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The Arabidopsis Homolog of Yeast TAP42 and Mammalian α4 Binds to the Catalytic Subunit of Protein Phosphatase 2A and Is Induced by Chilling[1]

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Type 2A serine/threonine protein phosphatases (PP2A) have been implicated as important mediators of a number of plant growth and developmental processes. In an effort to identify plant PP2A substrates and/or regulators, we performed a yeast two-hybrid screen using an Arabidopsis PP2A catalytic subunit cDNA as bait. All true positives identified by this screen were derived from the same gene, which we have named *TAP46* (2A phosphatase associated protein of 46 kD). The *TAP46* gene appears to be a single-copy gene and is expressed in all Arabidopsis organs. Transcripts derived from this gene are induced by chilling treatment but not by heat or anaerobic stress. Immunoprecipitation assays using antibodies generated to a peptide spanning amino acids 356 to 366 of TAP46 indicate that TAP46 is associated with a type 2A protein phosphatase in vivo. A search of the database identified TAP46 as a homolog of *Saccharomyces cerevisiae* TAP42 and mammalian α4. These two proteins are known to bind to the catalytic subunit of PP2A and to function in the target-of-rapamycin signaling pathway. Our results identify TAP46 as a plant PP2A-associated protein, with a possible function in the chilling response, and suggest that a target-of-rapamycin-like signaling pathway may exist in plants.

The type 2A Ser/Thr protein phosphatases (PP2A) are characterized by their sensitivity to inhibition by nanomolar amounts of the dinoflagellate toxin okadaic acid, by their lack of an absolute divalent cation requirement for activity, and by their preference for the α-subunit of phosphorylase kinase as a substrate (Wera and Hemmings, 1995). The enzyme has been implicated as a key control factor in numerous basic cellular processes such as metabolism, transcription, and signal transduction (Wera and Hemmings, 1995). The ability of the enzyme to control such diverse processes is thought to reside in its variable structure. PP2A exists in cells as either a heterodimer of the catalytic subunit (PP2Ac) and an A-regulatory subunit (PR65), or as a heterotrimer composed of PP2Ac, A, and various subunits with different masses. Extensive experimental evidence indicates that the subunit composition of PP2A is responsible for the specificity, activity, and subcellular localization of the enzyme (Wera and Hemmings, 1995).

Recently, a number of reports have described the association of PP2A subunits with a variety of cellular proteins other than the above-mentioned regulatory subunits. For example, PP2Ac binds to protein synthesis release factor 1 (eRF1), an association that may serve to recruit PP2Ac to the translational apparatus. In this fashion PP2A may function in controlling the phosphorylation state of various translation factors, and therefore might indirectly control protein synthesis (Andjelkovic et al., 1996). PP2Ac is also able to associate with the product of *Hox*11, a homeobox gene involved in genesis of the spleen (Kawabe et al., 1997). This association may control the G_2-to-M transition during the cell cycle, and supports the previously identified role of PP2A in the cell cycle (Mumby and Walter, 1993). In addition, PP2Ac associates with axin, a negative regulator of embryonic axis formation in vertebrates, and with the apoptosis suppressor Bcl2, although in the latter case the subunit composition of PP2A was not defined (Deng et al., 1998; Hsu et al., 1999). The A-regulatory subunit of PP2A is known to associate with caspase-3, a protease involved in apoptosis (Santoro et al., 1998). The A-subunit is cleaved by caspase-3 upon induction of the apoptotic pathway, and the degradation of the A-regulatory subunit without concomitant destruction of PP2Ac results in a rise of phosphatase activity. This alteration in phosphatase activity is coupled to alterations in the phosphorylation state of cellular proteins linked to cell proliferation, again supporting the role of PP2A in the cell cycle.

While PP2Ac or PP2A alone can associate with the above-mentioned proteins, associations among the PP2A heterodimer or heterotrimer with various signal transduction components have also been identified. For example, the PP2Ac/A heterodimer can bind to casein kinase 2α, while the PP2Ac/A/55-kD B heterotrimer associates with the Ca^{2+}/calmodulin-dependent protein kinase IV, p70 S6 kinase and with PAK1 and PAK3, two p21-activated kinases (Hériché et al., 1997; Westphal et al., 1998, 1999). These associations illustrate an emerging paradigm of phosphatase/kinase complex formation within cells, and suggest that such interactions may function to fine-tune the opposing activity of the associated enzymes and to integrate various signaling pathways.

In addition to the identification of the above-mentioned interaction of PP2A with various cellular proteins, PP2Ac has also been shown to associate with a protein called TAP42 in *S. cerevisiae* and α4 in mammals (Di Como and Arndt, 1996; Murata et al., 1997). While TAP42 and α4

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share only 24% identity (37% similarity), they appear to behave as homologs biochemically (Nanahoshi et al., 1998). In *S. cerevisiae* the association of TAP42 with PP2Ac (and its close relative SIT4) is regulated by the target-of-rapamycin (TOR) signaling pathway (Di Como and Arndt, 1996; Thomas and Hall, 1997). Specifically, stimulation of TOR1 and TOR2, two related protein kinases, in response to nutrient availability appears to cause, by an unknown mechanism, the association of TAP42 with PP2Ac and SIT4. This association, in a manner currently not understood, appears to positively control translation initiation through cap binding initiation factor 4E (eIF-4E). On the other hand, the immunosuppressant rapamycin, in conjunction with its immunophilin, targets TOR and causes a dissociation of TAP42 from PP2Ac, with a concomitant reduction in protein synthesis.

Mammalian TOR (mTOR, also known as FRAP, RAFT, and RAPT) stimulation appears to occur through growth factors and ultimately results in phosphorylation of eIF4E-binding protein (eIF4E-BP). Phosphorylation of eIF4E-BP prevents its association with eIF-4E and therefore stimulates the initiation of protein synthesis (Brunn et al., 1997; Burnett et al., 1998). The manner in which mTOR mediates this process remains unclear. While in vitro mTOR can phosphorylate eIF4E binding protein and directly activate p70 S6 kinase by phosphorylation, the in vivo relevance of these events is subject to controversy (Brunn et al., 1997; Burnett et al., 1998; Peterson et al., 1999). As is the case in yeast, treatment of mammalian cells with rapamycin results in reduced protein synthesis and appears to be mediated by mTOR.

The mammalian equivalent of TAP42 appears to be α4, a protein that was first identified as a component associated with Ig-α (MB-1) in the B cell receptor complex (Inui et al., 1995). Recently, this protein has been shown to bind to PP2Ac and its close relatives PP4 and PP6 (Chen et al., 1998). Association of α4 with PP2Ac is disrupted by rapamycin in some cell types (Murata et al., 1997; Inui et al., 1998; Nanahoshi et al., 1998). This suggests that, as is the case with TAP42 in yeast, the association of α4 with PP2Ac can be mediated by the TOR signaling pathway. In addition, α4 association with PP2Ac lowers its catalytic activity toward eIF4E-BP phosphorylated by mTOR and alters the phosphatase activity of PP2Ac toward various other substrates (Murata et al., 1997; Inui et al., 1998; Nanahoshi et al., 1998). These results indicate that TAP42/α4 serve as novel regulatory subunits of PP2Ac and are involved in a new (TOR) signaling pathway conserved among eukaryotes. However, the manner in which they function in this pathway has not yet been defined (Peterson et al., 1999). The association of TAP42/α4 with PP2Ac is especially unique, since it does not, as is the case for all B-subunits, require the presence of the A-regulatory subunit (Murata et al., 1997; Inui et al., 1998).

Here, we present data on the plant homolog of TAP42/α4, which we have called TAP46. TAP46 was identified in a yeast two-hybrid screen using Arabidopsis PP2Ac as bait. The Arabidopsis protein shows 38% to 42% similarity with TAP42 and α4 and appears to be induced by chilling treatment. Our results indicate that the interaction of PP2Ac with this novel subunit occurs in highly divergent eukaryotes, and suggest that a TOR-like signaling pathway may exist in plants.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen

The plasmid pGBT9 carrying a cDNA encoding a catalytic subunit of Arabidopsis (Columbia) PP2A (*PP2Ac-1*, Haynes et al., 1999) was co-introduced with an Arabidopsis cDNA library (prepared in the plasmid pGAD and kindly provided by Dr. V. Citovsky, State University of New York, Stony Brook) into competent *Saccharomyces cerevisiae* HF7c cells (Stratagene, La Jolla, CA). Co-transformants were selected by plating on medium lacking His, Leu, and Trp, and robust colonies were subsequently tested for β-galactosidase activity (Bartel et al., 1993). A total of 35 colonies were positive for both *HIS3* and *LacZ* reporter gene activity. Plasmids carrying the Arabidopsis cDNA were isolated from these positives and used in combination with the appropriate control DNAs to retransform yeast (Matchmaker two-hybrid system manual, CLONTECH, Palo Alto, CA). These assays revealed five of the original positives to be true.

cDNA Library Screening

A 550-bp *Eco*RI-*Pst*I fragment from the largest Arabidopsis cDNA positive identified in the yeast two-hybrid screen was used as a probe to screen 150,000 clones of a size-fractionated (1–2 kb) Arabidopsis cDNA library (Kieber et al., 1993), as described by Rundle et al. (1995). In addition, two ESTs (82E11T7 and 143E19T7) matching the sequence of the yeast two-hybrid positive were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). The complete sequence of both strands of the largest cDNA obtained from the library screen was established using nested deletions and custom oligonucleotide primers. In addition, partial sequence analysis was performed on the EST clones.

5′-RACE PCR

A cDNA amplification kit (Marathon, CLONTECH) was used according to the manufacturer's instructions to establish the 5′ end of the TAP46 mRNA using RACE-PCR. The substrate for cDNA synthesis was mRNA isolated from 5-d-old Arabidopsis seedlings, and the gene-specific primer for the 5′-RACE PCR reactions had the following sequence: 5′-CAGGCGCAGCTTCCCGATCATATCTTCG-3′. PCR products that hybridized with *TAP46* cDNA were excised from gels and cloned into plasmids. A total of 15 individual RACE clones were subjected to DNA sequence analysis.

Southern- and Northern-Blot Analysis

Southern-blot analysis was performed as indicated in Rundle et al. (1995). Blots were probed with a radiolabeled

fragment spanning nucleotides 111 to 558 of the *TAP46* cDNA. For northern-blot analyses poly(A^+) mRNA was isolated from appropriate Arabidopsis (ecotype Columbia) organs or whole seedlings using the Microfast track kit (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. RNA was separated by formaldehyde agarose electrophoresis and transferred to nylon membranes. Filters were probed as described by Rundle et al. (1995). Relative RNA levels were quantified by densitometry. For cold shock experiments, 14-d-old seedlings grown on Murashige and Skoog medium (Murashige and Skoog, 1962) with a 16-h light/8-h dark cycle were either kept at the growth temperature of 23°C or transferred to 4°C for 16 h. After this period, seedlings were quick frozen in liquid nitrogen and stored at −80°C until RNA isolation. For heat shock experiments, 10-d-old Arabidopsis seedlings grown on Murashige and Skoog medium with the same light/dark cycle as indicated above were either kept at the growth temperature of 23°C or placed at 37°C for 2 h. After treatment, seedlings were quick frozen in liquid nitrogen and stored at −80°C until RNA isolation.

Immunoprecipitation Assays

Plant extracts for immunoprecipitation assays were prepared from 4- to 5-week-old Arabidopsis plants grown at 23°C. All isolation and immunocomplex formation procedures were carried out at 4°C. Thirty grams of plant material was harvested, quick frozen in liquid nitrogen, and pulverized to a powder. The powder was mixed with 50 mL of grinding buffer (70 mM Tris-HCl, pH 8.3, 14 mM EDTA, 21 mM β-mercaptoethanol, 140 μM PMSF, 1.4 mM benzamidine, 2.1 mM DTT, and 14 μM leupeptin) and homogenized in a blender for 2 min. The homogenate was filtered through two layers of cheesecloth and centrifuged at 12,000*g* for 10 min. The supernatant was collected and centrifuged at 27,000*g* for an additional 10 min. Aliquots (1 mL) of supernatant from this last centrifugation were incubated with 25 μL of a 50% (w/v) slurry of protein A-agarose (Immunopure immobilized protein A, Pierce, Rockford, IL) in 10 mM Tris-HCl, pH 7.5, with 20 μL of preimmune IgGs, 20 μL of immune IgGs, or no addition. Antibodies were raised in rabbits against a KLH-coupled peptide spanning amino acids 356 to 366 of TAP46. Prior to use, preimmune and immune IgGs were purified from serum using a purification kit (ImmunoPure IgG protein A, Pierce) as instructed by the manufacturer.

After mixing the protein extracts with the appropriate IgGs, samples were incubated with shaking at 4°C for 4 h. After incubation the samples were centrifuged for 15 min at 2,500 rpm in an Eppendorf centrifuge. The supernatant was removed and the pellet resuspended in 1 mL of PBS (9.1 mM K_2HPO_4, 1.7 mM $KHPO_4$, and 150 mM NaCl, pH 7.4). The suspension was placed in a microfuge column and centrifuged for 5 min at 2,500 rpm. The column was then washed twice with 400 μL of PBS. After the final centrifugation, the agarose beads were resuspended in 400 μL of PBS and transferred to a standard microfuge tube. Upon centrifugation for 5 min at 2,500 rpm, the supernatant was removed and the pellet was resuspended in 20 μL of phosphatase assay buffer (Life Technologies, Cleveland).

Protein phosphatase assays were performed in duplicate using radiolabeled glycogen phosphorylase as a substrate, as indicated by the manufacturer (Life Technologies). Assays were performed at 30°C for 1 h. For assays including okadaic acid (1 nM final concentration) samples were preincubated with the inhibitor for 10 min on ice. The counts per minute released in each assay were calculated by subtracting from each sample the counts per minute released in a blank (a phosphatase assay performed using buffer and substrate only). At times, this yielded a negative counts per minute value, because treatment samples showed less radiolabeled phosphate release than the blank; such samples are presented with a value of 0 cpm released. In all assays, less then 30% of radiolabeled phosphorylase a was dephosphorylated.

Western-Blot Analysis

Soluble proteins were extracted from 4- to 5-week-old Arabidopsis seedlings as described for immunoprecipitation assay. Proteins were separated by SDS-PAGE (Mini-Protean II, Bio-Rad Laboratories, Hercules, CA), and the resulting gels were blotted to PVDF membranes. Blots were probed with either preimmune serum or equivalent amounts of antigen affinity-purified TAP46 peptide antibodies using the Protoblot western-blot system (Promega, Madison, WI) with two modifications. First, membranes were blocked for 1.5 h in 5% (w/v) BSA in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% [v/v] Tween 20), and second, the anti-rabbit IgG alkaline phosphatase conjugate consisted of a monoclonal antibody against rabbit IgG (Sigma, St. Louis).

RESULTS

Identification of a Novel Protein That Interacts with Arabidopsis PP2Ac

In an effort to identify novel substrates and/or regulators of Arabidopsis PP2A, we performed a yeast two-hybrid screen of an Arabidopsis cDNA library using *PP2Ac-1* cDNA as bait (Haynes et al., 1999). We obtained 35 colonies that were positive for both reporter genes (*LacZ* and *HIS3*). Plasmids encoding the interacting Arabidopsis cDNAs were isolated from each of the 35 positives and retested for specific interaction with PP2Ac-1 using the appropriate controls. These experiments yielded five surviving positives. Partial sequence analysis of these clones showed that, while their cDNA inserts were variable in length, they were all derived from the same gene (Table I). We have called the new gene *TAP46* (2A phosphatase associated protein). In an effort to determine if the TAP46 protein interacts with PP2A in a manner similar to the known B-regulatory subunit (Groves et al., 1999), we used the yeast two-hybrid system to test the interaction of the TAP46 protein with the A-regulatory subunit of Arabidopsis PP2A. Our results indicate that, unlike known B-regulatory subunits, there was no interaction between

TAP46 and A, suggesting a novel mechanism of interaction of PP2Ac with TAP46 (Table I).

TAP46 Is Homologous to *S. cerevisiae* TAP42 and Mammalian α4

To determine the structure of the TAP46 protein, we screened an Arabidopsis cDNA library with the 5′ region of the longest cDNA identified in our yeast two-hybrid experiments. This screen yielded three overlapping positive clones. The longest cDNA was completely sequenced on both strands. We also analyzed the sequence of two *TAP46* ESTs obtained from the Arabidopsis Biological Resource Center. Finally, we performed 5′-RACE-PCR to ensure that we identified the complete 5′ sequence of the *TAP46* transcript. Our results indicate that the mRNA encoded by the *TAP46* gene contains a 5′-UTR of 116 nucleotides, an ORF spanning 1,218 nucleotides, and a 3′-UTR of 145 or 218 nucleotides. The variable 3′-UTR indicates that two different polyadenylation sites may be operational in the *TAP46* gene, one of which results in an extension of the 3′-UTR by 73 nucleotides. The sequence of the longest combined *TAP46* cDNA has been deposited in the GenBank database under accession no. AF133708.

A search of the GenBank database with the complete *TAP46* cDNA identified a region on Arabidopsis chromosome V containing the *TAP46* gene (P1Clone MNB8, nucleotides 11,465–8,963; Y. Nakamura, unpublished data). The gene contains eight introns present at positions 213, 336, 423, 528, 690, 726, 798, and 1,079 in the *TAP46* cDNA with the following length and position in the genomic sequence: intron 1, 142 bp (11,254–11,112); intron 2, 290 bp (10,988–10,698); intron 3, 118 bp (10,610–10,492); intron 4, 105 bp (10,386–10,281); intron 5, 82 bp (10,118–10,036); intron 6, 83 bp (9,999–9,916); intron 7, 91 bp (9,843–9,752); and intron 8, 79 bp (9,470–9,391). All exon-intron boundaries contain the expected donor and acceptor GT and AG splice sites. Comparison of the genomic sequence with that of the 5′-RACE PCR products indicated that the transcription start site occurs at nucleotide 11,465 of the MNB8 P1 clone.

The predicted protein encoded by the *TAP46* cDNA is 46 kD and has an pI of 4.73. A search of the GenBank/EMBL database with the TAP46 protein sequence identified three proteins with significant similarity to TAP46 (Fig. 1). Of these three proteins, the closest homolog to Arabidopsis TAP46 is a chilling-induced protein from rice (BC601; Binh and Oono, 1992). This protein, of unknown function, is 43% identical and 59% similar to Arabidopsis TAP46. TAP46 and the rice protein show extensive homology throughout their amino acid sequence, with the exception of the center region of the proteins (spanning position 229–303 of TAP46 and 246–284 of the rice protein), which are highly variable in both length and sequence. In addition, our searches consistently identified two other proteins with significant homology to TAP46: *S. cerevisiae* TAP42 (Di Como and Arndt, 1996) and mammalian α4 (Inui et al., 1995).

TAP42 and α4 are known homologs of each other and both have been shown to associate directly with the catalytic subunit of PP2A, as well as with the catalytic subunit of related phosphatases (mammalian PP4 and PP6 in the case of α4, and the yeast homolog of PP6, SIT4, in the case of TAP42; Di Como and Arndt, 1996; Murata et al., 1997; Chen et al., 1998). In addition, both proteins are known to regulate the activity of PP2Ac upon complex formation (Murata et al., 1997; Inui et al., 1998; Nanahoshi et al., 1998). The overall similarity between TAP46 and its yeast and mammalian counterparts is limited to 38% and 42%, respectively. However, given the manner by which we identified TAP46 and the known PP2Ac-binding ability of TAP42 and α4, we believe these matches to be significant. As noted above, the central region of TAP42 and α4 show little homology in length or sequence to TAP46, however, their amino and carboxy termini show significant matches, with 10 residues near the amino termini and nine residues at the carboxy terminus being absolutely conserved. These results suggest that these residues may be critical for the interaction of TAP46 and its homologs with PP2Ac.

Table I. *Detection of interaction among TAP46 and components of Arabidopsis PP2A using the yeast two-hybrid system*

S. cerevisiae HF7c cells were cotransformed with the plasmids pGBT9 and pGAD424 (CLONTECH) encoding the GAL4 DNA-binding domain and activation domain, respectively. Appropriate Arabidopsis cDNAs encoding the TAP46, PP2Ac-1, or α isoform of the A-regulatory subunit (AtAα) were present in these plasmids for the production of GAL4 DNA-binding or activation domain fusion protein. Positive controls for interaction assays consisted of the murine p53 protein (VA3) and the large T antigen of SV40 (TD1) (CLONTECH). Protein interactions were tested by growth on medium lacking Trp, Leu, and His (His$^+$ assays results) and using a β-galactosidase assay. +, Positive assay result; −, negative assay result; no insert, introduction of either the pGAD or pGBT9 plasmid containing no insert.

GAL4-Binding Domain Fusion Protein	GAL4 Activation Domain Fusion Protein	His$^+$ Assay Result	β-Gal Assay Result
PP2Ac-1	TAP46	+	+
PP2Ac-1	No insert	−	−
No insert	TAP46	−	−
Aα	TAP46	−	−
Aα	No insert	−	−
No insert	No insert	−	−
VA3	TD1	+	+

Genomic Organization and Expression of TAP46

We performed genomic Southern blots to establish the copy number of the *TAP46* gene in the Arabidopsis genome. Genomic DNA digested with either *Eco*RI or *Hin*dIII was subjected to electrophoresis, blotted to a membrane, and probed with radiolabeled *TAP46* DNA. A single hybridizing band was detected in DNA digested with *Eco*RI, while two hybridizing bands of 6.5 and 0.95 kb were noted when DNA was digested with *Hin*dIII (Fig. 2A). The two bands detected in the *Hin*dIII digest were most likely both derived from *TAP46*, because the section of *TAP46* cDNA used as a probe spans a *Hin*dIII site present in intron 1 of the *TAP46* genomic sequence. Our results suggest that *TAP46* is a single- or low-copy gene.

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TAP46    .....................MGGLAMEEMPLSVLFEQARKIHLAASESGVDQDVVKKGCEMFQKCEDMIGKLALFSSNETKE.DIST    66
OsCip    MVEVEEVSNKMQVQMRLHPAAAAEEEDADL--PA--DK-SHL-SL--S-SL--EGIR--VDLLRR-DE-VS-VG--------D.----    87
α4       ..............MAAEDELQLPRLP-LFETGRQLLDEVEVATEPAG-RIV-EK-F--LDLLE-AAE-LSQ-D---R--DL-EIA--    74
TAP42    ....................MASVTEQFNDII-LYSTKLEHTS-RQDSPEYQGLLLSTIKKLLNLKTAIFDR-----T---ID.-V--    67

            * * *
TAP46    NNLKYLLVPYYLAELTEKIIQEDRIQIVKASYAKLKEFFSFCEAMELVPDEELEASSRGGSG..............APADRRALKIAR    140
OsCip    A----------G-M--RVA-----PVL---QDH----I-I---L--ISED---.I--QKNL..............I-WQIEEHRRLH    160
α4       .D-------AFQGA--M-QVNPSKRLDHLQRAREHFINYLTQCHCYH-AEF--PKTMNNSAENHTANSSMAYPSLV-M-SQ-QA--Q-    161
TAP42    ASI-F-A-D---GL-ISRRQSN-SDVAQRQSMKLIYLKK-VESFINFLTLLQDYKLLDPLV-EKLGNFKDRYNPQLSELYAQPKNNKD    155

TAP46    FKRQKAAEAKLLEIKERKERRGRSTKASALSTPVESGEDDIPDDDSEEEREAWLSSINLAICKAIDLLEMLKREEEMLSAIKERQLKD    228
OsCip    GSSA-RLQKQSSR--------.-RHES-C--A-I-L-.G-LL.RMM-K-R-GMV-YYLIGSIEGF---D---K---IVP-V--GKRRM    245
α4       Y-QK-EL-HR-SAM-SAV-SGQADDERVREYYLLHLQRWIDISLEEI-SIDQEIKILRERDSSREASTSNSS-Q-RPPVKPFILTRNM    249
TAP42    LSGAQLKRKEKI-LFQ-NKEI..---LHC-ELELKNNDE-HDH-ELLR-LYLMRLHHFSLDTINNIEQNLFEC-..---NFLKNSVHE    239

TAP46    GEGGFSRDALDDRTKKAETWHRDAAARIQYSKPAQPITCATFAQDVLEGRASVSQGHEHKNQPLIFGPASIVGGPLSTERERMIAQVF    316
OsCip    VMHLLVKCLMNVQKGLKHGTIMLPTVHHTPNQLI-SLVQ...................................-TS-----A----    297
α4       AQAKVFGAGYPSLPTMTVSDWYEQHRKYGALPD.......................................................    282
TAP42    VKSSGTQIRKESNDDDSTGFTDKLENINKPLIDKKG....................................................    275

                                                           *                *             *
TAP46    QPSHRMPTMCIEDAGLTEMNIMNDWQEQTKKAIEEATTSWYNDKPLRRKEEDEEDD....DE......DEEAVM........KARAF.    385
OsCip    ---Y-L---S--E---R--KM-EK---R-A-M-Q-SNSA-HK-GSRS....AQ--E....-A......E--..........---GW.    359
α4       .............................QG-AK-APEEFRKAAQQQ.--Q--KE....E-......-D-QTL........H.--RE    320
TAP42    -...VLRNFTLV-KRQ.........-L-Q-VRGYGQYGPTMSVEEFLD--F.--GRVLQGG-EPEQAP---N.-DWQDRETY---E..    347

          *  *    * **     *
TAP46    .DDWKDDNPRGAGNKKLT.PCG                                                                    405
OsCip    .-----------------.---                                                                    379
α4       W-----TH---Y--RQNM...-                                                                    339
TAP42    W-EF-ESHAK-S--...-MNR-                                                                    366
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Figure 1. Alignment of the amino acid sequence of TAP46 and its homologs from various organisms. The method of Pearson and Lipman (1988) was used to align the predicted amino acid sequences of TAP46, rice chilling-induced protein (OsCIP; Binh and Oono, 1992), mammalian α4 (Inui et al., 1995), and *S. cerevisiae* TAP42 (Di Como and Arndt, 1996). Dashes indicate amino acid identity; dots represent gaps introduced to maximize amino acid alignment; asterisks indicate amino acid residues conserved in all four of the compared proteins. The amino acids underlined in TAP46 represent the sequence of the peptide used for TAP46 antibody production.

Next we examined the expression pattern of *TAP46* in various Arabidopsis organs. Figure 2B shows that the primary *TAP46* transcript is 1.55 kb, in agreement with the size of the *TAP46* cDNA, and is ubiquitously expressed. In addition, a smaller transcript of approximately 1 kb accumulates primarily in flowers and roots, being most prominent in the latter. Subsequent probing of the same blot with radiolabeled actin cDNA shows the relative levels of RNA present in each lane (Fig. 2C) and indicates that the levels of the primary *TAP46* transcript are approximately equal in all organs. These results suggest that TAP46 performs an essential function within Arabidopsis cells.

Because the TAP46 protein shows extensive homology to a chilling-induced protein from rice, we examined the expression of the *TAP46* gene in response to various stresses. We began these experiments by examining the levels of *TAP46* transcripts in Arabidopsis seedlings maintained at control temperature (23°C) or exposed to 16 h of cold treatment at 4°C. RNA was isolated from the seedlings upon treatment, separated by electrophoresis, and blotted to a membrane. We first probed the membrane with radiolabeled actin to establish the relative levels of RNA in each lane (Fig. 3A). Next, we probed the same filter with a COR 6.6 cDNA to show that chilling treatment was performed successfully (Fig. 3B; Gilmour et al., 1992). Finally, we probed the filter with *TAP46* cDNA (Fig. 3C). Our results indicate that the levels of *TAP46* mRNA rise in response to chilling treatment (Fig. 3C), albeit not as dramatically as the *COR6.6* transcript levels.

Next we examined the expression of *TAP46* in response to heat stress. Arabidopsis seedlings were either kept at the control temperature (23°C) or placed at 37°C for 2 h. After treatment, RNA was isolated from the seedlings and used for northern-blot analyses. The relative levels of mRNA present in the control and treated sample lanes were determined using an actin probe (Fig. 3D). Our results indicate that *TAP46* mRNA levels do not increase in response to heat shock (Fig. 3F). Heat stress experiments were performed successfully, as shown by the dramatic rise of mRNA derived from the *HSP17.6* heat shock gene (Fig. 3E; Helm and Vierling, 1989). Finally, we also examined if

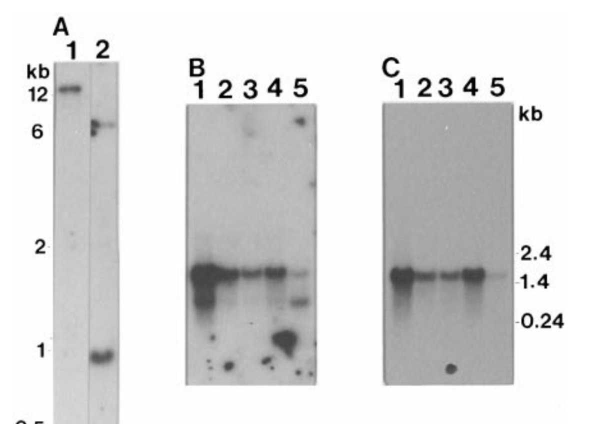

Figure 2. Genomic organization and expression of *TAP46*. A, Genomic Southern blot probed with a TAP46 fragment spanning nucleotides 111 to 558 of the TAP46 cDNA. Arabidopsis (Columbia) DNA was digested with either *Eco*RI (lane 1) or *Hin*dIII (lane 2). B, Northern blot of Arabidopsis mRNA isolated from flowers (lane 1), cotyledons (lane 2), leaves (lane 3), stems (lane 4), and roots (lane 5), and probed with nucleotides 111 to 558 of the TAP46 cDNA. C, Same blot as in B but probed with an actin fragment. Markers consist of a 1-kb ladder (A) and a RNA ladder (B and C) (Life Technologies).

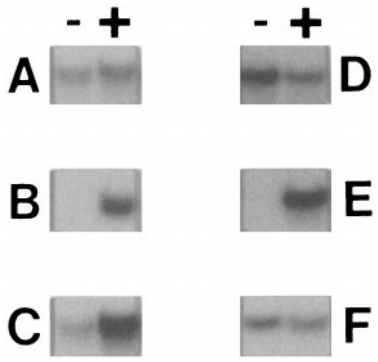

Figure 3. Effect of cold treatment and heat shock on *TAP46* expression. Arabidopsis seedlings were either kept at the control growth temperature of 23°C (−) or incubated at 4°C for 16 h (+, A–C) or heat shocked at 37°C for 2 h (+, D–F). Upon treatment, poly(A^+) RNA was isolated from the plants and used for northern-blot analysis. Filters were probed with the following DNAs: actin (A), *COR 6.6* (B), *TAP46* (C), actin (D), *HSP17.6* (E), and *TAP46* (F). Markers consist of a RNA ladder (Life Technologies).

TAP46 transcript levels could be affected by anaerobic stress, however, no such changes in mRNA levels were noted (data not shown). Our results indicate that *TAP46* mRNA levels increase specifically in response to chilling stress, as is the case for its homolog in rice (Binh and Oono, 1992). Other stress treatments appear to have little effect on *TAP46* mRNA levels, suggesting that the TAP46 protein might function specifically to aid plant survival during cold treatment.

PP2Ac and TAP46 Associate in Vivo

Extensive experiments in both yeast and mammals have confirmed the in vivo association of TAP42 and α4 with PP2Ac and its close relatives. We were interested in determining if PP2A is associated with TAP46 within Arabidopsis cells. For this purpose we prepared antibodies against a peptide of TAP46 spanning amino acids 356 to 366 (Fig. 1). The antibodies were characterized by probing a western blot of Arabidopsis whole-plant protein extracts. Figure 4 shows that the antibodies detect a protein of approximately 42 kD in molecular mass. No such reacting protein was detected if a similar membrane was probed with preimmune serum.

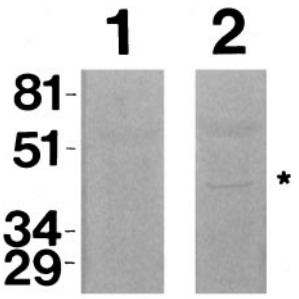

Figure 4. Immunological detection of TAP46 in Arabidopsis protein extracts. Arabidopsis whole-plant extracts were separated by SDS-PAGE and transferred to PVDF membranes. Blots were probed with either preimmune serum (lane 1) or an equivalent amount of affinity-purified immune serum prepared against a peptide spanning amino acids 356 to 366 of TAP46 (lane 2). Molecular mass standards consist of pre-stained broad-range markers (Bio-Rad). The asterisk indicates the TAP46 protein.

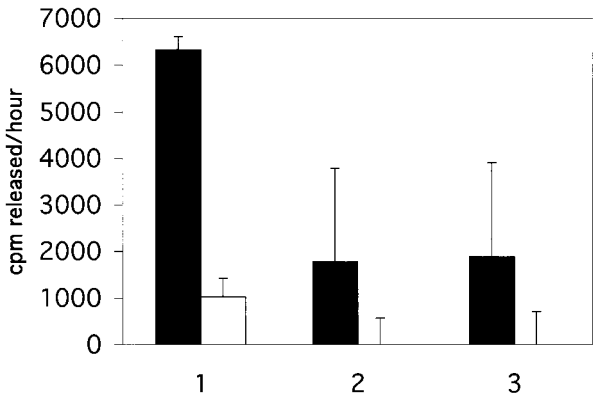

Figure 5. Co-immunoprecipitation of TAP46 and PP2Ac from Arabidopsis plant extracts. TAP46 was immunoprecipitated from Arabidopsis extracts using the anti-TAP46 antibody (1) described in Figure 4. Control immunoprecipitations included assays using preimmune serum (2) or no serum (3). Immune complexes were isolated using protein A-agarose and assayed for protein phosphatase activity in the absence (black bars) or presence (white bars) of 1 nM okadaic acid. Assays were performed in duplicate. Similar results were obtained in three independent experiments.

Our results indicate that the TAP46 protein, which has a predicted mass of 46 kD, either migrates in a slightly aberrant manner during SDS-PAGE (as has been noted for α4, Inui et al., 1995) or may be modified by proteolysis upon synthesis. The reaction of antibodies with TAP46 on western blots were generally weak, which have been due to low levels of TAP46 in plant extracts or because the antibodies used were not very effective for western blotting. Blots probed with preimmune or immune serum also show a band of approximately 55 kD. This band most likely represents the large subunit of Rubisco, which, due to its high level in extracts and despite extensive blocking of membranes, produces nonspecific binding of primary and/or secondary antibodies.

To establish the in vivo association of TAP46 and PP2Ac, we performed immunoprecipitation assays using the above antibody. Whole-plant extracts were incubated with preimmune, immune, or no serum, and immune complexes were isolated using protein A-agarose. Protein phosphatase assays were performed on the immune complexes in the presence or absence of 1 nM okadaic acid. This concentration of phosphatase inhibitor should affect only PP2A and not PP1 phosphatase activity. Figure 5 shows that immune serum complexes have approximately 3.6 times the level of phosphatase activity compared with preimmune serum complexes. Eighty-four percent of the phosphatase activity associated with immune serum complexes is inhibited by 1 nM okadaic acid, indicating that it is of the PP2A type. These data suggest that TAP46 and PP2Ac associate in vivo and confirm our yeast two-hybrid interaction results. Only a small fraction of total PP2A activity

(0.0013%–0.0024%) was removed from solution during immunoprecipitation experiments. In *S. cerevisiae* <2% of PP2A is associated with TAP42 (Di Como and Arndt, 1996). The low amount of PP2A activity obtained in our assays may be a reflection of the strength of our antibody or an indication of the limited amount of PP2Ac associated with TAP46 in plant cells. It is also possible that TAP46 inhibits PP2Ac activity toward phosphorylase a, resulting in low phosphatase activity in our immune complexes.

DISCUSSION

We used a yeast two-hybrid screen to identify a novel Arabidopsis protein that interacts with the catalytic subunit of PP2A. Our screen yielded five positives that were all derived from the same gene (*TAP46*). Given the manner in which we identified TAP46, it is clear that, unlike other previously characterized B-subunits of PP2A, TAP46 is able to interact directly with PP2Ac and does not require the A-regulatory subunit to do so (Goldberg, 1998; Haynes et al., 1999). This result is substantiated by the fact that TAP46 does not associate with the A-subunit in yeast two-hybrid assays, while other B-type regulatory subunits of Arabidopsis do (Haynes et al., 1999). These data identify TAP46 as a novel plant PP2A-binding protein. Given the growing number of known PP2A-binding proteins, it was surprising that our yeast two-hybrid screen yielded only TAP46. This may have been a reflection of the strength of the interaction between TAP46 and PP2Ac, or may also be because the effective binding of various other proteins to PP2Ac may require the A-subunit.

While the function of TAP46 in Arabidopsis remains to be elucidated, the putative *S. cerevisiae* (TAP42) and mammalian (α4) homolog of TAP46 have been clearly established as regulators of PP2Ac activity. The association of α4 with PP2A appears to displace the A-subunit and alter the activity ratio of PP2Ac toward myelin basic protein and phosphorylase a compared with the PP2Ac/A dimer (Murata et al., 1997). In addition, the association of α4 or TAP42 with PP2Ac lowers its phosphatase activity toward 4E-BP1, while it may increase the activity of PP2Ac toward histone H1 (Inui et al., 1998; Nanahoshi et al., 1998). Future experiments will be aimed at examining the effect of TAP46 on Arabidopsis PP2Ac activity. Because immunoprecipitates of TAP46 and PP2Ac showed phosphatase activity toward phosphorylase a, our results suggest that TAP46 does not cause complete inhibition of PP2Ac, at least toward this substrate. Most likely, as is the case for other PP2A-binding proteins and as indicated by the above PP2Ac activity data, it will cause an alteration in the activity, specificity, or subcellular localization of PP2A (Goldberg, 1998).

A comparison of the protein sequence of TAP46 with its rice homolog shows extensive sequence identity throughout the length of the two proteins. The main difference between the proteins resides in their midsection, where a region of high sequence divergence is followed by an insertion in the TAP46 protein relative to the rice polypeptide. While homology with the yeast and mammalian proteins is far less extensive, the overall pattern of conserved/diverged regions is the same as that noted in the two plant species. This suggests that the amino and carboxy termini of the proteins are critical for interaction with PP2Ac, while the core region might be tailored to the specific functional needs of each species (e.g. control of defined substrate specificity and/or specific response elements for interaction with different signal transduction cascades/components).

However, this theory is countered by the fact that Inui et al. (1998) defined the core region of α4, spanning amino acids 94 to 202, as being critical for the interaction of α4 with PP2Ac, while the protein termini appeared to merely enhance this interaction. This result is surprising in light of our findings and because the region spanning residues 94 to 202 also excludes all 19 of the amino acid residues absolutely conserved among all TAP46 homologs. Perhaps these conserved residues/conserved termini of the proteins function in the known interaction of TAP46 homologs with PP2A-related phosphatases (PP4 and PP6 in the case of α4; SIT4 in case of TAP42) and merely enhance interaction with PP2Ac (Di Como and Arndt, 1996; Chen et al., 1998). On the other hand, it is possible that the conserved regions function in the regulation of the activity of PP2Ac or related phosphatases, an effect that has not yet been examined. Future experiments will be aimed at deciphering the role of these conserved residues/regions in the interaction and effect of TAP46 with PP2A and the known plant homologs of PP4 (Pérez-Callejón et al., 1993).

The levels of Arabidopsis TAP46 mRNA increase in response to chilling stress, as is the case for its rice homolog (Binh and Oono, 1992). However, no increase in TAP46 mRNA levels was noted in seedlings subjected to heat or anaerobic stress. These results imply a possible specific role for TAP46 during chilling, but suggest that there is no overall stress response role for TAP46. Thus, TAP46 may modulate the specificity or subcellular localization of PP2Ac or related phosphatases during cold stress and thereby aid in plant survival. A possible role for PP2A in low-temperature signal transduction has been previously established in plants (Monroy et al., 1998).

In *S. cerevisiae* the association of TAP42 with PP2Ac and SIT4 is disrupted during nutrient starvation or treatment with the immunosuppressant rapamycin (Di Como and Arndt, 1996). In both cases, signal transduction occurs through the TOR pathway and results in the dissociation of TAP42 from the phosphatase, with a consequent reduction in protein synthesis. In mammals the association of α4 with PP2Ac is also reversed by rapamycin in certain cell types (Murata et al., 1997; Inui et al., 1998; Nanahoshi et al., 1998), presumably through mTOR signaling, and also results in a reduction of protein synthesis (Thomas and Hall, 1997).

Rapamycin affects signaling through the TOR pathway by associating with FK506- and rapamycin-binding proteins (FKBPs), and the rapamycin-FKBP complex subsequently affects the activity of PI kinase-related protein kinases (TOR in yeast and mTOR in mammals). mTOR may be directly responsible for the phosphorylation of p70 S6 kinase and 4E-BP1, thus regulating protein synthesis (Brunn et al., 1997; Burnett et al., 1998). Could a similar pathway be functional in plants? To our knowledge, the effect of rapamycin on plant cells is unknown, but numerous FKBPs and a putative, not yet well-characterized TOR

equivalent have been identified in plants (Luan et al., 1994; Blecher et al., 1996; Luan et al., 1996; Xu et al., 1998). In addition, an equivalent of the p70 S6 kinase has been identified in Arabidopsis, but when expressed in mammalian cells, this enzyme is resistant to rapamycin and is thus able to overcome the rapamycin block of S6 phosphorylation in mammalian cells (Turck et al., 1998).

While, overall, many of the possible components of a TOR signaling pathway appear to be present in plants, their true role in plant signal transduction remains to be defined. The identification of TAP46 strengthens the available evidence for the existence of this novel signaling pathway in Arabidopsis and suggests a role for PP2A in its function. Future experiments will be aimed at establishing the true presence and function of the TOR pathway in plants with the specific goal of determining its relationship to PP2A function and the role of PP2A in the plant stress response and in plant growth and development.

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