybean. Twenty-three putative GmPYLs can be found from soybean genome sequence and categorized into three subgroups. GmPYLs interact with AtABI1 and two GmPP2Cs in diverse manners. A lot of the subgroup I GmPYLs interact with PP2Cs in an

ABA-dependent manner, whereas most of the subgroup II and III GmPYLs bind to PP2Cs in an ABA-independent manner. The subgroup III GmPYL23, which cannot interact with any of the tested PP2Cs, differs from other GmPYLs. The CL2/gate domain is crucial for GmPYLs-PP2Cs interaction, and a mutation in the conserved proline (P109S) abolishes the interaction between GmPYL1 and AtABI1. Furthermore, the ABA dependence of GmPYLs-PP2Cs interactions are partially correlated with two amino acid residues preceding the CL2/gate domain of GmPYLs. We also show that GmPYL1 interacts with AtABI1 in an ABA-dependent manner in plant cells. Three GmPYLs differentially inhibit AtABI1 and GmPP2C1 in an ABA-dependent or -enhanced manner in vitro. In addition, ectopically expressing GmPYL1 partially restores ABA sensitivity of the Arabidopsis triple mutant *pyr1/pyl1/pyl4*. Taken together, our results suggest that soybean GmPYLs are ABA receptors that function by interacting and inhibiting PP2Cs.

Ge Bai, Da-Hai Yang and Yang Zhao have contributed equally to this work.

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GmPP2C · Abiotic stress

## Introduction

The plant hormone abscisic acid (ABA) regulates plant growth and development, including stomatal movements, seed and bud dormancy, seed germination, vegetative development, and fruit ripening (Cutler et al. 2010; Jia et al. 2011; Leung et al. 1994; Lumba et al. 2010; Nambara and Marion-Poll 2005). ABA also plays crucial roles in plant resistance to stresses such as drought, salinity, and pathogen infection (Chinnusamy et al. 2004; Lee and Luan 2012; Qin et al. 2011; Ton et al. 2009; Zhu 2002). Moreover, emerging evidence suggests that crosstalk and

interactions exist between components of ABA and other phytohormone signaling networks to ensure proper plant responses to developmental and environmental cues (Chinnusamy et al. 2004; Cutler et al. 2010; Himmelbach et al. 2003; Lee and Luan 2012).

Given the important physiological functions of ABA in plants, ABA signaling pathway has been intensively studied after the early chemical and biological discoveries of ABA. Genetic and biochemical approaches identified ABA Insensitive 1 (ABI1) and ABA Insensitive 2 (ABI2), two clade A protein phosphatase 2C (PP2C) proteins, as crucial negative regulators of ABA signaling (Leung et al. 1994, 1997; Merlot et al. 2001; Meyer et al. 1994; Rodriguez 1998). Hypersensitive to ABA 1 (HAB1), a homolog of ABI1 and ABI2, also negatively regulates ABA signaling (Saez et al. 2004). Furthermore, enhanced ABA sensitivity of the *abi1hab1* double mutants than that of *abi1* or *hab1* single mutants, and the constitutive ABA response phenotypes of the PP2C triple mutant *abi1-2hab1-lpp2ca-1* suggested that a combination of specific PP2Cs efficiently suppresses ABA signaling in plants (Fujii et al. 2009; Rubio et al. 2009; Saez et al. 2006).

Using yeast two-hybrid screens, several protein kinases in subfamily 2 of Snf1-Related protein Kinases (SnRK2 s), such as Open Stomata 1 (OST1)/SnRK2.6, SnRK2.2, and SnRK2.3, were identified as ABI1-interacting proteins and positive regulators of ABA signaling (Fujii et al. 2007; Fujii and Zhu 2009; Nakashima et al. 2009; Yoshida et al. 2006). In the absence of ABA, PP2Cs interact with SnRK2 s and dephosphorylate the kinase activation loop of SnRK2 s to inactivate the kinases (Umezawa et al. 2009; Vlad et al. 2009). Several studies showed that SnRK2 s can phosphorylate and activate ABA response element-binding factors (AREB/ABFs), a clade of basic-leucine zipper (bZIP) transcription factors that recognize the ABA Response Elements (ABREs) in the promoter region of many ABA-inducible genes (Choi et al. 2000; Fujii et al. 2007; Furihata et al. 2006; Johnson et al. 2002; Kobayashi et al. 2005; Uno et al. 2000). Thus, SnRK2 s and PP2Cs are central components of ABA signaling.

Recently, a group of proteins named Pyrabactin Resistance 1 (PYR1)/PYR1-Like (PYL)/Regulatory Component of ABA Receptor (RCAR), (hereafter referred to as PYLs) were identified as intracellular ABA receptors (Ma et al. 2009; Nishimura et al. 2010; Park et al. 2009; Santiago et al. 2009b). Upon binding ABA, PYL proteins physically interact with PP2Cs and inhibit their phosphatase activity, thus releasing the SnRK2 s from suppression by the PP2Cs (Hubbard et al. 2010; Joshi-Saha et al. 2011). Activation of SnRK2 s enables the phosphorylation of downstream effector proteins including transcription factors, NADPH oxidases, and ion channels (Klingler et al. 2010; Ravindra et al. 2010). Functional characterization of PYL proteins *in planta* and reconstitution of ABA signaling in

protoplasts and in test tubes using basic ABA signaling components provided evidences to support that PYL proteins are ABA receptors in higher plants (Fujii et al. 2009; Kim et al. 2012; Nishimura et al. 2010; Saavedra et al. 2010; Santiago et al. 2009a; Szostkiewicz et al. 2010). Hence, Arabidopsis core ABA signaling cascade consists of PYLs, PP2Cs, and SnRK2 s (Cutler et al. 2010; Hauser et al. 2011; Umezawa et al. 2010; Weiner et al. 2010).

Sequence analysis showed that PYLs share similarities to members of the START/Bet v I protein superfamily, which has a binding pocket to accommodate ligands such as lipids, hormones, and antibiotics (Iyer et al. 2001). Structural analysis of PYR1, PYL1, and PYL2 unraveled a helix-grip structure, which is a common structural feature for START/Bet v I proteins, consisting of 4  $\alpha$ -helices and seven-strand antiparallel  $\beta$ -sheet. Crystallographic studies of apo- and ABA-bound PYLs as well as ternary PYL-ABA-PP2C complexes further revealed the detailed molecular mechanisms of ABA recognition, PYL dimerization, and interactions of PYL-ABA-PP2C (Melcher et al. 2009; Miyazono et al. 2009; Nishimura et al. 2009; Santiago et al. 2009a; Soon et al. 2012; Yin et al. 2009).

In the absence of ABA, the C-terminal  $\alpha$ -helix and the  $\beta$ -sheets form a large open pocket for ligand binding. Two  $\beta$ -loops, which were named gate and latch,  $\beta$ 3– $\beta$ 4 and  $\beta$ 5– $\beta$ 6 loops (Miyazono et al. 2009), Pro-Cap and Leu-Lock (Nishimura et al. 2009), or CL2 and CL3 (Yin et al. 2009), respectively, flank around the entrance of the pocket (Melcher et al. 2010; Miyakawa et al. 2012; Weiner et al. 2010). ABA fits well in the cavity of PYLs, and interacts with the conserved residues distributed throughout the receptor sequence (Yin et al. 2009). Binding of ABA to the hydrophobic pocket of PYLs induces a series of conformational rearrangements in the receptors, including gate and latch closure, further forming a stable conformation by gate-latch interactions (Melcher et al. 2009, 2010; Miyakawa et al. 2012; Nishimura et al. 2009; Santiago et al. 2009a; Weiner et al. 2010; West et al. 2013; Yin et al. 2009). These findings provide useful insight into the critical amino acid residues in the ABA receptor for its activation. Recently, combinations of several mutations at certain amino acid residues of Arabidopsis ABA receptors (PYR1, PYL2, and PYL9) resulted in almost fully activation of the PYR1, PYL2, and PYL9 variants' basal activity, showing a potential for engineering ABA receptors to improve stress resistance in crops (Mosquna et al. 2011).

Soybean (*Glycine max* L.) provides an abundant protein source and vegetable oil for human beings as an important economic crop. Understanding ABA signal transduction in soybean will facilitate molecular breeding of stress-resistant soybeans. Currently, most of the studies of ABA receptors have been done in Arabidopsis with a few reports in other plant species, such as rice and strawberry (*Fragaria*

*ananassa*) (Jia et al. 2011; Kim et al. 2012). Functional analysis of PYLs in soybean has not been reported.

To obtain an overview of soybean ABA receptor family, 23 putative soybean ABA receptor genes were retrieved from recently released soybean genome sequence, and 21 soybean ABA receptor genes (*GmPYLs*) were cloned. We show that *GmPYLs* are localized to the cell cytoplasm and nucleus in *Nicotiana benthamiana*. Yeast two-hybrid assays revealed that *GmPYLs* interact with AtABI1 and two *GmPP2Cs* in an ABA-dependent or -independent manner. Importantly, the Arabidopsis triple mutant *pyr1/pyl1/pyl4* ectopically expressing *GmPYL1* shows less insensitivity to ABA compared with the *pyr1/pyl1/pyl4* mutant. Taken together, our data suggest that *GmPYLs* are ABA receptors that interact with and inhibit PP2Cs.

## Materials and methods

### Plant materials, growth conditions, and transformation

The Arabidopsis ABA receptor triple mutant *pyr1/pyl1/pyl4* was previously described by Sean Culter group (Park et al. 2009). Arabidopsis and *Nicotiana benthamiana* seeds were sterilized with 5 % commercial bleach for 7 min, and washed four times with sterile distilled water. The seeds were plated on half-strength Murashige and Skoog plates ( $\frac{1}{2}$  MS, supplemented with 3 % sucrose, 0.6 % agar and adjusted to pH 5.8). Seedlings were grown in a growth chamber at 24 °C under a 16 h light/8 h dark photoperiod. After two weeks, seedlings were transferred into soil and grown at 22 °C under a 16 h light/8 h dark photoperiod. Soybean cultivar Willam-82 was used in this research. Seeds were sowed into soil directly and grown in the same condition as Arabidopsis.

### Phylogenetic analysis of plant PYLs

*GmPYLs* protein sequences with high similarity to AtPYLs were obtained from soybean genome using BLAST tools from NCBI databases (<http://blast.ncbi.nlm.nih.gov>). AtPYLs, OsPYLs, and *GmPYLs* were aligned using ClustalW, and an unrooted phylogenetic tree was generated using MEGA 5.0. The phylogenetic tree was constructed by the neighbor-joining method, and the tree was evaluated with 1,000 replicates of bootstrap analysis.

### Analysis of *GmPYLs* expression

Total RNA was extracted from 100 mg of two-week-old Arabidopsis and soybean seedlings using Qiagen RNeasy Plant Mini Kit (Cat No. 74904) and treated with Ambion DNA-free kits (Cat No. 1906) following the manufacturer's

instructions. To generate first-strand cDNA, 1 µg of total RNA was reverse-transcribed using Invitrogen SuperScript® III First-Strand Synthesis SuperMix (Cat No. 18080400) and oligdT according to the manufacturer's protocol.

For measuring *RD29A* transcripts in Arabidopsis triple mutants expressing *GmPYL1*, total RNA was isolated from seedlings sprayed with 100 µM ( $\pm$ )-abscisic acid or distilled water for 3 h. Quantitative real-time RT-PCR were performed with Takara SYBR® Premix Ex Taq™ (Cat DRR420A), using an CFX96 Detector (Bio-Rad, USA) with the following thermocycling program: 95 °C for 3 min, followed by 40 cycles at 95 °C for 20 s, 58 °C for 20 s, followed by 71 cycles increasing from 60 to 95 °C at increments of 0.5 °C. Expression levels of Actin7 were used as an internal controls. Relative gene expression was analyzed by quantitative real-time PCR using the  $2^{-DDCT}$  method (Livak and Schmittgen 2001).

### Cloning of the *GmPYL1*

Coding sequence of *GmPYL1* was amplified by RT-PCR from soybean first-strand cDNA synthesized by Phusion enzyme (NEB Cat No. M0530L) and cloned into the pGEM T-easy vector and confirmed by sequencing, then subcloned into pCambia 1301. The resultant 35S::*GmPYL1* plasmid was transformed into *pyr1/pyl1/pyl4* triple mutant mediated by *Agrobacterium tumefaciens* GV3101 and then T0 seeds were selected by (50 mg/L concentration) hygromycin.

### Germination assay

The surface-sterilized seeds were planted on  $\frac{1}{2}$  MS with or without 1 µM ABA (Sigma-Aldrich Cat A1049). Seeds formed green shoots were scored as germinated.

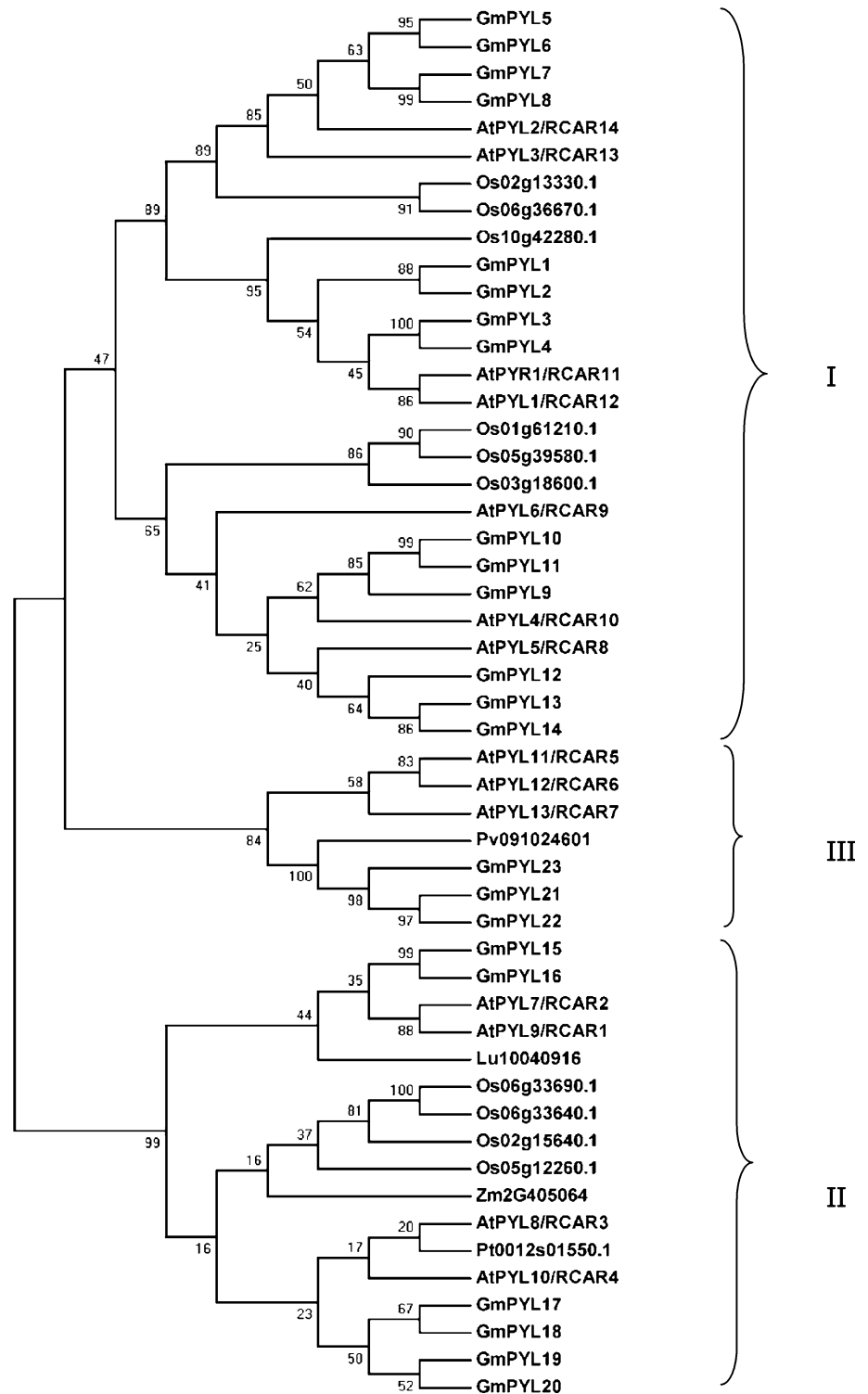
### Yeast two-hybrid assay

Coding sequences of *GmPYLs*, *GmPP2Cs* and AtABI1 were cloned into pDONR™/Zeo vector (Invitrogen, Cat No. 12535-035), then *GmPYLs* were subcloned into invitrogen pDEST32 vector and *GmPP2Cs* and AtABI1 were subcloned into pDEST22 vector via LR reaction (Invitrogen, Cat No. 11791100).

*GmPYL1* point mutation was constructed by site-directed mutagenesis using pGEM T-easy *GmPYL1* as template, then the wild type *GmPYL1* and *GmPYL1*<sup>P109S</sup> were subcloned into PBDGAL4 vector, and AtABI1 into PGADT7 vector.

Using the LiOAC method, each pair of AD and BD constructs were co-transformed into *Saccharomyces cerevisiae* strain AH109. As controls, pDEST32-*GmPYLs* and pDEST22-PP2Cs (pSITE-*GmPYL1*-nEYFP-N1 with

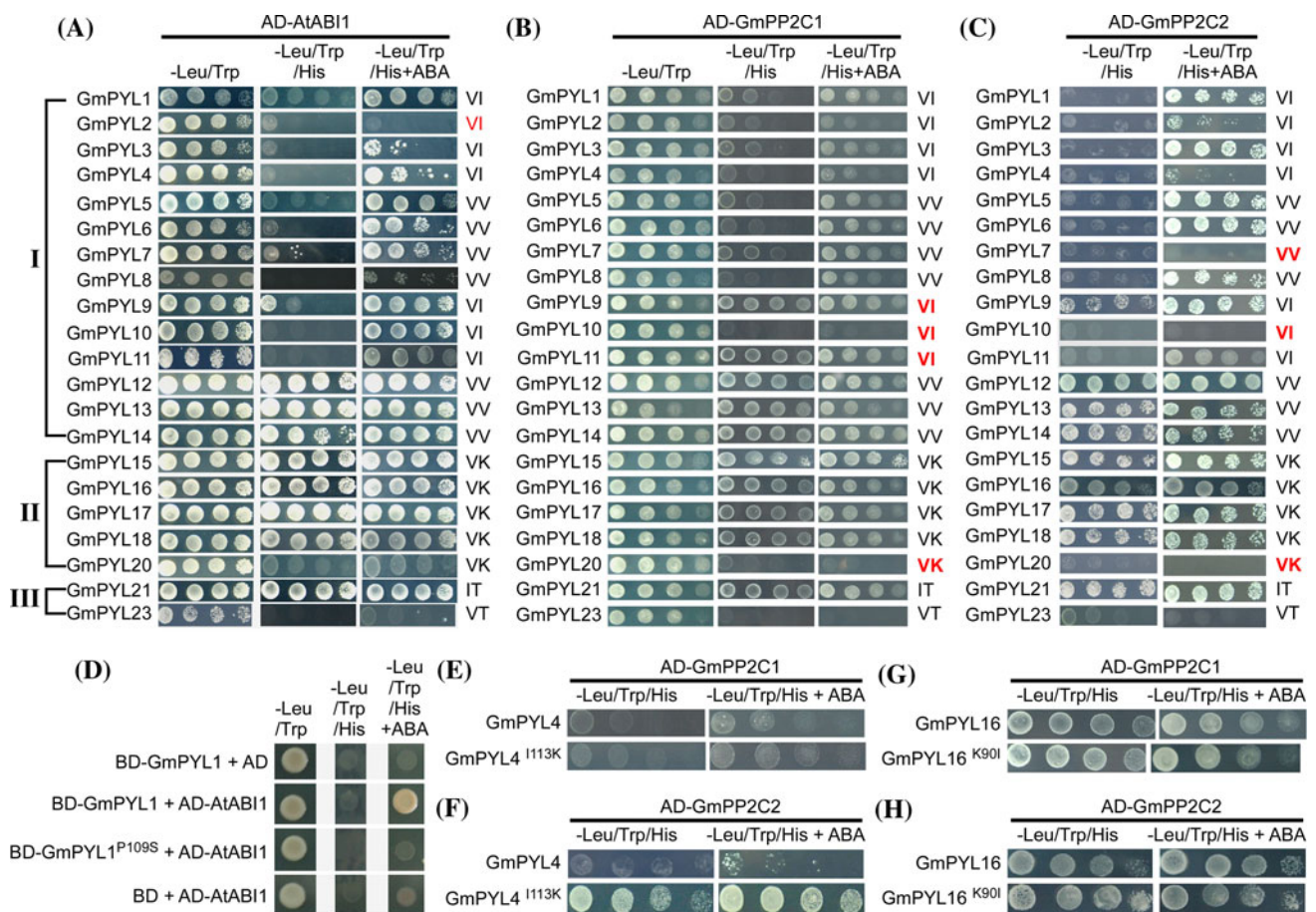
**Fig. 1** Phylogenetic tree of plant PYLs. An unrooted phylogenetic tree showing three plant PYL subfamilies and four other plant PYLs, including 23 soybean PYLs, 14 *Arabidopsis* PYLs, 10 rice PYLs. At, *Arabidopsis thaliana*; Gm, *Glycine max*; Os, *Oryza sativa*; Pt, *Populus trichocarpa*; Pv, *Phaseolus vulgaris*; Zm, *Zea mays*; Lu, *Linum usitatissimum*. Phylogenetic analysis was conducted using MEGA version 5 (Tamura et al. 2007). Accession numbers are shown in Supplementary Table 1 and the alignment of these PYLs is available as Supplementary Fig. 1



pSITE-AtABI1-cEYFP-N1, pSITE-GmPYL1-nEYFP-N1 with pSITE- GmPYL1-cEYFP-N1, pSITE-GmPYL4-nEYFP-N1 with pSITE- GmPYL4-cEYFP-N1, pSITE-GmPYL16-nEYFP-N1 with pSITE-GmPYL16-cEYFP-N1) were used for co-transformation with empty pDEST22 and pDEST32 vectors, respectively. Transforms were

plated on the SD minimal media without leucine (Leu) and tryptophan (Trp). The positive yeasts were grown in liquid SD-Leu/Trp medium at 30 °C for 1 day and 10 µl of dilutions of 1:10, 1:100, 1:1,000 and 1:10,000 were pipetted onto various plates (SD-Leu/Trp, SD-Leu/Trp/His, and SD-Leu/Trp/His supplementary with 10 µM ABA).





**Fig. 2** Interactions between GmPYLs and PP2Cs in yeast. **a–c** BD-GmPYL fusions were co-expressed together with **a** AD-AtABI1, **b** AD-GmPP2C1, and **c** AD-GmPP2C2 in yeast. Interaction was determined by growth assay on media lacking Leu, Trp, and His in the presence and absence of 10  $\mu$ M ABA. These marked residues on the right side of **a**, **b**, and **c** are the two residues preceding the conserved ABA receptor CL2/gate domain coding by consensus SGLPA sequence. **d** CL2 region (SGLPA) in PYLs is crucial for GmPYL1-AtABI1 interactions. Replacement of P109 by S in GmPYL1

(GmPYL1<sup>P109S</sup>) abolished its binding activity with AtABI1 in the presence of 10  $\mu$ M ABA. **e–f** Replacement of I113 by K in GmPYL4 (GmPYL4<sup>I113K</sup>) did not affect the binding between **e** GmPYL4 and GmPP2C1, but increased its binding affinity with **f** GmPP2C2 either in the presence of 10  $\mu$ M ABA or absence of ABA. **g–h** Replacement of K90 by I in GmPYL16 (GmPYL16<sup>K90I</sup>) did not affect the interaction of **g** GmPYL16-GmPP2C1 and **h** GmPYL16-GmPP2C2 in the presence of 10  $\mu$ M ABA or absence of ABA

These plates were incubated in 30 °C for 2 days and then photographed.

#### Subcellular localization and bimolecular fluorescence complementation

To generate yellow fluorescent protein (YFP)-GmPYL constructs, coding sequences of *GmPYLs* were cloned into pDONR vector via BP reaction, and subcloned into PEARLEYGATE 104 vector via LR reaction. To generate BiFC vector, coding sequences of soybean *GmPYL1* and *AtABI1* were cloned into pDONR via BP reaction, and then coding sequence of *GmPYL1* was subcloned into pSITE-nEYFP-N1 vector, and coding sequence of *AtABI1* was subcloned into pSITE-cEYFP-N1 vector via LR reaction.

All these vectors were transformed into *Agrobacterium tumefaciens* GV3101 via chemical transformation method.

For transient expression assay, the *A. tumefaciens* GV3101 harbouring PEARLEYGATE104 vector were employed with the p19 strain infiltration in 6-week-old *Nicotiana benthamiana* leaves.

For bimolecular fluorescence complementation, *A. tumefaciens* GV3101 harboring pSITE-nEYFP-N1 or pSITE-cEYFP-N1 fusion constructs were employed with the p19 strain co-infiltration into 6-weeks-old *N. benthamiana* leaves. After 2 days, *N. benthamiana* leaves were sprayed with 100  $\mu$ M abscisic acid (ABA). The infiltrated leaves sprayed with distilled water served as controls. After 3 days, the lower epidermis cells of *N. benthamiana* were

analyzed under confocal microscopy (Zeiss 510 Meta) using LSM5 Image Browser.

### Protein purification

To generate *GST-GmPYL* and *His-GmPP2C1* constructs, GmPYLs and GmPP2C1 were cloned into pDONR vector via BP reaction, and then subcloned into pDEST15 vector and pDEST 17 vector via LR reaction respectively.

Protein expression was induced by 0.1 mM IPTG for 5 h at 30 °C. Proteins were purified from the soluble fraction using Glutathione Sepharose 4B (GE Cat No. 17-0756-01) or Ni-NTA Agarose (Invitrogen Cat No. R901-15) under native conditions according to the manufacturer's instructions.

### PP2C phosphatase activity assay

GmPP2C1 phosphatase activity was measured using the colorimetric substrate p-nitrophenyl phosphate (pNPP, Sigma). Reactions were performed in 1 × reaction buffer (50 mM Tris-HCl, pH 7.5, 25 mM Mg(OAc)<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 0.5 mM EGTA, 0.5 % β-mercaptoethanol, and 0.5 % BSA) with 1 μM His-GmPP2C1 and increasing amounts of GST-GmPYLs (1, 2.5, and 5 μM) as described. Reactions were initiated by the addition of pNPP to a final concentration of 50 mM. The hydrolysis of pNPP was measured by following the absorbance at 405 nM (A<sub>405</sub>).

AtABI1 phosphatase activity was measured using the BioVision Phosphate Assay Kit (BioVision Incorporated Cat No. 410–500). Reactions were performed in tubes by adding 50 nM His-AtABI1 and 250 nM of each soybean ABA receptor (GmPYL1, GmPYL16, and GmPYL21) in 270 μl 1 × PP2C buffer (50 mM imidazole, pH 7.2, 5 mM MgCl<sub>2</sub>, 0.1 % β-mercaptoethanol and 0.5 μg/ml BSA) in the absence of ABA (named as 0 μM ABA treatment) or in the presence of 10 μM (+)-ABA (named as 10 μM ABA treatment).

In parallel, the reaction without AtABI1 with the same setting served as controls (named as background). Reaction only adding 50 nM AtABI1 and 270 μl 1 × PP2C buffer (without adding any ABA receptor) served as positive control (named as positive control). The final volume of each reaction was adjusted to the volume of 10 μM ABA treatment using 1 × PP2C buffer. Then these tubes were incubated at room temperature for 30–35 min.

Reactions were initiated by adding 10 μl phosphopeptides (N'-HSQPK[pSer]TVGTP-C') as substrates to a final concentration of 35 μM. After incubating at room temperature for 20 min, the reactions were stopped by adding 40 μl of the colorimetric dye working solution.

Subsequently, 100 μl of solution in each reaction was transferred into each of three adjacent wells of a 96 well

microtiter plate. The absorbance at 650 nm was measured in (BioTek). The relative phosphatase activity was calculated by the formula  $[(A_{650} \text{ value of } 0 \mu\text{M ABA treatment} - A_{650} \text{ value of background}) / (A_{650} \text{ value of positive control} - A_{650} \text{ value of background})] \times 100 \%$  for phosphatase activity without ABA and  $[(A_{650} \text{ value of } 10 \mu\text{M ABA treatment} - A_{650} \text{ value of background}) / (A_{650} \text{ value of positive control} - A_{650} \text{ value of background})] \times 100 \%$  for phosphatase activity without 10 μM ABA.

## Results

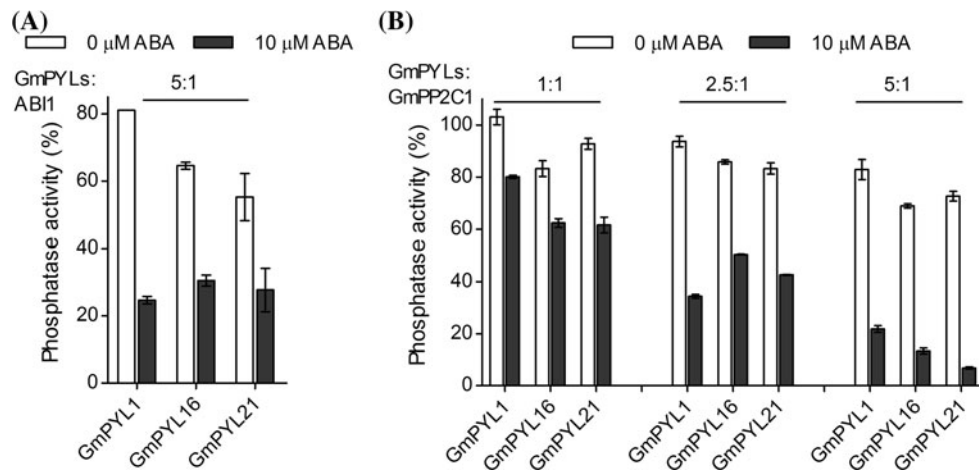
The soybean genome encodes 23 putative ABA receptors

Based on sequence similarity to the Arabidopsis PYR/PYL/RCAR (hereafter referred to as PYLs) family of ABA receptors, twenty-three genes encoding putative soybean PYL homologs (GmPYLs) were retrieved from recently released soybean genome sequence in NCBI database (Supp. Table 1). We cloned the cDNAs for 21 of the GmPYLs, and GmPYL proteins share high similarity with corresponding PYL proteins in other plant species (Supp. Fig. 1). GmPYLs were designated according to their corresponding orthologous PYL proteins in Arabidopsis. We have been unsuccessful in obtaining the cDNAs for *GmPYL19* and *GmPYL22*, likely because that the expression levels of these two genes are too low.

To analyze these GmPYL proteins further, an unrooted phylogenetic tree was constructed using protein sequences of the GmPYLs and other plant PYLs (Fig. 1). PYLs can be categorized into three subgroups. Subgroup I included GmPYL1–14, which were closely clustered with AtPYR1 and AtPYL1–6. Subgroup II contains GmPYL15–20 and AtPYL7–10. Subgroup III consists of GmPYL21–23 and AtPYL11–13 (Fig. 1).

GmPYLs interact with clade A PP2Cs in diverse manners

PYLs can bind PP2Cs in yeast two-hybrid (Y2H) assays in an ABA-dependent or -independent manner (Hao et al. 2011; Park et al. 2009). To determine whether GmPYLs may interact with clade A PP2Cs, we examined the interaction of GmPYLs with AtABI1 in Y2H assays in the presence or absence of ABA in yeast. In this experiment, GmPYLs differentially interacted with AtABI1 (Fig. 2a). GmPYL1 and GmPYL3–11 interact with AtABI1 in an ABA-dependent manner. These interactions were enhanced in the presence of 10 μM ABA (Fig. 2a). Among these GmPYLs, GmPYL8, GmPYL10 and GmPYL11 interact with AtABI1 only in the presence of 10 μM ABA. In contrast, GmPYL12–



**Fig. 3** GmPYL1, GmPYL16, and GmPYL21 inhibit PP2Cs in different manners. **a** The activity of ABI1 was differentially inhibited by GmPYL1, GmPYL16, and GmPYL21. The phosphatase activity of ABI1 was measured in absence or presence of 10  $\mu$ M ABA. Phosphatase activity was measured using BioVision Phosphate Assay. Values represent the mean  $\pm$  SD of three biological replicates. **b** The

activity of GmPP2C1 was differentially inhibited by GmPYL1, GmPYL16, and GmPYL21. The phosphatase activity of GmPP2C1 was measured in the absence or presence of 10  $\mu$ M ABA using colorimetric substrate p-nitrophenyl phosphate. Values represent the mean  $\pm$  SD of three biological replicates

18 and GmPYL20–21 interact with AtABI1 in an ABA-independent manner (Fig. 2a). These GmPYLs constitutively bind to AtABI1 although the binding intensity differs among the GmPYLs. GmPYL12–18 and GmPYL21 bind strongly to AtABI1, but GmPYL20 binds weakly to AtABI1. Additionally, GmPYL2 and GmPYL23 showed nearly no interaction with AtABI1 even in the presence of ABA (Fig. 2a). In these Y2H experiments, all GmPYLs showed no obvious self activation (Supp. Fig. 2). These results demonstrate that GmPYLs bind to AtABI1 in diverse manners and with different intensities.

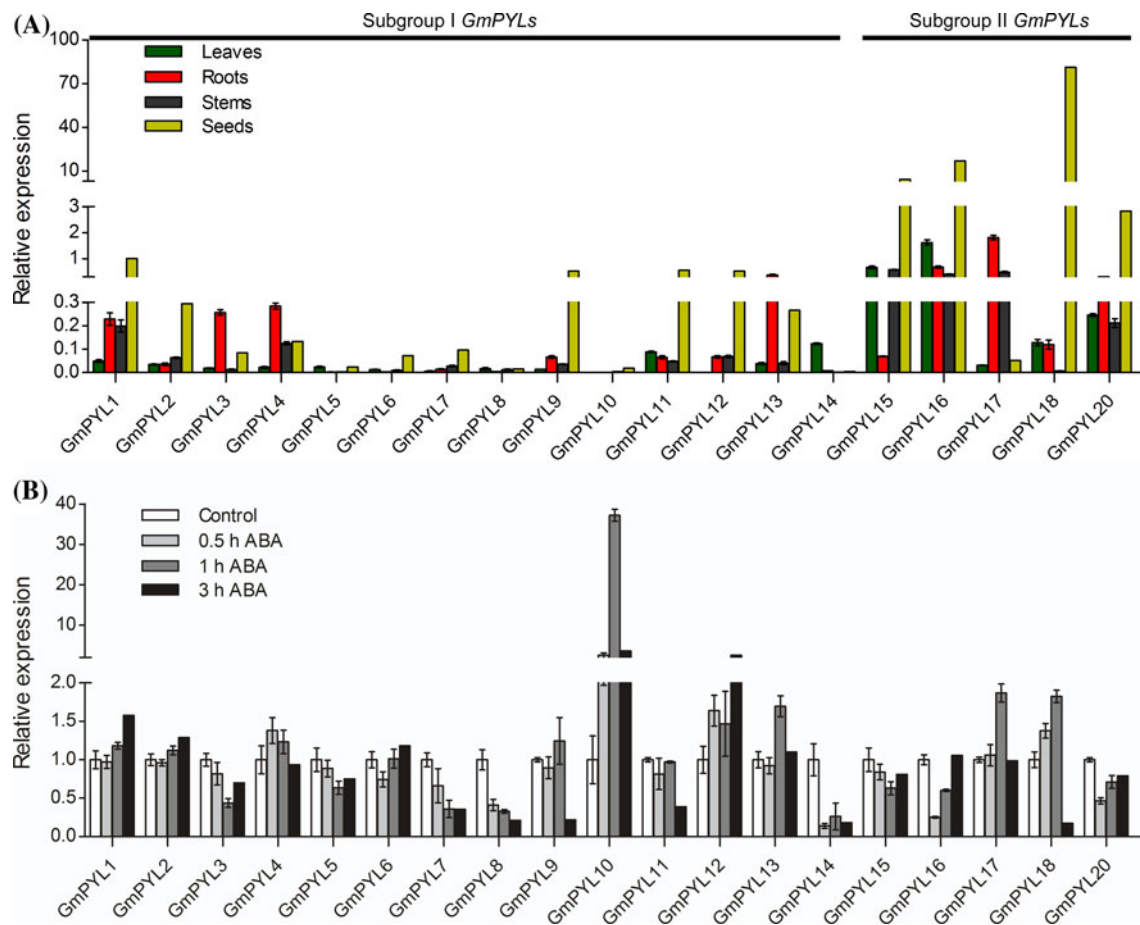
To investigate whether GmPYLs can interact with soybean PP2Cs (GmPP2Cs), we investigated the interactions between GmPYLs and two soybean PP2Cs GmPP2C1 (LOC100789207) and GmPP2C2 (LOC100791754), which share high identities with AtABI1 (58 % for GmPP2C1 and 65 % for GmPP2C2, Supp. Fig. 3). In this experiment, GmPYLs differentially interacted with GmPP2C1 and GmPP2C2 (Fig. 2b, c). GmPYL1–8 interact with GmPP2C1 in an ABA-dependent manner, whereas GmPYL9, GmPYL11–18, and GmPYL21 interact with GmPP2C1 in an ABA-independent manner. GmPYL10, GmPYL20, and GmPYL23 showed nearly no interaction with GmPP2C1 even in the presence of ABA (Fig. 2b). In comparison, GmPYL1–6, GmPYL8–9 and GmPYL11 bind to GmPP2C2 in an ABA-dependent manner, whereas GmPYL12–18 and GmPYL21 interact with GmPP2C2 in an ABA-independent manner. GmPYL7, GmPYL10, GmPYL20, and GmPYL23 showed nearly no interaction with GmPP2C2 even in the presence of ABA (Fig. 2c). These results demonstrate that GmPYLs bind to GmPP2Cs in diverse manners and in differing intensities.

In general, subgroup I GmPYLs (except for PYL12–14) bind to PP2Cs mainly in an ABA-dependent manner, whereas subgroup II GmPYLs bind to PP2Cs in an ABA-independent manner (Fig. 2a–c). Among the subgroup III GmPYLs, GmPYL21 binds to PP2Cs in an ABA-independent manner, but GmPYL23 appears to have lost the ability to bind to PP2Cs, either in the absence or presence of ABA (Fig. 2a–c). The interactions between subgroup I GmPYLs and different PP2Cs appear selective. GmPYL10 binds to ABI1 in a typical ABA-dependent manner, but it cannot bind to the tested GmPP2Cs. GmPYL11 binds to ABI1 and GmPP2C2 in an ABA-dependent manner, although it interacts with GmPP2C1 in an ABA-independent manner. GmPYL1–8 also bind to these three PP2Cs with different intensities in the presence of ABA.

The CL2 region is crucial for GmPYLs–PP2Cs interactions

Previous reports showed that the conserved proline (P88) in the CL2/gate domain plays a crucial role in the PYR1–HAB1 interaction in Arabidopsis (Park et al. 2009). This residue is also conserved in GmPYLs (Supp. Fig. 1). We mutated the conserved proline (P109) in the CL2/gate domain of GmPYL1, and subjected the mutated protein (GmPYL1<sup>P109S</sup>) to Y2H analysis for interaction with AtABI1. In the presence of 10  $\mu$ M ABA, GmPYL1 can interact with AtABI1 in yeast, and the interaction between GmPYL1 and AtABI1 was very weak (Fig. 2a) and not observed (Fig. 2d) in the absence of ABA. However, GmPYL1<sup>P109S</sup> is not able to interact with AtABI1 either in the presence or





**Fig. 4** Tissue-specific and ABA-regulated expression of *GmPYL* genes. **a** Tissue-specific expression of *GmPYL*s were analyzed by quantitative real-time PCR in leaves, roots, stems, and seeds. The expression level of *GmPYL1* in seeds was set as 1. **b** ABA-induced expression of *GmPYL* genes. Two-week-old seedlings were sprayed

with 100  $\mu$ M ABA for 0, 0.5, 1, and 3 h. Quantitative real-time PCR was carried out in triplicate and the fold change was analyzed via the  $2^{-\Delta\Delta CT}$  method using the soybean *GmFbox* gene as an internal control. Values represent the mean  $\pm$  SD of three biological replicates. Error bars indicate SD ( $n = 3$ )

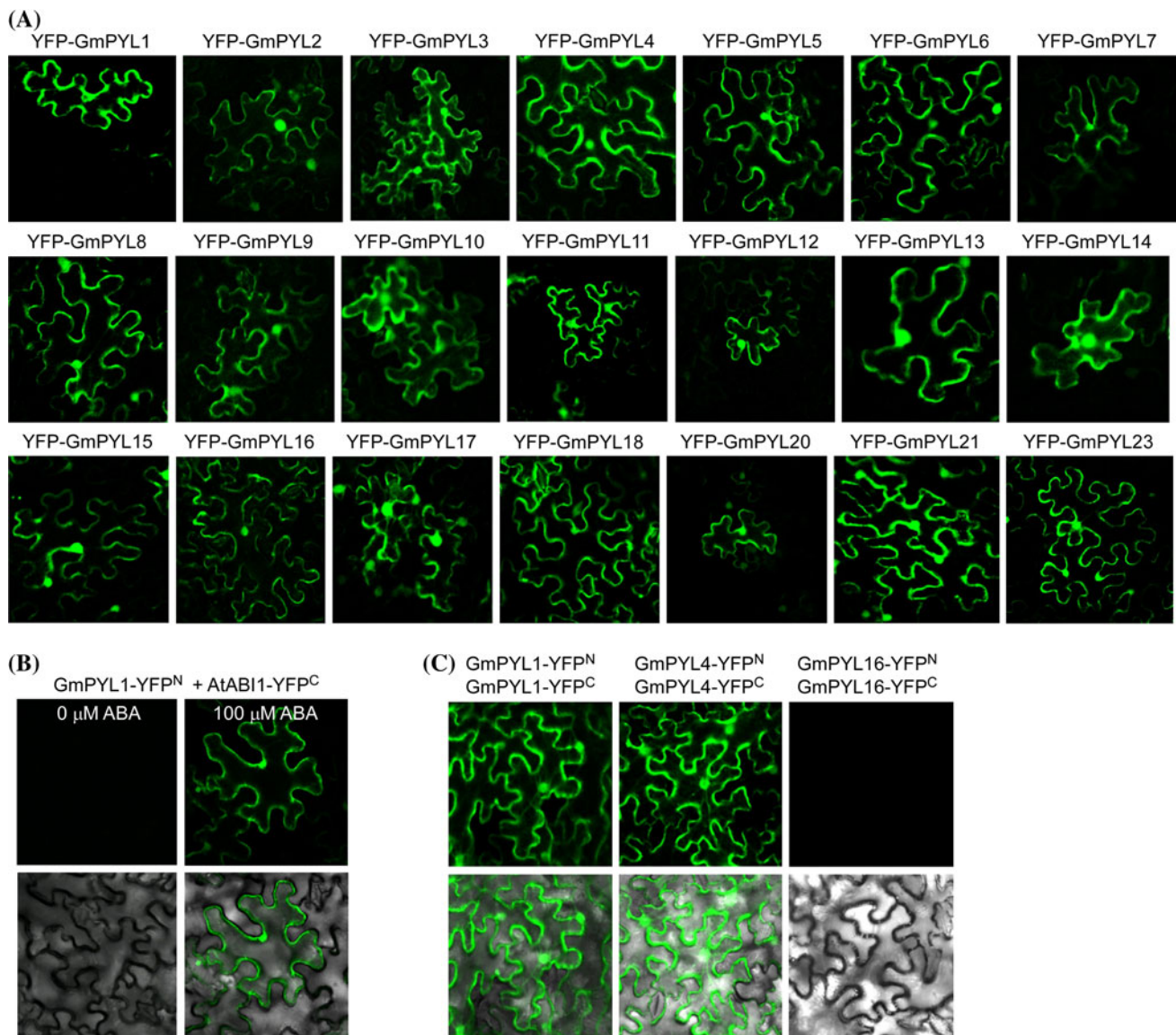
absence of ABA in the Y2H assays (Fig. 2d). The results show that the conserved proline in CL2/gate domain is critical for *GmPYL1*-AtABI1 interaction.

ABA-dependence of *GmPYL*s-PP2Cs interactions are partially correlated with two amino acid residues preceding the CL2/gate domain of *GmPYL*s

Two amino acid residues (L79K80, hereafter referred to as LK) in CL2 of AtPYL10 is critical for its ABA-independent inhibition of PP2Cs. The mutated PYL2, PYL2-V87L/I88 K (two amino acid residues mutated in AtPYL2 at the corresponding positions of LK in AtPYL10), constitutive inhibits several PP2Cs activity in the absence of ABA, thus mimic the ABA-independent PP2C inhibition of AtPYL10 (Hao et al. 2011). Since the inhibition of PP2Cs by PYLs needs the physical interactions between PYLs and PP2Cs, we speculated that these two amino acid residues may also affect the ABA dependence of other PYLs-PP2Cs interactions.

To gain further insights into the molecular determinants of the ABA dependence of *GmPYL*-PP2C interactions, we performed amino acid sequence alignment of various PYL proteins, and found that *GmPYL*s, AtPYLs, and rice PYLs have VI, VV, and VK polymorphisms at the two residues preceding the conserved CL2/gate domain consensus SGLPA sequence. Moreover, there are some exceptions (VQ, VT, VR, VN and IT, LV, LK) present in Arabidopsis, rice, populus, flax (*Linum usitatissimum*), and maize, which suggests divergent combinations of these two amino acid residues exist in plants (Supp. Fig. 4). Y2H assays clearly show that certain correlations exist between the two amino acid residues preceding the SGLPA sequence and ABA dependence of *GmPYL*s-PP2Cs interactions (Fig. 2a–c). Generally, interactions between *GmPYL*1–11 and PP2Cs are dependent on or at least enhanced by ABA; whereas *GmPYL*12–21 interact with PP2Cs in an ABA-independent manner (Fig. 2a–c). *GmPYL*1–4, and *GmPYL*9–11 (*subgroup I* in the *GmPYL* family) all share the identical VI





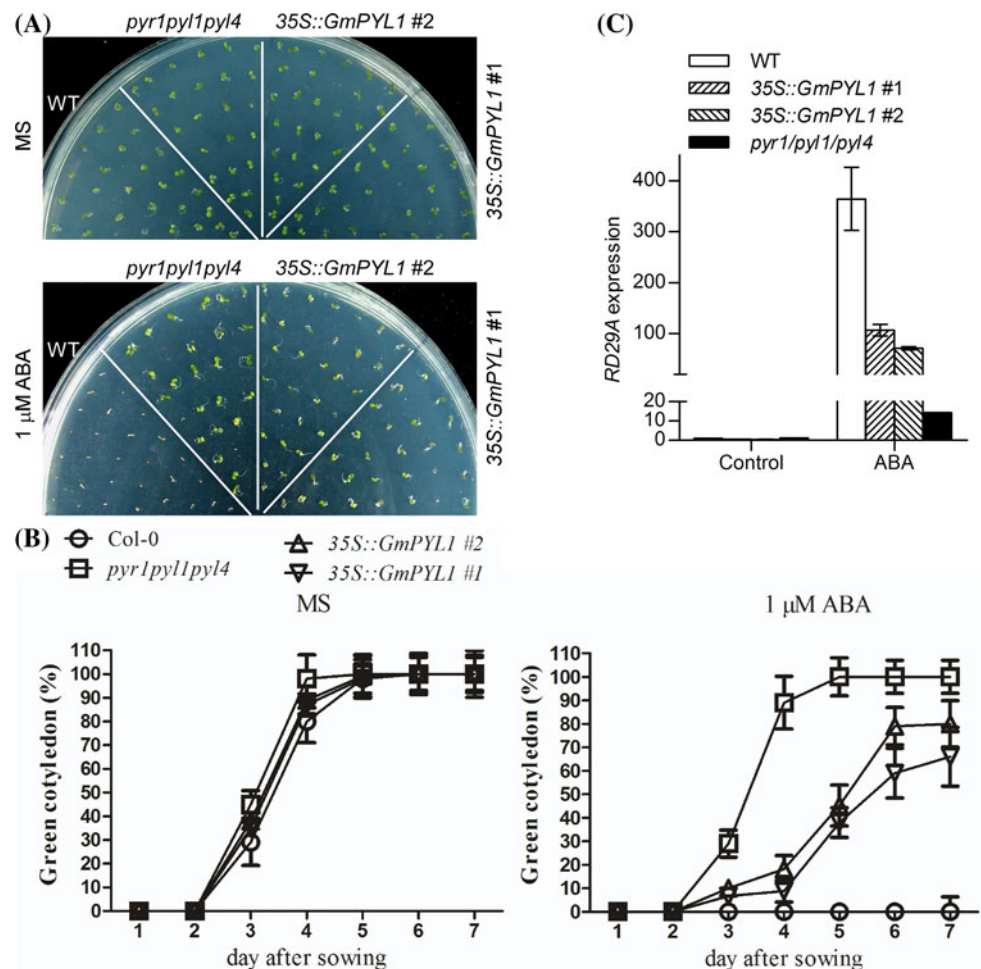
**Fig. 5** Subcellular localization of GmPYLs, interactions of GmPYL1-AtABI1, and GmPYLs self-interactions. **a** Confocal images of epidermal cells taken from the *Nicotiana benthamiana* leaves transiently expressing yellow fluorescent protein (YFP)-GmPYLs driven by the 35S promoter using agro-infiltration. **b** The interaction between GmPYL1 and AtABI1 was detected using bimolecular fluorescence complementation (BiFC) assay. *N. benthamiana* leaves transiently expressed *GmPYL1-YFP<sup>N</sup>* and *AtABI1-YFP<sup>C</sup>* were treated

with 100 μM ABA. Confocal images of epidermal cells were taken before ABA treatment (*left column*) and after ABA treatment (*right column*). **c** The interactions between GmPYLs with themselves were detected using BiFC assay. GmPYL1 (*left column*), GmPYL4 (*middle column*), and GmPYL16 (*right column*) were analyzed. For **b** and **c**, *upper panel* shows YFP signals, and *bottom panel* are bright-field images

preceding the SGLPA sequence, and show an ABA-dependent interaction with PP2Cs (Fig. 2a–c, Supp. Fig. 4). GmPYL15–20 (*subgroup II in the GmPYL family*) have VK at the corresponding position, and interact with PP2Cs in an ABA-independent manner (Fig. 2a–c, Supp. Fig. 4). In addition, GmPYL5–8 and GmPYL12–14 (*subgroup I in the GmPYL family*), in which VV can be found at the corresponding position, interact with PP2Cs in an ABA-dependent and -independent manner (Fig. 2a–c, Supp. Fig. 4), respectively, suggesting that residues in

other regions of GmPYL5–8 and GmPYL12–14 also participate in the regulation of these ABA-dependent and -independent interactions. *Notably, the presence of IT in GmPYL21 and VT in GmPYL23 (two members of subgroup III in the GmPYL family) is correlated with their distinct PP2C interactions* (Fig. 2a–c, Supp. Fig. 4), *although GmPYL21 and GmPYL23 share high amino acid sequence identity (94 %)* (Supp. Fig. 5). Therefore, we conclude that the VI preceding the SGLPA sequence is correlated with ABA-dependence of the GmPYLs-PP2Cs interactions in

**Fig. 6** Ecotopically expressing *GmPYL1* partially rescues the phenotypes of the *AtPYL* triple mutant. **a–b** Seed germination of the wild type (WT), *AtPYL* triple mutant (*pyr1/pyl1/pyl4*), and transgenic *pyr1/pyl1/pyl4* seedlings harboring 35S::*GmPYL1* (35S::*GmPYL1*) in 1/2 MS medium and 1/2 MS plus 1  $\mu$ M ABA. The panels show the percentage of seeds that developed green cotyledons. Error bars indicate s.e.m. ( $n = 3$ ). **c** Expression of *RD29A* in the WT, *pyr1/pyl1/pyl4*, and 35S::*GmPYL1* transgenic seedlings sprayed with distilled water or 100  $\mu$ M ABA. Quantitative real-time PCR was conducted to analyze samples treated with distilled water (control) or ABA for 3 h. Values represent the mean  $\pm$  SD of three biological replicates. Error bars indicate SD ( $n = 3$ )



yeast, and VK or IT are correlated with ABA-independence of the GmPYLs-PP2Cs interactions.

However, the ABA dependence of some GmPYL-PP2C interactions does not follow these rules. The presence of VI in GmPYL9 and GmPYL11, for example, is not correlated with ABA dependence of GmPYL9-GmPP2C1, GmPYL11-GmPP2C1, and GmPYL9-GmPP2C2 interactions (Fig. 2a–c). Moreover, changing the VI in GmPYL4 to VK results in the ABA-independent interaction between GmPYL4<sup>I113K</sup> and GmPP2C2 (Fig. 2f), but the interaction between GmPYL4<sup>I113K</sup> and GmPP2C1 is still ABA-dependent (Fig. 2e). When VK in GmPYL16 is changed to VI, the interactions between GmPYL16<sup>K90I</sup> and GmPP2Cs are still ABA-independent (Fig. 2g, h). These results suggest that both GmPYLs and GmPP2Cs contribute to the ABA dependence of GmPYLs-GmPP2Cs interactions in soybean.

### Three GmPYLs differentially inhibit the phosphatase activities of AtABI1 and GmPP2C1

GmPYLs differentially interact with PP2Cs in Y2H assays (Fig. 2). To investigate whether GmPYLs may inhibit

PP2Cs, we examined the effects of three GmPYLs on the phosphatase activity of purified AtABI1 and GmPP2C1 with and without ABA. The results demonstrate that all GmPYLs tested show an ABA enhanced inhibition on ABI1 and GmPP2C1 activity (Fig. 3), consistent with the notion that all of the tested GmPYLs are functional ABA receptors. In the absence of ABA, the inhibition by GmPYL1 (subgroup I) was weaker, whereas the inhibitions by GmPYL16 (subgroup II) and GmPYL21 (subgroup III) were stronger (Fig. 3), which was correlated with their binding intensity in Y2H assays (Fig. 2a). We examined the inhibitory effect on GmPP2C1 by GmPYLs with the GmPYL:GmPP2C1 ratios of 1:1, 2.5:1, and 5:1, and found that the ABA independent inhibition of GmPYLs to GmPP2C1 was enhanced by the addition of more GmPYLs (Fig. 3b).

In the presence of 10  $\mu$ M ABA, the extents of inhibitory effect for all three GmPYLs on ABI1 and GmPP2C1 activity were similar, and the inhibition of GmPP2C1 was enhanced by the addition of more GmPYLs. These results suggest that GmPYLs are functional ABA receptors that differentially inhibit the phosphatase activities of AtABI1 and GmPP2C1.

### Tissue-specific and ABA-regulated expression patterns of *GmPYL* genes

To obtain an overview of the tissue-specific and ABA-regulated expression patterns of *GmPYL* genes, the steady state levels of *GmPYLs* transcripts in different tissues and in ABA-treated leaves were measured by quantitative RT-PCR (qRT-PCR) (Fig. 4).

The expression level of *GmPYL1* in seeds was set as 1. Most of the *GmPYL* transcripts can be detected in all of the tissues checked; many *GmPYLs* transcripts accumulated highly in seeds, such as *GmPYL1-2*, *GmPYL6-7*, *GmPYL9-12*, *GmPYL15-16*, *GmPYL18*, and *GmPYL20* (Fig. 4A). *GmPYL3*, *GmPYL4*, *GmPYL13*, and especially *GmPYL17* showed highest expression levels in roots, whereas transcripts of *GmPYL5*, *GmPYL8*, and *GmPYL14* mainly accumulated in leaves (Fig. 4a). Generally, subgroup I *GmPYLs* showed relatively lower expression levels than subgroup II *GmPYLs*.

The expression levels of some *GmPYLs*, such as *GmPYL3*, *GmPYL5*, *GmPYL7*, *GmPYL8-9*, *GmPYL11*, *GmPYL14*, and *GmPYL15*, are reduced after ABA treatment. *GmPYL6*, *GmPYL16*, and *GmPYL20* expression levels are reduced in the early time points after ABA treatment (0.5 h, for example) and gradually increased in the later time points (1 and 3 h after ABA application) (Fig. 4b). In contrast, the transcripts of *GmPYL1*, *GmPYL10*, *GmPYL12-13*, and *GmPYL17* increased after ABA treatment. No clear expression pattern exists for members from the same subgroups of soybean ABA receptor family after ABA treatment.

The expression level of *GmPYL19* was too low to be detected. *GmPYL21-23* all have very high similarity and identity with each other (Supp. Fig. 5). This made it difficult to design specific real time PCR primers to analyze their expression profiles.

### Subcellular localization of *GmPYLs* and *GmPYL1*-AtABI1 interaction in plant cells

It has been shown that the fluorescence of AtRCAR1/PYL9-GFP fusion protein can be detected in both the cytosol and nucleus in Arabidopsis protoplasts (Ma et al. 2009). To investigate the subcellular localization of *GmPYLs*, 35S promoter-driven yellow fluorescent protein (YFP)-*GmPYLs* fusion proteins were transiently expressed in *Nicotiana benthamiana* leaves by agroinfiltration. Expressing YFP-*GmPYLs* resulted in fluorescence signals in both the cytoplasm and nucleus (Fig. 5a).

To test whether *GmPYLs* can interact with PP2Cs in plant cells, we performed bimolecular fluorescence complementation (BiFC) experiments in *N. benthamiana* leaves using *GmPYL1* and AtABI1. ABA treatment clearly

promoted the interaction between *GmPYL1* and AtABI1 in plant cells. No fluorescence signal could be detected in the same experimental setting without adding ABA (Fig. 5b). These data indicate that *GmPYL1* interacts with AtABI1 in an ABA-dependent manner *in planta*.

*GmPYL1* and *GmPYL4*, but not *GmPYL16* can form dimers in plant cells

It was suggested that that Arabidopsis PYR1, PYL1, and PYL2 form dimers, whereas AtPYL4-10 exist as monomers in solution (Hao et al. 2011). To investigate the monomeric or dimeric state of *GmPYLs* *in planta*, we studied the dimer formation of *GmPYL1* and *GmPYL4* (two orthologs of AtPYR1 and AtPYL1), and *GmPYL16* (an ortholog of AtPYL7 and AtPYL9) in *N. benthamiana* using the BiFC approach.

*GmPYL1* and *GmPYL4* are able to form dimers in the transfected cells; in contrast, *GmPYL16* cannot form dimers in *N. benthamiana* leaves, suggesting that *GmPYL16* exists as monomers *in planta* (Fig. 5c). These results are consistent with the notion that AtPYR1, AtPYL1 and AtPYL2 form dimers and that AtPYL7-10 exist as monomers in solution to impair PP2C activity to various extents even without adding ABA (Hao et al. 2011; Yin et al. 2009).

### *GmPYL1* attenuates the ABA insensitivity of Arabidopsis *pyr1/pyl1/pyl4* mutant

To begin to examine the functions of *GmPYLs* in plants, *GmPYL1* that is closely clustered with AtPYR1 and AtPYL1 in the phylogenetic analysis (Fig. 1) was chosen for further investigation. Park et al. (2009) showed that the Arabidopsis triple mutant *pyr1/pyl1/pyl4* is insensitive to ABA during early seedling establishment. Therefore, a construct of 35S promoter-driven *GmPYL1* was introduced into *pyr1/pyl1/pyl4* plants through agrobacterium transformation.

Transgenic Arabidopsis *pyr1/pyl1/pyl4* seedlings harboring 35S::*GmPYL1* showed normal growth compared with the wild-type (WT) control and *pyr1/pyl1/pyl4* seedlings on media without ABA, indicating that ectopic expression of *GmPYL1* in Arabidopsis does not affect seedling growth (Fig. 6a–b). In the presence of 1  $\mu$ M ABA, the growth of WT was almost totally suppressed, while the growth of *pyr1/pyl1/pyl4* seedlings was only slightly inhibited. Notably, the ABA insensitive phenotype of the *pyr1/pyl1/pyl4* triple mutant was partially rescued by expressing *GmPYL1* (Fig. 6a–b).

We further quantified the expression level of *RD29A*, a typical marker gene that is induced by ABA, in WT, *pyr1/*



*pyl1/pyl4*, and 35S::*GmPYL1* seedlings by qRT-PCR. WT, *pyr1/pyl1/pyl4*, and 35S::*GmPYL1* seedlings have similar levels of *RD29A* transcripts without adding ABA. However, ABA treatment induces a high level of expression of *RD29A* in WT but not *pyr1/pyl1/pyl4* seedlings. The expression level of *RD29A* in 35S::*GmPYL1* seedlings is moderately induced by supplementary of ABA (Fig. 6c). Thus, *GmPYL1* expression partially rescued the ABA insensitive phenotype in *pyr1/pyl1/pyl4* seedlings, which suggests that GmPYL1 is a functional ABA receptor in vivo.

## Discussion

In this study, based on amino acid sequence similarity with *AtPYR/PYL/RCAR*, we found 23 orthologous genes (*GmPYLs*) encoding putative ABA receptors in soybean. The deduced GmPYLs can be classified into three subgroups according to phylogenetic analysis, and twenty-one *GmPYLs* were cloned. *GmPYLs* are differentially expressed in leaves, stems, roots, and seeds, and transcripts of some *GmPYLs* are regulated by ABA treatment. All 35S promoter-driven YFP::*GmPYLs* are localized to the cytoplasm and nucleus, which is consistent with previous reports of the subcellular localization of *AtRCAR/PYLs* (Ma et al. 2009).

Notably, most GmPYLs interact with PP2Cs in an ABA-dependent or -independent manner in yeast, and three representative GmPYLs (GmPYL1, 16, and 21 from subgroup I, II, III, respectively) can impair AtABI1 and GmPP2C1 activity in an ABA-enhanced manner in vitro. In the absence of ABA, the inhibition of PP2C activity by GmPYL1 was weaker, while the inhibitions of PP2C activity by GmPYL16 and GmPYL21 were stronger, which was correlated with their binding intensity in Y2H assays without ABA. These data suggest that GmPYLs are soybean ABA receptors.

Functional characterization of GmPYL1 revealed that GmPYL1 interacts with AtABI1 in an ABA-dependent manner in *N. benthamiana*, and expressing GmPYL1 partially recovers ABA sensitivity of Arabidopsis *pyr1/pyl1/pyl4* seedlings. Moreover, AtABI1 activity assays showed that GmPYL1 can impair AtABI1 activity in an ABA-dependent manner in vitro, and quantification of *RD29A*, an ABA-responsive marker gene by qRT-PCR revealed that ABA induces modest transcripts of *RD29A* in transgenic *pyr1/pyl1/pyl4* seedlings harboring 35S promoter-driven *GmPYL1* construct. All together, these evidences indicate that GmPYL1 is a functional ABA receptor in soybean, and molecular mechanisms of ABA perception and signaling are highly conserved in soybean.

Results from yeast two-hybrid and protoplast reconstitution assays combined with biochemical analysis suggested

that some PYLs interact with PP2Cs to suppress PP2C activity even in the absence of ABA (Fujii et al. 2009; Nishimura et al. 2010; Park et al. 2009). Hao et al. (2011) reported that PYL10, a representative of a subclass of PYLs (PYL4-10), impairs some PP2C (ABI1, HAB1, HAB2) activity with the PYL10:PP2C ratio of 1:1 in the absence of ABA. Further substitution of the two amino acid residues (VI to LK) preceding the conserved SGLPA sequence in the CL2/gate domain in PYL2 resulted in strong inhibition of some PP2C (ABI1 and HAB1) activity with the mutated PYL2 (V87L, I88 K):PP2C ratio of 100:1 in the absence of ABA (Hao et al. 2011). These observations suggest that the two residues preceding the CL2/gate domain may play an important role for the constitutive activity of PYLs and thus PYLs-PP2Cs interactions in the absence of ABA.

Similar to AtPYLs-HAB1 interactions (Park et al. 2009), GmPYLs interact with AtABI1 and GmPP2Cs in an ABA-dependent or -independent manner. The presence of VI preceding the SGLPA sequence is correlated with ABA-dependent interaction between GmPYL1-4, GmPYL9-11 and AtABI1; whereas GmPYL15-20, which share the VK at the corresponding position, interact with AtABI1 in an ABA-independent manner. Particularly, GmPYL21 and GmPYL23, in which IT and VT can be found at the corresponding position, respectively, showed an opposite pattern of binding to ABI1. GmPYL21 interacts with ABI1 in an ABA-independent manner, whereas GmPYL23 cannot bind to ABI1. Thus, our results support that the ABA dependence of GmPYLs-AtABI1 interactions are correlated with the identity of the two residues preceding the SGLPA sequence of the CL2/gate domain of GmPYLs: VI for ABA dependent interaction, VK or IT for ABA independent interaction, and VT for no interaction.

However, further investigation of GmPYLs-GmPP2Cs interactions (for example, GmPYL9 or GmPYL11 with GmPP2C1) revealed some exceptions to these rules. Furthermore, site-directed mutagenesis experiments showed that substitution of I by K, and K by I did not always result in the expected change of ABA dependence. Therefore, the ABA dependence of GmPYLs-PP2Cs interactions are only partially correlated with the two amino acid residues preceding the CL2 domain of GmPYLs. Further experiments are necessary to clarify the molecular determinants for the basal activity of PYLs in the absence of ABA and the ABA dependency of GmPYLs-GmPP2Cs interactions.

Arabidopsis PYLs were proposed to fall into two subclasses based on the oligomeric state of apo PYLs (Dupeux et al. 2011; Hao et al. 2011). Recently, Zhang et al. (2012) reported that AtPYL3 can form *trans*-homodimer, and suggested that AtPYR/PYL/RCAR proteins can be separated into three groups: homodimeric (AtPYR1, AtPYL1, and AtPYL2), *trans*-homodimeric (AtPYL3), and monomeric (AtPYL4-6 and AtPYL8-10) PYLs. GmPYL1 and



GmPYL4, two orthologs of AtPYR1 and AtPYL1, form homodimers in plant cells. In contrast, GmPYL16, an ortholog of AtPYL9, is not able to form homodimers in *N. benthamiana* leaves. Whether the oligomeric status of GmPYLs correlates with basal activity of GmPYLs and ABA dependency of GmPYLs-PP2Cs interactions deserves further study.

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