



Research Report

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Functional Analysis of a type-2C Protein Phosphatase (AtPP2C52) in *Arabidopsis thaliana*

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Abstract AtPP2C52 is a plasma membrane type-2C protein phosphatase. In this study, AtPP2C52 promoter-GUS analysis revealed that AtPP2C52 gene was found in a broad expression spectrum with a higher level in the vascular and meristem. AtPP2C52 can interact with multiple proteins, including a proteasome maturation factor, UMP1, and a cysteine proteinase, RD21a, as well as the heterotrimeric G proteins β subunit, AGB1. By mutational analysis of AtPP2C52, it was identified that some residues were essential for AtPP2C52 to bind AGB1, UMP1 and RD21a, suggesting that these proteins should be potential substrates of AtPP2C52.

Keywords Protein phosphatase; Vascular; Protein-protein interaction, *Arabidopsis thaliana*

Background

Protein phosphorylation regulates almost all aspect of cell life (Hunter, 1998; Cohen, 1997). In *Arabidopsis* genome, 112 protein phosphatases have been identified (Kerk et al., 2002). Protein phosphatases have been considered to be much more flexible enzymes, which have a larger number of substrates and present with overlapping activities (Lammers and Lavi, 2007).

Based on the substrate specificity and on the conservation of the catalytic domain, protein phosphatases were grouped into protein serine/threonine (Ser/Thr) phosphatases and protein tyrosine phosphatases. Protein Ser/Thr phosphatases were classified into phosphoprotein phosphatases (PPPs) and metal-dependent protein phosphatases (PPMs). The PPM family contains type-2C protein phosphatase (PP2C) subfamily and pyruvate dehydrogenase phosphatase (Cohen, 1997).

PP2Cs were found in all organisms, such as plants, bacteria, yeast, nematodes, insects, and mammals (Schweighofer et al., 2004). A distinguishing feature of PP2Cs is the requirement of bivalent cation (Mn^{2+} or Mg^{2+}) for their catalytic activity. Meanwhile, the intracellular concentrations of Mg^{2+} and Mn^{2+} do not fluctuate substantially under physiological conditions.

Therefore, the activities of PP2Cs may controlled predominantly by their tissue- or cell type-specific expression, subcellular compartmentalization, post-translational modification, or/and degradation (Lammers and Lavi, 2007).

In *Arabidopsis*, seventy-six PP2C genes were identified (Kerk et al., 2002). These genes were clustered into several groups, based on their sequence similarity (Schweighofer et al., 2004; Xue et al., 2008). Group A PP2C genes are annotated as negative regulators of the ABA response in plant (Hirayama and Shinozaki, 2007). On the other hand, SNF1-related protein kinase 2 (SnRK2) family, which is activated by ABA or osmotic stress, positively regulates the ABA response in various tissues (Mustilli et al., 2002; Yoshida et al., 2002; Fujii et al., 2007). Group A PP2Cs interacted physically with SnRK2s in various combinations, and efficiently inactivated SnRK2s via dephosphorylation of multiple Ser/Thr residues in the activation loop (Umezawa et al., 2009). In response to ABA, PP2C-dependent negative regulation can be canceled by ABA receptors, RCAR/PYRs, leading to activation of positive regulatory pathways (Ma et al., 2009; Park et al., 2009). Group A PP2Cs interacted physically with RCAR/PYRs. Members of Group B PP2C were shown to regulate stomata aperture, seed germination,

abscisic acid inducible gene expression, and interact and inactivate mitogen-activated protein kinase (MAPK, Umbrasaite et al., 2010).

AtPP2C52 was clustered into Group E (Xue et al., 2008). The interaction between the heterotrimeric G proteins β subunit (AGB1) and AtPP2C52 has been confirmed by Y2H analysis and an *in vitro* pull-down assay in our previous work (Tsugama et al., 2012a). Here we proved that AtPP2C52 is expressed in almost all the plant organs with a higher level in the vascular and meristem. AtPP2C52 can interact with UMP1 and RD21a as well as AGB1.

1 Results

1.1 Interaction between AtPP2C52 and AGB1 *in vitro*

AtPP2C52 (GenBank Accession No.: NP_680572) was identified as an AGB1-interacting protein (Tsugama et al., 2012a). Coimmunoprecipitation (Co-IP) was used to confirm the interaction of AtPP2C52 and AGB1 *in vitro* (Figure 1). Myc-tagged AGB1 (Myc:AGB1), HA-tagged AtPP2C52 (HA: AtPP2C52) and HA epitope tag (HA) were synthesized *in vitro* in a rabbit reticulocyte lysate system. Either HA epitope tag or HA:AtPP2C52 was mixed with Myc:AGB1, and then precipitated by anti-HA antibody. Subsequently, G Sepharose was added. After incubation, Myc:AGB1 in the elutant from the G Sepharose was analyzed by immunoblotting using anti-Myc antibody. Specific signals of Myc:AGB1 were detected only when AtPP2C52 was present (Figure 1), indicating that AtPP2C52 interacts with AGB1 *in vitro*.

	Input		IP:HA	
	+	-	+	-
HA	+	-	+	-
HA: AtPP2C52	-	+	-	+
Myc: AGB1	+	+	+	+
WB: Myc				
WB: HA				

Figure 1 AtPP2C52 interacts with AGB1 *in vitro*

Note: HA epitope tag or HA-tagged AtPP2C52 (HA: AtPP2C52) was immunoprecipitated with anti-HA antibody; Western-blot using anti-Myc antibody revealed that anti-HA antibody co-precipitated with Myc-tagged AGB1 (Myc:AGB1) in the presence of HA:AtPP2C52 but not in the presence of HA epitope tag

1.2 P_{AtPP2C52}::GUS analysis

To analyze the temporal-spatial expression pattern of AtPP2C52, transgenic plants expressing a promoter-reporter fusion gene (P_{AtPP2C52}::GUS) were used. P_{AtPP2C52}::GUS was expressed in almost all the plant organs (Figure 2).

In 4-day-old seedlings, P_{AtPP2C52}::GUS was predominantly expressed in vascular, root tip and apical meristem (Figure 2A; Figure 2B). In 3-week-old plants, P_{AtPP2C52}::GUS was evident in the whole plant (Figure 2C). The expression of P_{AtPP2C52}::GUS was still higher in vascular and apical meristem in this stage. In adult plants, P_{AtPP2C52}::GUS was found in all the organs of flower, excepting the anther (Figure 2E; Figure 2F). The expression level of P_{AtPP2C52}::GUS was lower in the sporangia (Figure 2G; Figure 2H).

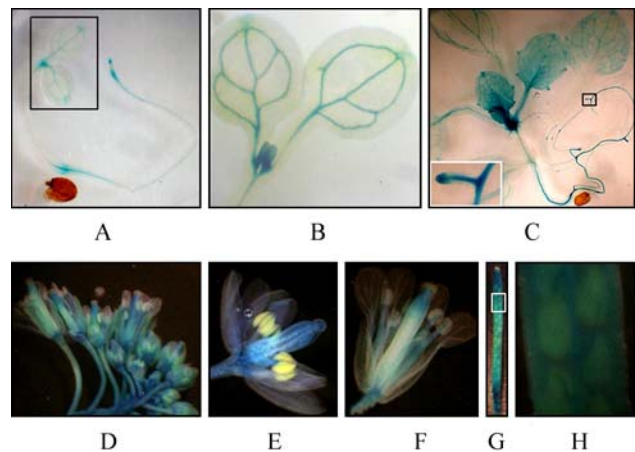


Figure 2 Temporal and spatial expression of AtPP2C52

Note: A: GUS staining showing the expression of P_{AtPP2C52}::GUS in 4-day-old seedlings cultured under SDs; B: Enlarged view indicated by blank rectangle in panel A; C: Three-week-old plants cultured under short day photoperiods; D: Inflorescence; E and F: Flowers at stage 12 and 15, respectively; G: Silique; H: Enlarged view indicated by white rectangle in panel G

1.3 Interaction between AGB1 and site-directed mutants of AtPP2C52

Three site-directed mutants of AtPP2C52 (AtPP2C52^{G99D}, AtPP2C52^{G105D} and AtPP2C52^{DGH102-104ERN}) were generated (Figure 3A). These mutated sites were highly conserved, and they are involved in the PP2C active site (Das et al., 1996; Sheen, 1998). The mutated sequences encoded unrelated amino acids. None of these mutations affected the molecular weight of these mutant proteins (Figure 3B). However, all of these mutations abolished the interaction between AGB1 and AtPP2C52 (Figure 3C).

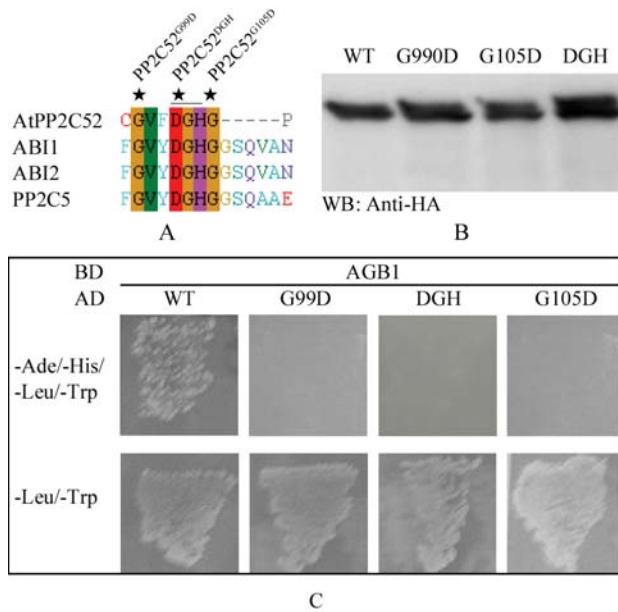


Figure 3 Site-directed mutations abolished the interaction between AtPP2C52 and AGB1

Note: A: AtPP2C52, ABI1 (At4g26080), ABI2 (At5g57050), and PP2C5 (AT2G40180) possess two uniquely conserved G residues around the DGHG (underlined) active site; B: Proteins were expressed by TNT Quick Coupled Transcription/Translation Systems and separated by SDS/PAGE, Anti-HA antibody was used for western blot; C: Y2H analysis; When the reporter genes were activated in the condition that the baits (BD) interacted with the pray (AD), yeast cells can grow on quadruple dropout medium (SD/-Ade/-His/-Leu/-Trp); WT: Wild-type AtPP2C52; G99D: AtPP2C52^{G99D}; G105D: AtPP2C52^{G105D}; DGH: AtPP2C52^{DGH102-104ERN}

1.4 Potential substrates of AtPP2C52

To further identify compartments of the signaling pathway mediated by AtPP2C52, full-length of AtPP2C52 was used as the bait in Y2H screening. Even on high-stringency selection media, more than 2500 positive clones were obtained and 300 clones were sequenced. Among them, a proteasome maturation factor, UMP1, and a cysteine proteinase, RD21a, were used for further analysis.

The interaction of AtPP2C52 with either UMP1 or RD21a was confirmed by Y2H (Figure 4). AtPP2C52^{G99D} and AtPP2C52^{DGH102-104ERN} mutants failed to interact with UMP1 in Y2H (Figure 4A). G105D mutation did not affect the Y2H interaction

between AtPP2C52 and UMP1 (Figure 4A). All of these mutations abolished the Y2H interaction between AtPP2C52 and RD21a (Figure 4B). The interactions were examined by a BiFC assay in Arabidopsis protoplast.

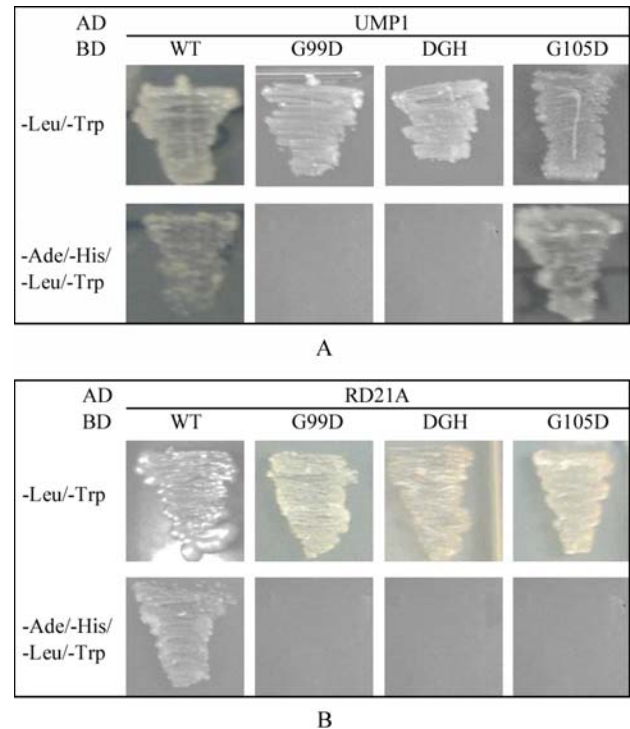


Figure 4 AtPP2C52 interacted with UMP1 and RD21a in Y2H

Note: When the reporter genes were activated in the condition that the baits (BD) interacted with the pray (AD), yeast cells can grow on quadruple dropout medium (SD/-Ade/-His/-Leu/-Trp); WT: Wild-type AtPP2C52; G99D: AtPP2C52^{G99D}; G105D: AtPP2C52^{G105D}; DGH: AtPP2C52^{DGH102-104ERN}

The ORFs of *AGB1*, *UMP1* and *RD21a* were fused downstream of the nYFP and the ORF of *AtPP2C52* was fused upstream of the cYFP. BiFC signals of nYFP-fused *AGB1* and cYFP-fused *AtPP2C52* were detected in the peripheral region of Arabidopsis mesophyll protoplasts (Figure 5) as previously described (Tsugama et al., 2012a). BiFC signals of nYFP-fused *UMP1* and cYFP-fused *AtPP2C52* were also detected in the peripheral region (Figure 5), suggesting that *AtPP2C52* interacted with *UMP1* in the plasma membrane. *AtPP2C52* interacted with *RD21a* not only in the plasma membrane but also in the nucleus (Figure 5). These results suggest that *RD21a* and *UMP1* are the potential substrates of *AtPP2C52*.

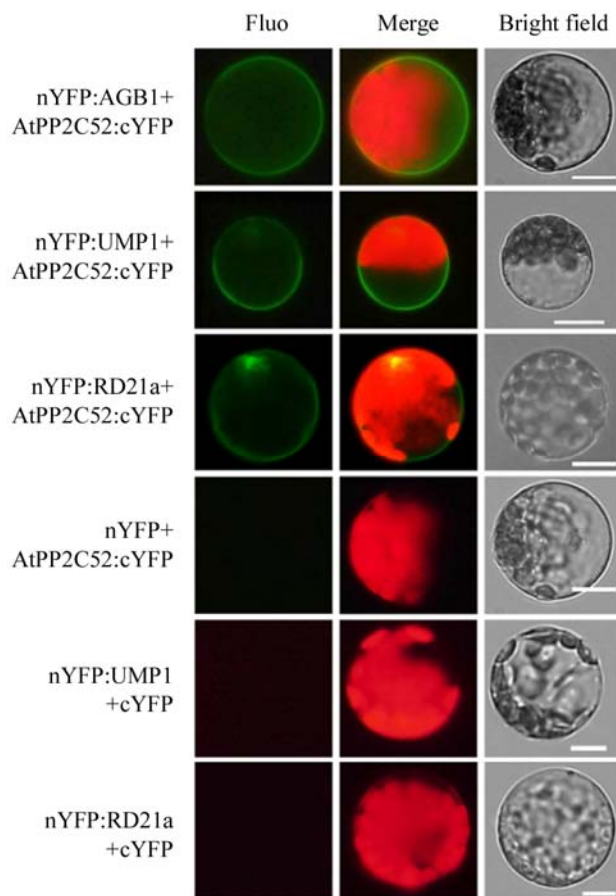


Figure 5 AtPP2C52 interacted with UMP1 and RD21a in BiFC assays in Arabidopsis protoplasts

Note: The combination of AGB1:nYFP+AtPP2C52:cYFP was used as a positive control; The combinations of nYFP+AtPP2C52:cYFP, UMP1:nYFP+cYFP, and RD21a:nYFP+cYFP were used as negative controls to show that these proteins cannot interact with half-YFP; Fluo: fluorescence from YFP; Merge: overlap of Bright field and YFP signal; Scale bars=25µm

2 Discussion

2.1 Expression pattern of *AtPP2C52* gene

Here we proved that $P_{AtPP2C52}::GUS$ was evident in the whole plant in 3-week-old plants (Figure 2C), and $P_{AtPP2C52}::GUS$ was found in most of the flower organs (Figure 2D~F), indicating that *AtPP2C52* gene has a broad expression pattern as other PP2C genes in Arabidopsis (Xue et al., 2008). On the other hand, the expression of $P_{AtPP2C52}::GUS$ was predominantly found in vascular, root tip and apical meristem in 4-day-old seedlings (Figure 2A; Figure 2B). The expression of $P_{AtPP2C52}::GUS$ was lower in the anther

(Figure 2D~2F) and sporangia (Figure 2G; Figure 2H). These results suggest that the expression of *AtPP2C52* gene was in a tissue specific manner at some level.

2.2 Mutational analysis of *AtPP2C52*

These mutated sites of *AtPP2C52* were highly conserved in PP2C family. ABI1 is a well-known member belonging to PP2C Group A (Sheen, 1998). Three *AtPP2C52* mutants, G99D, DGH102-104ERN and G105D, corresponded to the ABI1 mutants, G174D, DGH177-179KLN and G180D, respectively (Sheen, 1998). ABI1 mutations (G174D and DGH177-179KLN) abolished the ability of ABI1 to block ABA-inducible transcription. G174D and DGH177-179KLN were identified as true null mutations of ABI1 owing to dramatic changes in amino acids charges near to or at the PP2C active site. G180D mutation abolished PP2C activity of ABI1. G180D mutant was as effective as wild-type ABI1 in abolishing the ABA-inducible transcription, suggesting G180D mutant blocks ABA responses through a dominant interfering effect without PP2C activity.

Here, G99D and DGH102-104ERN mutations of *AtPP2C52* abolished the ability of *AtPP2C52* to interact with AGB1, UMP1 and RA21a in Y2H assay (Figure 3C, Figure 4). G105D mutation did not affect the Y2H interaction between *AtPP2C52* and UMP1 (Figure 4A). These results suggest that these residues may be essential for *AtPP2C52* to bind specific targets, such as UMP1, RA21a and AGB1. G105D may play a different role from G99D and DGH102-104ERN.

In conclusion, physical interactions between *AtPP2C52* with UMP1, RA21a and AGB1 were confirmed. Mutational analysis shown that G99D, DGH102-104ERN and G105D are essential for *AtPP2C52* to bind specific targets, such as UMP1, RA21a and AGB1.

3 Materials and Methods

3.1 Plant materials and growth conditions

Arabidopsis Col-0 plants were used. Seeds were surface sterilized in 70% (v/v) ethanol solution for 30s, then sterilized in 0.25% (v/v) sodium hypochlorite containing 1% (v/v) Tween X-100 and washed four times in sterile distilled water. Afterward, seeds were sowed on MS plates containing half MS basal salts (Wako) and 1% (w/v) sucrose, pH5.7, solidified with



0.8% (w/v) agar (Wako). Plates were sealed and incubated in 4°C for 72 h in darkness, then transferred to growth chamber incubated at 22°C. Light intensity is 120 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. After ten days of growth, plants were transferred onto rockwool cubes and grown further with 0.2×MS solution regularly supplied.

3.2 Yeast two-hybrid (Y2H)

Y2H experiments were performed as previously described (Tsugama et al., 2012a). cDNA clone RAFL-18-04-O14 of *AtPP2C52* (AT4G03415) was obtained from RIKEN BRC Experimental Plant Division (Seki et al., 2002). The open reading frame (ORF) of *AtPP2C52* was amplified by PCR using the cDNA clone as template and the following primer pair: 5'-CCCGAATTCTCTAGAATGGGGGGTTGTGTGTCGAC-3' (*EcoRI* and *XbaI* sites are underlined) and 5'-GGGCTCGAGGAGTCTTCGATTCTCTTCAGAG-3' (*XhoI* site is underlined). The PCR products were digested by *EcoRI* and *XhoI*, and cloned into the *EcoRI/XhoI* site of pGADT7-rec, generating pGAD-*AtPP2C52*.

The point mutations of *AtPP2C52* gene were generated by PCR using PrimeSTAR (TaKaRa) with wild type *AtPP2C52* cDNA as template. For the point mutation of *AtPP2C52*^{G99D}, PCR was performed using the following primer pair: G99D-Fw 5'-GTGACATTTGTGATGTATTTGATGGTCATGGTCC-3' and *AtPP2C52*-Rv 5'-GAGTCGGATCCTCAAGTCTTCGATTTCTCTTC-3' (*BamHI* site is underlined), generating 3'-terminus of *AtPP2C52*^{G99D}. To generate 5'-terminus of *AtPP2C52*^{G99D}, PCR was performed using the following primer pair: *AtPP2C52*-Fw 5'-GAGTCGAATTCATGGGGGGTTGTGTGTC-3' (*EcoRI* site is underlined) and G99D-Rv 5'-GACCATCAAATACACCACAAAATGTCACATCTCAGAC-3'. Subsequently, the mixture of 3'- and 5'-terminus of *AtPP2C52*^{G99D} was used as template for PCR using primer pair *AtPP2C52*-Fw and *AtPP2C52*-Rv, generating full-length *AtPP2C52*^{G99D}. The PCR products of *AtPP2C52*^{G99D} were digested by *EcoRI* and *BamHI*, and cloned into the *EcoRI/BamHI* site of pGADT7-rec, generating pGAD-*AtPP2C52*^{G99D}. For the point mutation of *AtPP2C52*^{G105D}, PCR was performed using the following primer pair: G105D-Fw 5'-GATGGTCATGATCCTTATGGCCATCTTGTTGCTCG-3' and *AtPP2C52*-Rv, generating

3'-terminus of *AtPP2C52*^{G105D}. To generate 5'-terminus of *AtPP2C52*^{G105D}, PCR was performed using the following primer pair: *AtPP2C52*-Fw and G105D-Rv 5'-GCCATAAGGATCATGACCATCAAATACACCACAAAATG-3'.

Subsequently, the mixture of 3'- and 5'-terminus of *AtPP2C52*^{G105D} was used as template for PCR using primer pair *AtPP2C52*-Fw and *AtPP2C52*-Rv, generating full-length *AtPP2C52*^{G105D}. The PCR products of *AtPP2C52*^{G105D} were digested by *EcoRI* and *BamHI*, and cloned into the *EcoRI/BamHI* site of pGADT7-rec, generating pGAD-*AtPP2C52*^{G105D}. For the point mutation of *AtPP2C52*^{DGH102-104ERN}, PCR was performed using the following primer pair: DGH102-104ERN-Fw 5'-GGTGTATTTGAACGTAATGGTCCTTATGGCCATCTTG-3' and *AtPP2C52*-Rv, generating 3'-terminus of *AtPP2C52*^{DGH102-104ERN}. To generate 5'-terminus of *AtPP2C52*^{DGH102-104ERN}, PCR was performed using the following primer pair: *AtPP2C52*-Fw and DGH102-104ERN-Rv 5'-CATAAGGACCATTACGTTCAAATACACCACAAAATGCG-3'. Subsequently, the mixture of 3'- and 5'-terminus of *AtPP2C52*^{DGH102-104ERN} was used as template for PCR using primer pair *AtPP2C52*-Fw and *AtPP2C52*-Rv, generating full-length *AtPP2C52*^{DGH102-104ERN}. The PCR products of *AtPP2C52*^{DGH102-104ERN} were digested by *EcoRI* and *BamHI*, and cloned into the *EcoRI/BamHI* site of pGADT7-rec, generating pGAD-*AtPP2C52*^{DGH102-104ERN}. These mutations were confirmed by sequencing and then used for yeast transformation.

Full-length cDNA of *UMP1* (AT1G67250) and *RD21a* (AT1G47128) were ordered from ABRC and used as PCR templates for following plasmids construction. The ORF of *UMP1* was amplified by PCR using the cDNA clone as template and the following primer pair: 5'-GAGTCGAATTCATGGAGTCTGAGAAAAAGATAGCTCATG-3' (*EcoRI* site is underlined) and 5'-GAGTCGGATCCTTACATGAACTTGGGTAATCGG-3' (*BamHI* site is underlined). The PCR products were digested by *EcoRI* and *BamHI*, and cloned into the *EcoRI/BamHI* site of pGADT7-rec, generating pGAD-*UMP1*. The ORF of *RD21a* was amplified by PCR using the cDNA clone as template and the following primer pair: 5'-GAGTCGAATTCATGGGGTTCCTTAAGCCAACCATGGC-3' (*EcoRI* site is underlined) and 5'-GAGTCCCTAGGTTAGGC



AATGTTCTTTCTGCCTTGTGACCAG-3' (*Bam*HI site is underlined). The PCR products were digested by *Eco*RI and *Bam*HI, and cloned into the *Eco*RI/*Bam*HI site of pGADT7-rec, generating pGAD-*RD21a*.

Yeast transformation using yeast strain AH109 and screening were performed by Matchmaker Gold Yeast Two-Hybrid System (Clontech). At least 5 colonies grown on the SD media lacking leucine and tryptophan (SD/-Leu/-Trp), were streaked on the SD/-Leu/-Trp and the SD media lacking leucine, tryptophan, adenine, and histidine (SD/-Trp/-Leu/-His/-Ade). Photos were taken after the yeasts were cultured for 3-5 days. The experiments were performed for three times.

3.3 *In vitro* Coimmunoprecipitation (Co-IP)

Proteins were expressed by TNT Quick Coupled Transcription/Translation Systems (Promega). Plasmid DNA pGBK-*AGB1*, pGAD-*AtPP2C52*, pGAD-*AtPP2C52*^{G99D}, pGAD-*AtPP2C52*^{G105D}, pGAD-*AtPP2C52*^{DGH102-104ERN} and pGADT7-rec vector were used to synthesize proteins Myc-tagged *AGB1*, HA-tagged *AtPP2C52* and HA epitope tag, respectively. Co-IP was carried out using anti-HA antibody (MBL), anti-Myc antibody (MBL) and protein G Sepharose (GE Healthcare) following the MATCHMAKER Co-IP Kit User Manual (Cat No. 630449, 2003).

3.4 Bimolecular Fluorescence Complementation (BiFC) assay

The vectors for BiFC assay were constructed by replacing GFP in the vector pBS-35SMCS-GFP (Tsugama et al., 2012b) with the N-terminus (154 amino acids) or the C-terminus (80 amino acids) of YFP, generating pBS-35SMCS-nYFP and pBS-35SMCS-cYFP, respectively. The pGAD-*AtPP2C52* was digested by *Xba*I and *Xho*I, and the resultant ORF fragments of *AtPP2C52* were inserted into the *Xba*I/*Sal*I site of pBS-35SMCS-cYFP, generating pBS-35S-*AtPP2C52*-cYFP. The ORF of *UMP1* without stop codon was amplified by PCR using the cDNA clone as template and the following primer pair: 5'-GAGTCGGTACCATGGGGTTCCTTAAGCCAC-3' (*Kpn*I site is underlined) and 5'-CTCGA ACTAGTGGCAATGTTCTTTCTGC-3' (*Spe*I site is underlined). The PCR products were digested by *Kpn*I and *Spe*I, and cloned into the *Kpn*I/*Spe*I site of

pBS-35SMCS-nYFP, generating pBS-35S-nYFP-*UMP1*. The ORF of *RD21a* without stop codon was amplified by PCR using the cDNA clone as template and the following primer pair: 5'-GAGTC GGTACCATGGAGTCTGAGAAAAAGATAGC-3' (*Kpn*I site is underlined) and 5'-CTCGAACTAGT CATGAAACTTGGGTAAATCGG-3' (*Spe*I site is underlined). The PCR products were digested by *Kpn*I and *Spe*I, and cloned into the *Kpn*I/*Spe*I site of pBS-35SMCS-nYFP, generating pBS-35S-nYFP-*RD21a*. Arabidopsis protoplast isolation and transformation were conducted as described (Wu et al., 2009). Plasmids DNA were introduced into onion epidermal cells by a bombardment system (Bio Red, PDS-1000). Images were processed using Canvas X software (ACD Systems) and enhanced using Photoshop CS4 software (Adobe).

3.5 Preparation of chimeric constructs

An approximately 2 kb upstream promoter sequence of *AtPP2C52* ($P_{AtPP2C52}$) was cloned from an Arabidopsis genetic DNA by using primer pair: 5'-GGATCCCCGGGATGAATCATGTAGGTGAC-3' (*Sma*I site is underlined) and 5'-CCCTCTAGA TGTTTAATCCCAGCCTAGA-3' (*Xba*I site is underlined). The PCR products were double-digested with *Sma*I/*Xba*I and used to replace the CaMV 35S promoter of pBI121 vector (Clontech, Bevan, 1984), generating pBI121- $P_{AtPP2C52}$::*GUS*.

3.6 Plant transformation

Arabidopsis Col-0 plants were transformed by *Agrobacterium tumefaciens*-mediated transformation using the floral-dip method (Clough and Bent, 1998). The *Agrobacterium* strain used was EHA105 harboring binary vector pBI121 carrying the $P_{AtPP2C52}$::*GUS* fusion gene.

3.7 GUS histochemical analysis

For GUS histochemical characterization of the $P_{AtPP2C52}$::*GUS* lines, several developmental stages were examined. Samples were treated with 90% acetone for 30 min at 4°C, immersed in a staining solution (5 mmol/L X-gluc, 0.5 mmol/L $K_3Fe(CN)_6$, 0.1% Triton X-100, 0.5 mmol/L $K_4Fe(CN)_6$, 10 mmol/L Na_2EDTA and 50 mmol/L sodium phosphate buffer, pH 7.0), vacuum infiltrated for more than 1 hour, then incubated at 37°C. After staining, samples were cleared by several changes of 70%



ethanol. Photographs were enhanced using Photoshop CS4 software (Adobe).

Author Contributions

TetsuoTakano and Shenkui Liu are responsible for overall planning and management. Collecting data and article writing were completed by Hua Liu and Daisuke Tsugama. Manuscript was completed by TetsuoTakano and Hua Liu.

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