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# A putative myristoylated 2C-type protein phosphatase, PP2C74, interacts with SnRK1 in Arabidopsis

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**_N_-myristoylation is a lipid modification of many signaling proteins in which myristate is added to an N-terminal glycine residue. Here we show that PP2C74, a putative myristoylated 2C-type protein phosphatase (PP2C) in Arabidopsis, is transcribed in various tissues and has protein phosphatase activity. GFP-fused PP2C74 localized to the plasma membrane, but not when a glycine residue at position 2, which is the putative myristoylation site, was substituted with an alanine residue. Yeast two-hybrid analysis and GST pull-down analysis showed that PP2C74 interacts with AKIN10, the catalytic α subunit of the SnRK1 protein kinase complex, the β subunits of which are known targets of myristoylation.**

*Structured summary of protein interactions*:
**AKIN10** physically interacts with **PP2C74** by two hybrid (View interaction)
**AKIN10** physically interacts with **PP2C74** by pull down (View interaction)

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## 1. Introduction

*N*-myristoylation (MYR) is a process in which myristate, a C: 14 fatty acid, is added to the N-terminal glycine of a subset of proteins. Myristoylated proteins are likely to be localized to the plasma membrane, where they regulate membrane properties and signal transduction on the plasma membrane ([1], for a review). MYR is catalyzed by *N*-myristoyltransferase (NMT). Arabidopsis has two *NMT* homologs, *AtNMT1* and *AtNMT2*. *AtNMT1* knockout causes severe growth arrest in the early stage of development, while *AtNMT2* knockout has less severe effects, suggesting that *AtNMT1* and *AtNMT2* have different functions [2]. The NMT activity of AtNMT1 was confirmed [2,3] while AtNMT2 has not been biochemically characterized. Based on the biochemical properties of AtNMT1, 422 proteins in Arabidopsis were predicted to be substrates of AtNMT1 ([3], listed in http://www.isv.cnrs-gif.fr/tm/maturation/myristoylome2007am.htm). Many of these proteins are protein kinases and protein phosphatases as well as other signaling molecules. Phosphorylation relays on the plasma membrane initiate signal transduction from extracellular ligands to downstream pathways in brassinosteroid signaling ([4], for a review) and MAMP (microbe-associated molecular pattern) signaling [5,6]. Functional membrane proteins such as SOS1 and AtbohF are regulated by their phosphorylation states [7,8]. Myristoylated protein kinases and protein phosphatases may be involved in such processes.

One of the AtNMT1 substrates is PP2C74, which is a putative 2C-type protein phosphatase (PP2C). In this study, we examined the transcription pattern of *PP2C74* and its biochemical function and subcellular localization. We found that PP2C74 interacts with the catalytic subunit of SnRK1, which is a target of regulation by AtNMT1 [2]. To our knowledge, this is the first report on functions of a potential myristoylated PP2C in plants.

## 2. Materials and methods

### 2.1. Reverse-transcription-PCR (RT-PCR)

*Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used as the plant material. Seeds were surface sterilized and sown on 0.8% agar (Wako) containing 0.5× MS salts (Wako), 1% w/v sucrose and 0.5 g/l MES, pH 5.8, chilled at 4 °C in the dark for 48 h (stratified), and germinated at 22 °C. Plants were grown at 22 °C under 16-h light/8-h dark condition (light intensity 120 $\mu$mol $m^{-2}$ $s^{-1}$). After 3 weeks of growth, plants were transferred onto rockwool cubes and grown further with 0.2× MS solution regularly supplied. For

*Abbreviations:* SnRK, sucrose non-fermenting-1-related protein kinase; PP2C, 2C-type protein phosphatase; AKIN10, Arabidopsis SNF1 kinase homolog 10; MYR, *N*-myristoylation; NMT, *N*-myristoyltransferase; MAMP, microbe-associated molecular pattern; qRT-PCR, quantitative reverse transcription-PCR; Y2H, yeast two-hybrid; AKINβ1, Arabidopsis SNF1 kinase β subunit 1

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RNA extraction, leaves, roots, flowers and flower stalks from mature plants and 2-week-old seedlings were sampled.

Total RNA was prepared using RNeasy Plant Mini Kit (Qiagen) and cDNA was synthesized from 3 µg of the total RNA with PrimeScript Reverse Transcriptase (Takara Bio) using an oligo (dT) primer. The reaction mixtures were diluted 20 times with distilled water and used as a template for PCR. Real-time quantitative RT-PCR (qRT-PCR) analysis of *PP2C74* (AT5G36250, [9]) expression was performed using the following primer pair: 5′-GGGAGTTGC CCGTCTGTGGC-3′ and 5′-CGCACTGCAGCCTCCACCAA-3′, GoTaq qPCR Master Mix (Promega) and a StepOne Real-Time PCR System (Applied Biosystems). Relative expression levels were calculated by the comparative $C_T$ method using *UBQ5* as an internal control gene.

### 2.2. Protein phosphatase assay

For protein expression in *Escherichia coli* and purification, the open reading frame (ORF) of *PP2C74* was amplified by RT-PCR using the following primer pair: 5′-CCCGTCGACCATATGGGGTCCTG CTTATCATC-3′ as the forward primer and 5′-CCCGTCGACACT-CTTTGGTTGGGACATATAC-3′ as the reverse primer (*Sal*I sites are underlined). The PCR products were digested by *Sal*I and inserted into the *Sal*I site of pGEX-6P-3 (GE Healthcare) in-frame to the coding sequence of glutathione S-transferase (GST). The resultant plasmid was transformed into the *E. coli* strain, BL21 (DE3). To induce GST-fused PP2C74 (GST-PP2C74), transformed *E. coli* cells were cultured at 37 °C in LB medium until $OD_{600}$ reached 0.5, and incubated at 28 °C for 2 h after addition of IPTG to a final concentration of 0.1 mM. The cells were then harvested by centrifugation and resuspended in 1× TBS (150 mM NaCl in 20 mM Tris–HCl, pH 7.5) with 20 µg/ml lysozyme (Wako). The cell suspension was frozen at −80 °C and thawed at room temperature. Freezing and thawing were repeated two more times to lyse the cells, and 2 units of recombinant DNase I (Takara Bio) was added to the solution. The solution was incubated at room temperature until the solution became fluid due to DNA degradation. The solution was then centrifuged at 12 000×*g* for 5 min and the supernatant was used as crude protein extracts. Different volumes (25, 50 and 75 µl) of the crude extracts were adjusted to a final volume of 75 µl by adding 1× TBS. Forty microliters of 50% slurry of Glutathione Sepharose 4 Fast Flow was added to each tube and the solutions were incubated for 30 min at room temperature with gentle shaking to bind GST-PP2C74 to the resin. The resin was washed 4 times by an excess amount of 1× TBS. This resin-bound GST-PP2C74 was used for the protein phosphatase assay.

Protein phosphatase assay was performed using Non-Radioactive Serine-Threonine Phosphatase Assay System (Promega). The slurry of the resin with GST-PP2C74 was centrifuged briefly at 6000×*g* and the supernatant was carefully removed. The resin was then resuspended in 50 µl of the phosphatase assay solution (20 mM $MgCl_2$, 0.1 mM phosphopeptide (RRApTVA), 50 mM Tris–HCl, pH 7.5). The slurry was incubated at room temperature for 60 min with gentle shaking, centrifuged, and the supernatant (50 µl) was transferred to another tube. Fifty microliters of Molybdate dye/Additive solution was then added to the supernatant and the solution was incubated for 20 min at room temperature to fully develop a color in the solution. $A_{600}$ of the solution was measured and the amounts of released phosphate were calculated by a standard curve obtained from known concentrations of phosphate standard solutions. The resin remained in each tube was boiled for 5 min in 2× SDS sample buffer (0.125 M Tris–HCl, pH 6.8, 10% v/v β-mercaptoethanol, 4% w/v SDS, 10% w/v sucrose and 0.01% w/v bromophenol blue), and the supernatant was subjected to immunoblot analysis using an anti-GST antibody (GE Healthcare) to compare the amounts of GST-PP2C74 used for the phosphatase assay. For a negative control, GST alone, which was derived from the empty pGEX-6P-3 vector, was used for the phosphatase assay as described above.

### 2.3. Subcellular localization study of GFP-fused proteins

To create plasmids for expressing GFP-fused proteins, pBI121 was digested by *Hin*dIII and *Eco*RI to obtain the DNA fragment containing CaMV35S promoter-*GUS*-Nos terminator. This fragment was blunted by T4 DNA polymerase (Takara Bio) and cloned into pBluescript II SK$^-$ which was digested by *Sac*I and *Kpn*I and blunted by T4 DNA polymerase. The resultant plasmid was digested by *Xba*I and *Sac*I and ligated with the mixture of the following oligonucleotides: 5′-CTAGACTGGTACCCGGGTCGACTGACTAGTACGAGCT-3′ and 5′-CGTACTAGTCAGTCGACCCGGGTACCAGT-3′ (sequences that make an end to be ligated to *Xba*I or *Sac*I are underlined). The ORF of *GFP* (*EGFP*) was amplified by PCR using pEGFP-N1 (Clontech) as a template and the following primer pair: 5′-AAACTAGTATGGT GAGCAAGGGCGAGGAGC-3′ and 5′-AAGAGCTCTTACTTGTACAG CTCGTCCATG-3′ (*Spe*I and *Sac*I sites are underlined), and inserted into the *Spe*I-*Sac*I site of the plasmid above. Thus pBS-35SMCS-GFP, which has the CaMV35S promoter and restriction sites upstream of *EGFP*, was generated. The ORF of *PP2C74* was amplified as described above, digested by *Sal*I, and inserted into the *Sal*I site of pBS-35SMCS-GFP, generating pBS-35S-PP2C74-GFP. To introduce the glycine→alanine mutation at position 2 (G2A mutation) of PP2C74, the ORF of *PP2C74* was amplified by RT-PCR using the same *PP2C74*-specific reverse primer as described above and the following forward primer: 5′-GAGGTCGACCATATGGCGTCCTGCT TATCATCATCTGG-3′ (the *Sal*I site is underlined). The PCR products were digested by *Sal*I, and inserted into the *Sal*I site of pBS-35SMCS-GFP. To create a tonoplast marker plasmid to transform Arabidopsis protoplasts, vac-rk, which contains a tonoplast marker gene, *γ-TIP-mCherry* [10], was obtained from the Arabidopsis Biological Resource Center (ABRC, http://www.arabidopsis.org).

Transient expression of GFP-fused proteins in Arabidopsis mesophyll protoplasts was performed as previously described [11,12]. Transient expression of GFP-fused proteins in onion cells was achieved by particle bombardment and was detected by fluorescence microscopy as previously described [13]. To co-express PP2C74-GFP and γTIP-mCherry in onion cells, the cells were bombarded with particles coated with a mixture of 500 ng each of pBS-35S-PP2C74-GFP and vac-rk. Images were processed with Canvas X software (ACD Systems).

### 2.4. Yeast two-hybrid (Y2H) assay and GST pull-down assay

Y2H experiments were performed using Matchmaker Two-Hybrid System (Clontech). The ORF of *PP2C74* was amplified as described above, digested by *Nde*I and *Sal*I, and inserted into the *Nde*I-*Xho*I site of pGADT7-rec, generating pGADT7-PP2C74. The ORF of *PP2C1* (AT1G03590, [9]) was amplified by RT-PCR using the following primer pair: 5′-GAGGTCGACCATATGGGAGGTTG TATCTCTAAG-3′ and 5′-GAGGTCGACAAGTCTTTGGTTCCTCTCCAG GG-3′ (*Nde*I and *Sal*I sites are underlined). The PCR products were digested by *Nde*I and *Sal*I, and inserted into the *Nde*I–*Sal*I site of pGADT7-rec. The ORF of *AKIN10* (AT3G01090) was amplified by RT-PCR using the following primer pair: 5′-GAGGTCGACCAT ATGTTCAAACGAGTAGATGAG-3′ and 5′-GAGGTCGACAGAGGACTCG GAGCTGAGCAAG-3′ (*Nde*I and *Sal*I sites are underlined). The PCR products were digested by *Nde*I and *Sal*I, and inserted into the *Nde*I–*Sal*I site of pGBKT7. The ORF of *AKIN11* (AT3G29160) was amplified using the following primer pair: 5′-GAGGTCGACGCCAT GGATCATTCATCAAATAG-3′ and 5′-CCCGTCGACACTCTTTGGTTGG GACATATAC-3′ (*Nco*I and *Sal*I sites are underlined). The PCR products were digested by *Nco*I and *Sal*I, and inserted into the *Nco*I–*Sal*I

site of pGBKT7. Constructs were co-introduced into the *Saccharomyces cerevisiae* strain, AH109. The combinations of constructs are shown in Fig. 4A. After transformation, at least 4 colonies grown on the SD media lacking leucine and tryptophan (SD/-Leu/-Trp), were streaked on the SD/-Leu/-Trp and the SD media which lacks leucine, tryptophan and histidine and contains 10 mM 3-amino-1,2,4-triazole (Sigma) to check reporter gene (*HIS3*) expression activated by a positive interaction between proteins of interest.

For the GST pull-down assay, the ORF of *AKIN10* was amplified as described above, digested by *Sal*I, and inserted into the *Sal*I site of pGEX-6P-3. Crude protein extracts containing GST-fused AKIN10 (GST-AKIN10) were prepared as described above. To express HA-tagged PP2C74 (HA-PP2C74) in *E. coli*, the ORF of *HA-PP2C74* was amplified by PCR using pGADT7-PP2C74 as a template, the same *PP2C74*-specific reverse primer as described above, and the following forward primer: 5′-CCCACTAGTATGGAGTACCCATACGACGTA-3′ (the *Spe*I site is underlined). The PCR products were digested by *Spe*I and *Sal*I, and inserted into the *Nhe*I-*Xho*I site of $pRSET_B$ (Invitrogen). The resultant plasmid was introduced into BL21 (DE3). Transformed *E. coli* cells were cultured overnight at 37 °C, collected by centrifugation, and lysed to prepare crude protein extracts as described above. Addition of IPTG was not necessary to express HA-PP2C74. GST-AKIN10 was bound to Glutathione Sepharose 4 Fast Flow and washed 4 times by 1× TBS as described above. After removing 1× TBS, the resin was resuspended in the crude extracts containing HA-PP2C74 and incubated at room temperature for 60 min with gentle shaking. The resin was then washed 4 times by 1× TBS and resuspended in 20 mM reduced glutathione in Tris–HCl, pH 8.0. The suspension was incubated at room temperature for 15 min to elute GST-AKIN10. The slurry of the resin was centrifuged for a few minutes at 12000×*g* and the supernatant was transferred to another tube. GST-AKIN10 and HA-PP2C74 in the solution were analyzed by immunoblotting using an anti-GST antibody and an anti-HA antibody (MBL), respectively. To detect signals of HA-PP2C74 in the GST pull-down assay, SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific) was used. For the other immunoblot experiments, SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) was used.

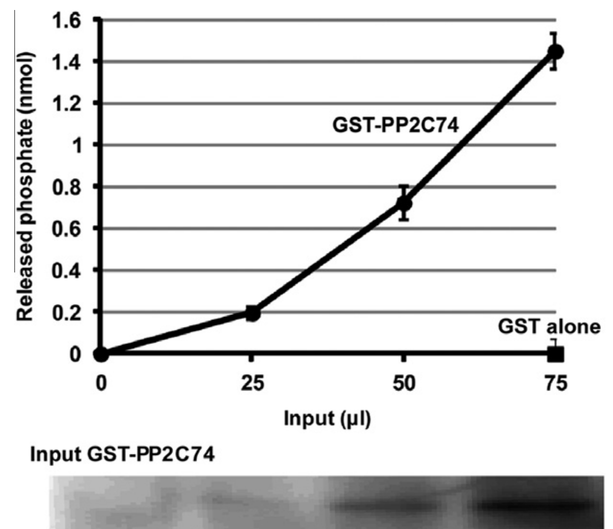


**Fig. 2.** Effect of GST-PP2C74 on phosphate release from the substrate. GST-PP2C74 was expressed in *E. coli* and used as a resin-bound form. Inputs of crude *E. coli* extracts for protein purification are shown on the *x* axis. GST-PP2C74 was eluted from the resin after the protein phosphatase assay and detected by immunoblotting for verification of the amounts of GST-PP2C74 (lower panel). Experiments were performed in triplicate. Values are means ± S.E.

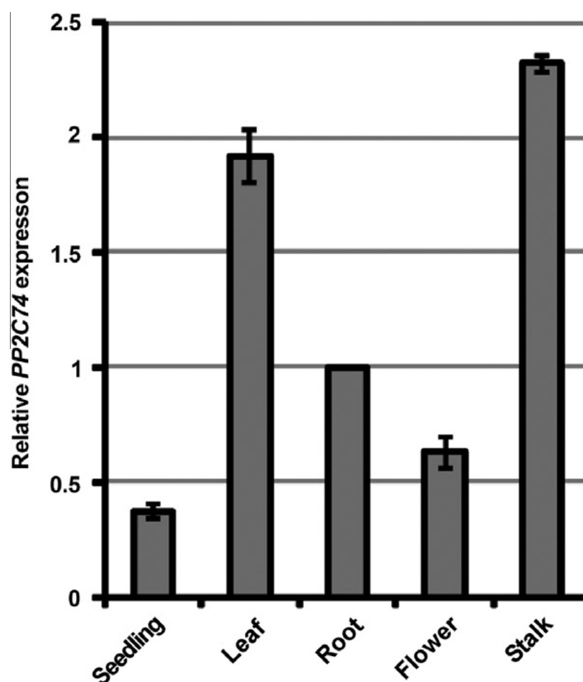


**Fig. 1.** Quantitative RT-PCR analysis of *PP2C74* mRNA expression in various tissues. Relative expression levels were calculated by the comparative $C_T$ method using *UBQ5* as an internal control gene and root sample as a reference sample. Experiments were performed in triplicate. Values are means ± S.E.

## 3. Results and discussion

### 3.1. Expression of PP2C74 mRNA

*PP2C74* mRNA expression was observed in all the tissues studied, being highest in flower stalks and lowest in seedlings (Fig. 1).

### 3.2. PP2C74 has protein phosphatase activity

To confirm that PP2C74 functions as a protein phosphatase, an in vitro phosphatase assay was performed using GST-PP2C74 expressed in *E. coli*. The amounts of phosphate released from a phosphatase substrate correlated with the inputs of GST-PP2C74 (Fig. 2), confirming the protein phosphatase activity of PP2C74.

### 3.3. PP2C74 is localized to the plasma membrane by MYR

Subcellular localization of PP2C74 was examined using GFP-fused PP2C74. A previous study showed that PP2C74 is localized to the nucleus when GFP is fused to the N-terminus of PP2C74 [14]. However, because PP2C74 has a putative MYR site in its N terminus, GFP was fused to the C terminus of PP2C74 in this study. PP2C74G2A, in which the putative MYR site, a glycine at position 2, was substituted with alanine, was also examined. When expressed in Arabidopsis mesophyll protoplasts, the fluorescence of wild-type PP2C74-GFP was limited to the peripheral region of cells (Fig. 3A, upper panels), while the fluorescence of PP2C74G2A-GFP was dispersed throughout the cytoplasm (Fig. 3A, lower panels). These results are consistent with the finding that the same mutation in two other AtNMT1 substrates, AKINβ1 and AKINβ2, disrupted their plasma-membrane localization [2]. PP2C74-GFP and PP2C74G2A were also expressed in onion epidermal cells. PP2C74-GFP fluorescence was detected in the cell periphery and PP2C74G2A

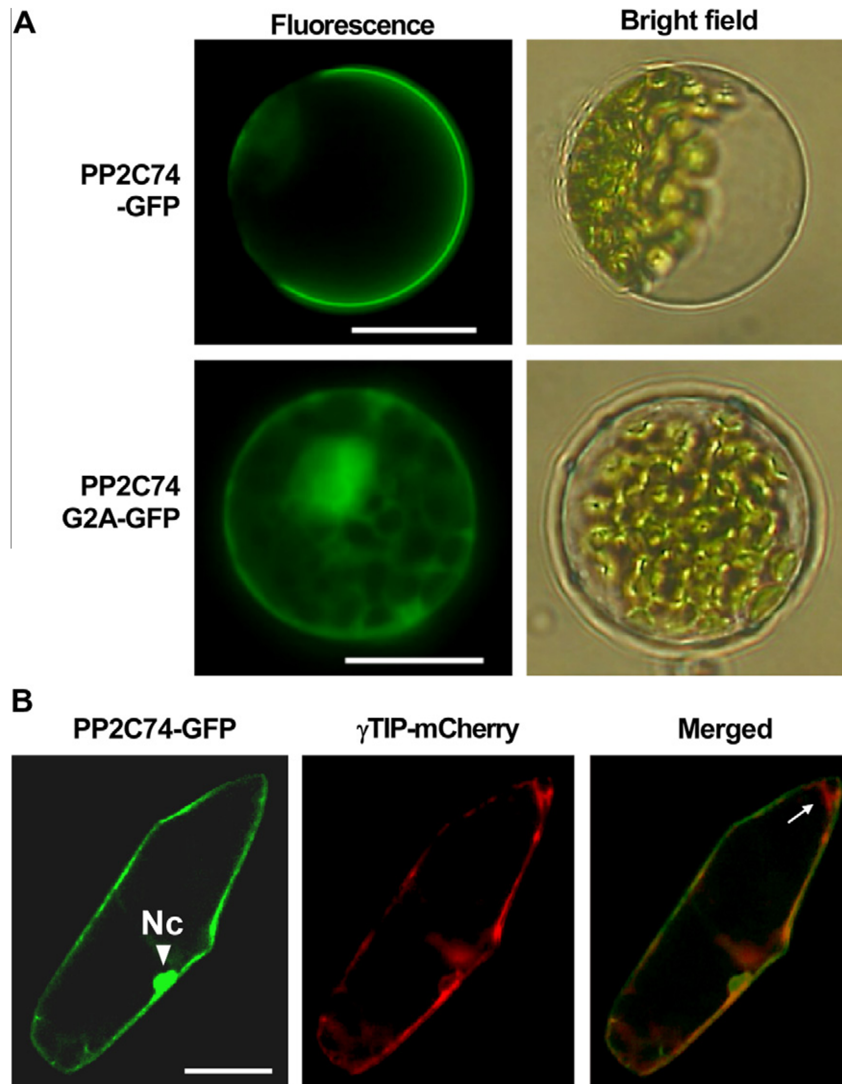


**Fig. 3.** (A) Subcellular localization of PP2C74-GFP in Arabidopsis mesophyll protoplasts. PP2C74G2A is a mutated PP2C74 with a glycine→alanine substitution at position 2. More than 20 transformed cells were observed and a representative cell is shown for each construct. Scale bars = 20 μm. (B) Subcellular localization of PP2C74-GFP in onion epidermal cells. The nucleus is indicated as Nc in the left panel. The arrow in the right panel shows separation of the γTIP-mCherry (tonoplast marker) fluorescence from the plasma membrane. Scale bar = 100 μm.

fluorescence was detected throughout the cytoplasm as in Arabidopsis protoplasts, but in onion cells, the fluorescence of wild-type PP2C74-GFP was detected not only on the plasma membrane but also in the nucleus (Supplementary Fig. 1, upper panel, and Fig. 3B). Although the nuclear localization of PP2C74-GFP in onion cells may be an artifact of heterologous overexpression, a motif-scanning program (cNLS Mapper, http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi [15]) predicted the presence of three nuclear localization signals in the N-terminal region (positions 11–57) of PP2C74. Thus the nuclear localization of PP2C74 would be possible under physiological conditions. To further examine the subcellular localization of PP2C74, PP2C74-GFP was co-expressed with a tonoplast marker, γTIP-mCherry [10], in onion cells. The fluorescence of γTIP-mCherry was detected from the inner side of the cell periphery while that of PP2C74-GFP was not (Fig. 3B), suggesting that PP2C74 exists predominantly in the plasma membrane rather than in the tonoplast. PP2C74 has three potential palmitoylation sites (positions 4, 377 and 381) (predicted by CSS Palm, http://css-palm.biocuckoo.org/prediction.php [16]). Palmitoylation reversibly attaches palmitate, a C: 16 fatty acid, to specific cysteine residues of target proteins. The plasma membrane localization of myristoylated proteins can be stabilized by palmitoylation ([1], for a review), thus the localization of PP2C74 may be controlled by palmitoylation as well as by MYR.

### 3.4. PP2C74 interacts with AKIN10

Among the 422 substrates of AtNMT1, SnRK1 is thought to be especially important [2]. SnRK1 is activated by phosphorylation of its catalytic α subunit [17–19]. To examine whether PP2C74 is involved in regulation of SnRK1, physical interactions between PP2C74 and AKIN10, one of the α subunits of SnRK1, were analyzed. AKIN10 was chosen for analyses because AKIN10 is the best-characterized α subunit of SnRK1 in Arabidopsis and has been shown to have significant effects on plant growth [17–22].

First Y2H interactions between PP2C74 and AKIN10 were studied. GAL4 activation domain-fused PP2C74 and GAL4 DNA-binding domain-fused AKIN10 were co-expressed in AH109 cells and reporter gene activation was checked by culturing cells on the selection medium which lacked histidine. Yeast cells could grow on the selection medium when both PP2C74 and AKIN10 were present, but not when either of them was absent, indicating that PP2C74 and AKIN10 interact with each other in yeast cells. PP2C1, another putative myristoylated PP2C in Arabidopsis, and

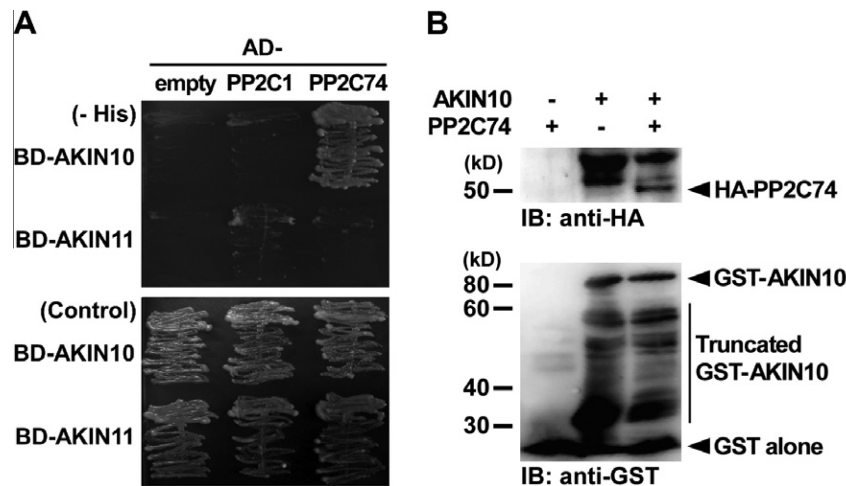


**Fig. 4.** Interactions between PP2C74 and AKIN10. (A) Y2H interactions. The pGADT7-rec plasmid containing no insert, *PP2C1* or *PP2C74* (AD-empty, AD-PP2C1 or AD-PP2C74, respectively) and the pGBKT7 plasmid containing no insert, AKIN*10* or *AKIN11* (BD-empty, BD-AKIN10 or BD-AKIN11, respectively) were co-introduced into AH109 in the indicated combinations. Yeast cells were cultured on media with histidine (Control) or without histidine (-His) to check activation of the reporter gene, *HIS3*. (B) In vitro GST pull-down assay. GST-AKIN10 and HA-PP2C74 were expressed in *E. coli* and used for the analysis. The presence or absence of each protein in the reaction mixture is shown as + or −, respectively. Experiments were performed 4 times and a representative result is shown. Antibodies used for immunoblotting are shown as IB: anti-.

AKIN11, a homolog of AKIN10, were also subjected to the Y2H assay, but none of the combinations except the combination of PP2C74 and AKIN10 showed a positive interaction (Fig. 4A).

To examine whether PP2C74 interacts with AKIN10 in vitro, a GST pull-down assay was performed. GST-fused AKIN10 (GST-AKIN10) was bound to resin and mixed with a solution containing HA-tagged PP2C74 (HA-PP2C74). After incubation, GST-AKIN10 was eluted from the resin and HA-PP2C74 in the elutant was analyzed by immunoblotting. Specific signals of HA-PP2C74 were detected only when both PP2C74 and AKIN10 were present (Fig. 4B), indicating that PP2C74 interacts with AKIN10 in vitro.

A previous study showed that a human PP2C can dephosphorylate and inactivate $\alpha$ subunits of SnRK1 from spinach in vitro [17]. Our results suggest that the interaction between PP2C74 and AKIN10 is specific (Fig. 4). This kind of specific interaction may enhance the efficiency of dephosphorylation of the $\alpha$ subunits of SnRK1 by PP2Cs. Because PP2C74 is localized to the plasma membrane (Fig. 3), the PP2C74–AKIN10 interaction probably occurs on the plasma membrane. Previous studies suggest that the SnRK1 complex is sequestered at the plasma membrane via MYR of its $\beta$ subunit [2]. Therefore PP2C74 may contribute to inactivation of the SnRK1 complex on the plasma membrane.

To our knowledge, this is the first report on the functions of a potential myristoylated PP2C in plants. Although direct MYR of PP2C74 by NMT remains to be demonstrated, the plasma membrane localization of PP2C74 and its disruption by the G2A mutation strongly support the idea that PP2C74 is myristoylated. It is notable that PP2C74 interacts with the SnRK1 protein kinase complex, which is also a target of MYR. Further study of PP2C74 may contribute to the elucidation of the signaling network among myristoylated proteins and other membrane proteins.

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## Appendix A. Supplementary data

Supplementary data (subcellular localization of PP2C74-GFP in onion epidermal cells. PP2C74G2A is a mutated PP2C74 with a glycine→alanine substitution at position 2. For wild-type PP2C74-GFP (upper panel), the nucleus is shown as Nc. More than 20 transformed cells were observed and a representative cell is shown for each construct. Scale bars = 100 μm) associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.02.019.

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