Expression of a Gene for Cyclophilin Which Contains an Amino-Terminal Endoplasmic Reticulum-Targeting Signal

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We isolated a novel gene for cyclophilin (CyP) first identified as an intracellular target of the immunosuppressant cyclosporin A and also known to have peptidyl-prolyl cis-trans isomerase (PPIase) activity, named ATCYP5 from Arabidopsis thaliana. ATCYP5 encoded a polypeptide with 201 amino acids with a putative ER-targeting signal sequence at its N-terminal, but without the typical ER-retention signal in its C-terminal. In addition, ATCYP5 protein contained a seven amino-acid long sequence which has been found previously only in cytosolic CyPs from plants. The synthetic mutant green fluorescent protein (sGFP; S65T) was fused to the N-terminal part of ATCYP5, and expressed in tobacco BY-2 cells. The fluorescence derived from the fusion protein was detected mainly around the nucleus, indicating translocation into ER. ATCYP5 was expressed mainly in young stems especially in the apical region and weakly in leaves and roots.

Key words: Arabidopsis thaliana — Cyclophilin — Endoplasmic reticulum — Green fluorescent protein — Peptidyl-prolyl cis-trans isomerase.

Cyclophilin (CyP) is ubiquitous, and constitutes a highly conserved protein family which was first discovered as an intracellular receptor for the immunosuppressant cyclosporin A (CsA) (Handschumacher et al. 1984). The complex composed of CsA and CyPA, which is the major cytosolic CyP in mammalian cells, binds and inhibits the Ca2+- and calmodulin-dependent protein phosphatase calcineurin, that is responsible for the signal transduction pathway leading to expression of lymphokine genes (Liu et al. 1992) and inactivation of the potassium channel in plant guard cells (Luan et al. 1993), suggesting the implication of calcineurin or calcineurin-like protein phosphatase in these signal transduction pathways.

CyP is also known as peptidyl-prolyl cis-trans isomerase (PPIase). Because cis-trans isomerization of peptidyl-prolyl bond is a slow rate-limiting step for folding of some proteins, PPIase activity is required to accelerate formation of active proteins (Fischer and Schmid 1990, Freskgard et al. 1992). Studies on the collagen triple helix formation (Steinmann et al. 1991), rhodopsin isofom folding (Stammes et al. 1991) and transferrin folding (Lodish and Kong 1991) indicated significant roles of CyPs in protein folding in vivo. In many cells, CyPs are widely distributed in cellular compartments. For example, mammalian cells have CyPA in cytosol, CyPB in endoplasmic reticulum (ER) (Price et al. 1991, Hasel et al. 1991, Spik et al. 1991), CyPC in secretory pathways (Friedman and Weissman 1991) and CyPD in mitochondria (Bergsma et al. 1991). Each CyPB, CyPC and CyPD has the targeting sequence for their compartments at the N-terminal and the amino acid sequences of their mature region are highly similar to that of CyPA.

Additionally, CyPs are known to be involved in various intracellular events, such as apoptosis (Montague et al. 1994), replication cycle of human immunodeficiency virus type-1 (Luban et al. 1993, Franke et al. 1994, Thali et al. 1994) and conformational modification of transcription factor YY-1 (Yang et al. 1995).

In plants, cDNAs or genes for CyP have been isolated from various species, for example, maize, tomato and Brassica (Gasser et al. 1990), Arabidopsis thaliana (Bartling et al. 1992, Lippuner et al. 1994, Hayman and Mierzyk 1994, Saito et al. 1993), Phaseolus vulgaris (Marivet et al. 1992) and rice (Buchholz et al. 1994). Some cytosolic CyPs have been reported to be induced under various stress conditions and to be preferentially expressed in actively growing cells (Gasser et al. 1990, Marty et al. 1993, Marivet et al. 1992, 1994). cDNAs for a chloroplast-localized CyP have been cloned from Arabidopsis thaliana (Lippuner et al. 1994) and fava bean (Luan et al. 1994). The deduced precursor of chloroplast CyP has a transit peptide for
chloroplast-targeting and the mRNAs has been detected mainly in above ground organs (Lippuner et al. 1994, Luan et al. 1994). We are interested in the existence of various CyPs in higher plants, especially in the expression and function of distinct CyP genes. Previously, we reported cloning of two cytosolic CyP genes, ATCYP1 and ATCYP2 (Saito et al. 1995), from Arabidopsis thaliana. In this study, we describe the isolation and expression analysis of the gene for ATCYP5, a newly isolated CyP which has an ER-targeting signal sequence from Arabidopsis thaliana. We also describe translocation of synthetic green fluorescent protein (sGFP) fused with the signal sequence of ATCYP5 to ER in tobacco cultured cells.

Materials and Methods

Plant material and preparation of genomic DNA—Arabidopsis thaliana (L.) Heynh. ecotype Landsberg erecta was sown on germination medium (GM; valvekens et al. 1988) and kept at 22°C under continuous light conditions. Fourteen days later (day 14), they were frozen in liquid nitrogen. Total high-molecular-weight DNA was prepared from frozen tissues as described by Liu et al. (1995).

PCR and library screening—Arabidopsis genomic DNA was completely digested with HindIII and ligated with HindIII cassette (TakaRa Shuzo, Kyoto, Japan), which have annealing sites for cassette primers and 3' terminal compatible to the HindIII site, and then used as template DNA. PCR was performed using two sets of primers. As cassette primers, we synthesized C1 (5'-GTACATATTGCCTGAACGGCGAATACGACCTCA-3') and C2 (5'-CGTTAGAACGGCGTAATACGACTAGAG-3'), which were suitable for the HindIII cassette. Two highly conserved regions of plant CyPs, GKHVVFG and GFMQQGGDFT (Fig. 2) were chosen for synthesis of antisense (5'-CGTTAGAACGCGTAATACGACTCACTATAGGGAGA-3'), which were suitable for the HindIII cassette.

DNA amplification reaction mixture contained 50 mM dNTPs, 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 25 mM KCl, 2× Taq buffer, 0.02 units of Taq DNA polymerase (Takara Shuzo), 1× Taq buffer (100 µg/ml BSA and 0.02 units of Taq DNA polymerase (Takara Shuzo)). The first reaction was performed using 500 ng of Arabidopsis genomic DNA treated for cassette PCR, and 200 nM of each primer C1 and S1. The second reaction mixture contained a 1 µl aliquot of the first reaction, and 200 nM of each primer C2 and S2. Both reactions were carried out under the following conditions: preincubation at 94°C for 2 min, and then 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and polymerization at 72°C for 2 min 30 s. The 500 bp amplification product of PCR was purified by gel electrophoresis using a 1% agarose gel and blunted with T4 DNA polymerase (Takara Shuzo), and then cloned into the EcoRV site of pBluescriptII (Clontech, Palo Alto, CA, U.S.A.) by HindIII and XbaI. The obtained plasmid had a BamHI-XbaI site only on the 3' side of the 35S promoter. Furthermore, the HindIII site of the resulting plasmid was replaced by the SacI site by introducing SacI linker (5'-CGAGCTCG-3'). Because the BamHI-XbaI sites were repeated on both the 5' and 3' sides of cauliflower mosaic virus (CaMV) 35S promoter of pTH-2, the 35S promoter of the resulting plasmid was replaced by the 35S promoter of pBI112 (Clontech, Palo Alto, CA, U.S.A.) by HindIII and XbaI. The obtained plasmid had a BamHI-XbaI site only on the 3' side of the 35S promoter. Furthermore, the HindIII site of the resulting plasmid was replaced by the SacI site by introducing the SacI linker, and named pSA501. The N-terminal region (1-48th amino acids) of ATCYP5 was amplified by PCR using the obtained cDNA as template with the following primers 5'-CTTCTAGATGGCGAAAGCAAGCTTT-3' (sense) and 5'-CTGGATCCAACGCGACCAGCGGATTT-3' (antisense) corresponding to the 5' and 3' putative untranslatable regions of ATCYP5 (Fig. 1). The 500 bp fragment obtained was blunt-end ligated and cloned into the EcoRV site of pBluescriptII.

DNA sequencing—Double stranded plasmids were sequenced with a AmpliCycle Sequencing Kit (Perkin-Elmer, Norwalk, CT, U.S.A.) and A.L.F. DNA Sequencer (Pharmacia, Uppsala, Sweden) according to the directions of the manufacturer. DNA SIS (HITACHI Software Engineering, Yokohama, Japan) was used for subsequent analysis.

Construction of synthetic green fluorescent protein expression plasmids and transformation of tobacco BY-2 cells—Plasmid pTH-2 (Chiu et al. 1996) which contained sGFP (S5ST) was modified as below. The Nol site was replaced by the SacI site by introducing SacI linker (5'-CGAGCTCG-3'). Because the BamHI-XbaI sites were repeated on both the 5' and 3' sides of cauliflower mosaic virus (CaMV) 35S promoter of pTH-2, the 35S promoter of the resulting plasmid was replaced by the 35S promoter of pBI112 (Clontech, Palo Alto, CA, U.S.A.) by HindIII and XbaI. The obtained plasmid had a BamHI-XbaI site only on the 3' side of the 35S promoter. Furthermore, the HindIII site of the resulting plasmid was replaced by the SacI site by introducing the SacI linker, and named pSA501. The N-terminal region (1-48th amino acids) of ATCYP5 was amplified by PCR using the obtained cDNA as template with the following primers 5'-CTTCTAGATGGCGAAAGCAAGCTTT-3' (sense) and 5'-CTGGATCCAACGCGACCAGCGGATTT-3' (antisense) which have XbaI and BamHI sites at the 5' region, respectively (Fig. 1), to fuse with sGFP. The PCR product was double-digested with XbaI and BamHI and then in-frame inserted in the XbaI-BamHI site of pSA501. The resulting plasmid was named pSA502. The binary vector pAHB-Hml, constructed from pBI101 by introducing the hygromycin phosphotransferase (HPT) gene and the Arabidopsis β-amylase promoter was a gift from Dr. S. Mita (Mita et al. 1995), and modified as below. The HindIII fragment containing β-amylase promoter was removed and self-ligated. This resulting plasmid was named pH1. Then HindIII site was replaced by the SacI site by introducing the SacI linker. The SacI fragment containing the β-glucuronidase (GUS) gene was replaced by SacI fragments of pSA501 and pSA502 to construct pSA701 (35S-sGFP) and pSA702 (35S-A5sGFP), respectively (Fig. 4). Stable transformation of Nicotiana tabacum cv. BY-2 cells mediated by Agrobacterium tumefaciens EHA101 was carried out as described by Matsuoka and Nakamura (1991). Microscopy—When BY-2 cells were viewed using a fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan), the following filters were used: B2 (excitation filter 450-490 nm, dichroic mirror 510 nm, barrier filter 520 nm). To analyze confocal sections of sGFP in BY-2 cells, an LSM-G200 system (Olympus, Tokyo, Japan) was used. A 25 mW Argon laser was used for blue
Excitation light at 488 nm (band path mirror 535 nm, dichroic mirror 488 nm).

Preparation of protein fractions—Protein extraction from E. coli and BY-2 cells was carried out as follows. E. coli DH5α harboring pSA702 cultured in LB medium containing 50 μg ml⁻¹ hygromycin for 20 h was spun briefly and the pellet was suspended with extraction buffer containing 100 mM Tris-HCl (pH 7.5), 0.1% Triton X-100, 1 mM PMSF. Subsequently, the sample was sonicated twice for 30 s at intervals of 1 min on wet ice and then centrifuged at 15,000 rpm for 15 min. The supernatant was stored at —80°C. BY-2 cells transformed with pSA702 were liquid-cooled for 4 d and spun briefly, followed by resuspension in 100 mM Tris-HCl (pH 6.8), 4% SDS, 200 mM dithiothreitol (DTT), 20% glycerol. Then cells were homogenized with a pellet pestle and centrifuged at 15,000 rpm, 10 min. The supernatant was stored at —80°C. For protein preparation from culture medium of BY-2, day 4 cultured media were filtered through 4-layered Miracloth (Calbiochem, La Jolla, CA, U.S.A.) and centrifuged to remove insolubles. The proteins were precipitated from the supernatant by 70% saturation with ammonium sulfate and dissolved in H₂O, then dialyzed overnight against 5 mM Tris-HCl (pH 6.8). The solution of crude protein was stored at —80°C. Proteins were quantified using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, U.S.A.).

SDS-PAGE and immunoblot analysis—Proteins were denatured in sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 1.5% SDS, 5% glycerol, 2.5% 2-mercaptoethanol and 5 μg ml⁻¹ bromophenol blue. For separation of polypeptides, we performed SDS-PAGE according to Laemmli (1970), then electrotransferred the peptides onto a nitrocellulose filter (0.45 μm, Advantech, Tokyo, Japan). Immunoblot analyses were performed with an ECL. Western blotting detection system (Amersham, U.K.), using GFP Polyclonal Antibody (Clontech) and anti-rabbit IgG-horseradish peroxidase conjugate (Amersham) as a second antibody.

RNA gel blot analysis—For analysis of organ specific expression of ATCYP5, roots, leaves, stems and flowers were collected from day 28 Arabidopsis plants (ecotype Landsberg erecta) and frozen in liquid nitrogen. For analysis of the effects of cold, heat or salt stress, plants grown on GM medium for 2 weeks at 22°C were transferred to an incubator kept at 4°C or 37°C, or were placed on 3-layered Whatman 3MM paper that had been immersed in 200 mM NaCl. Plants were treated for various periods and then frozen in liquid nitrogen. Total RNA was isolated with total RNA isolation reagent ISOGENT (Nippon Gene, Tokyo, Japan) according to the directions of the manufacturer. Ten micrograms of total RNA samples were fractionated on 1.2% agarose gel containing 2 M formaldehyde and blotted onto Hybrid-N⁺ nylon membrane (Amersham) (Sambrook et al. 1989). Prehybridization was performed in a solution containing 50% formamide, 7% SDS, 2% blocking reagent (Boehringer Mannheim), and 0.1% sodium N-lauroyl sarcosinate, in 50 mM sodium phosphate (pH 7.0) at 50°C for 2 h and hybridization was performed with prehybridization buffer containing 1 ng ml⁻¹ DIG-labeled ATCYP5 cDNA as a probe at 50°C for 16 h. Then the filter was washed twice with 2 × SSC, 0.1% SDS at room temperature for 5 min, and twice with 0.1 × SSC, 0.1% SDS at 68°C for 15 min. For detection, we used anti-DIG-alkaline phosphatase conjugate and the chemiluminescent substrate CSPD™ (TROPIX).

Construction of the promoter-GUS plasmid—For histochemical analysis, we made the ATCYP5 promoter-GUS construct as follows. HindIII-EcoRI fragment of pBI221 (Clontech) containing 35S promoter and GUS gene was inserted into the same site of pUC119 to make pBI211. Then Xhol linker (5'-CTTGGATCCGAATTCATCTCAAAA-TA-3' (sense) and 5'-CTTCTCGAGCCGTGCCGATGCTG-3' (antisense) (Fig. 1) which have BamHI and Xhol sites at the 5' region respectively. The 1.6 kbp amplification fragment was isolated and double-digested with BamHI and Xhol and then inserted in the same site of pBI241. Subsequently, the Xbal-Sacl fragment was inserted in the same site of pHM1. The resulting plasmid, named pSA601 (Fig. 8), was transformed into Agrobacterium tumefaciens C58C1 (pMP90) by electroporation. Transformation of Arabidopsis (ecotype Wassilewskija; WS) was carried out as described by Bechtold et al. (1993). Seeds were screened on 20 mg liter⁻¹ hygromycin-containing growth media (0.5 × MS salts, 0.5 × B5 vitamins, 0.5 g liter⁻¹ 2-[N-morpholino]ethanesulfonic acid, pH 5.7).

Histochemical GUS analysis—Transgenic Arabidopsis plants were immersed in reaction buffer containing 50 mM sodium phosphate (pH 7.0), 0.2% Triton X-100, 0.5 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). Samples were subjected to vacuum infiltration for 15 min, slowly vented, and then incubated at 37°C for 20 h. Then the reaction buffer was removed and samples were destained through several changes of 70% ethanol.

Results

Isolation and sequence analysis of ATCYP5—In a previous PCR experiment using a set of primers corresponding to the highly conserved regions among known CyPs, we obtained four kinds of partial DNA fragments derived from different CyP genes (Saito et al. 1995). They corresponded to ATCYP1, ATCYP2 (Saito et al. 1995), ATCYP4 (Saito et al. accession no. U31370) and ATCYP3 (Saito et al. unpublished) which was identical to ROC3 (Chou and Gasser, accession no. U40399). They were thought to be cytosolic CyPs, because they lacked any known signal for transport to specific compartments. In this study, we tried to isolate other types of CyP genes using the cassette PCR technique with different sets of primers (see Materials and Methods). As a result, we obtained the 500 bp PCR product, which seemed to be a single band. We cloned the DNA fragment and sequenced more than 10 recombinant plasmids with the 500 bp fragment. They were found to be identical, and derived from a novel CyP gene. Thus we used the 500 bp fragment as a probe for screening of the Arabidopsis genomic library. Of 1 × 10⁶ plaques, five positive clones were isolated. Since Southern blot analysis of the inserted fragment of these five phage clones indicated that they were overlapping clones of the same gene (data not shown), the overlapping EcoRI-PstI and HindIII-HindIII fragment of one clone was subcloned into pBluescriptII and sequenced. Figure 1 shows the nucleotide sequence of the EcoRI-HindIII region of the clone. Because CyPs are highly conserved in the
The predicted amino acid sequence of ATCYP5 included five introns, and the ORF started at amino acid position 1 of the exon in Figure 1. The in-frame termination codon existed in the open reading frame (ORF) in the nucleotide sequence. This prediction was substantiated by the sequence of Arabidopsis expressed sequence tags (EST) T46293 and H37581 (Newman et al. unpublished), the sequence of which corresponded to the capital nucleotides in —29 to 1016 and 622 to 1419 of the cDNA clone isolated in this work. Furthermore the sequence of expressed sequence tags (EST) T46293 and H37581 was substantiated by the sequence of Arabidopsis microsomal CyP (Sheldon et al. 1996) showing significant identity with the sequence from Lys-24 of ATCYP5 (Fig. 3). The program that predicts protein sorting signal and localization site (PSORT; Nakai, K., Osaka University) showed that ATCYP5 is localized in ER and/or vesicle compartments and the cleavage site of the signal peptide is indicated that ATCYP5 is localized in ER and/or vesicle compartments and the cleavage site of the signal peptide is.

Fig. 1 Nucleotide and deduced amino acid sequence of ATCYP5. The nucleotides and amino acids are numbered from the first nucleotide of the initiation codon. Small letters indicate introns. Restriction sites used for cloning are indicated in bold. Nucleotide sequences used to generate primers for cassette PCR, cDNA cloning, or amplification of the promoter region are underlined, double-underlined or dotted-underlined, respectively.

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The prediction amino acid sequence, we could assign the open reading frame (1–23th amino acids) which contained a hydrophobic stretch preceded by a positively charged amino acid (Lys-3) as compared with known plant CyPs (Fig. 2), and this region was thought to act as the ER-targeting signal sequence. The program that predicts protein sorting signal and localization site (PSORT; Nakai, K., Osaka University) showed that ATCYP5 is targeted to ER with a high degree of probability. The finding that the N-terminal amino acid sequence of maize microsomal CyP (Sheldon et al. 1996) showed significant identity with the sequence from Lys-24 of ATCYP5 (Fig. 3) indicated that ATCYP5 is localized in ER and/or vesicle compartments and the cleavage site of the signal peptide is between Ala-23 and Lys-24.
Fig. 2 Alignment of amino acid sequences of plant Cyps. Dots indicate amino acids that are identical to those in ATCYP5 and dashes indicate gaps introduced for maximal matching. Asterisks indicate the codon for termination of translation. The two conserved regions used to design primers for cassette PCR are boxed. The sources of the sequences are Arabidopsis (ATCYP1, ATCYP2, Saito et al. 1995; ATCYP4, Lippuner et al. unpublished; ROC1 and ROC4, Lippuner et al. 1994; ROC3, Lippuner et al. unpublished; ATHCYC, Bartling et al. 1992), tomato, maize and Brassica (Gasser et al. 1990), bean (Marivet et al. 1992), rice (CYP1 and CYP2, Buchholz et al. 1994) and fava bean (pCyPB; Luan et al. 1994).

sequence of the deduced ATCYP5 protein with that of other plant Cyps. The sequence of the mature region of ATCYP5 exhibited 65% to 70% identity to those of plant cytosolic Cyps including those from other species. On the other hand, 65% and 62% identities were observed to the mature region of ROC4 and pCyPB, chloroplast-localized Cyps in Arabidopsis and fava bean, respectively. Interestingly, ATCYP5 contained an insertion of seven amino acids (KSGKPLH, 76-82th amino acids) which were previously pointed out as a characteristic of plant cytosolic Cyps (Lippuner et al. 1994) (Fig. 4). The evolutionary origins of plant CyP genes is discussed below.

Translocation of ATCYP5-sGFP fusion protein into ER—To examine whether ATCYP5 is translocated to ER, the synthetic green fluorescent protein (sGFP; S65T) (Chiu et al. 1996) fused with the putative signal peptide of ATCYP5 was expressed in tobacco BY-2 cells. As a control, BY-2 cells transformed with pSA701 (35S-sGFP) (Fig. 5) were viewed by fluorescent microscopy. Green fluorescence of sGFP was detected mainly in the nucleus (Fig. 6a) as reported by Baulcombe et al. (1995). Because tobacco BY-2 cells were highly vacuolated, only weak fluorescence was observed in the cytosol. Accumulation of sGFP in the nucleus was confirmed by the images of confocal sections and we also observed an sGFP-free space, probably a nuleolus, in the nucleus as described by Kohler et al. (1997) (Fig. 6c-f). Then the location of the sGFP fused

ATCYP5 MAKASFILLGTLFLFGA1ASIQAIIKELKEIE 30
Maize  KROILTEIV/ 7

ATCYP5 THKYPFIDVEIDGKSVRGTVGGFLF 53
Maize  THKYPFIDVEIDGRPAGRIVGFLF 30
with the first to 48th amino acids containing the putative signal peptide of ATCYP5 (A5sGFP) was analyzed in the BY-2 cells transformed by pSA702 (35S-A5sGFP) (Fig. 5). As shown in Figure 6b, circular green fluorescence was observed around the nucleus. In addition, by confocal microscopic analysis, the diameter of the circles was shown to change gradually in proportion to the depth of confocal sections (Fig. 6g–j). These results indicated that A5sGFP accumulated in the nuclear envelope region and supported our speculation that A5sGFP was translocated to the ER, and that the N-terminal region of ATCYP5 could act to translocate the fusion protein to the lumen of ER. We could not observe clearly the reticularly dispersed fluorescence in the cytosolic space, probably because of the presence of highly developed vacuoles in the cells as mentioned above.

**Immunoblot analysis**—To ascertain whether the signal peptide of ATCYP5 was processed in transformed BY-2 cells, A5sGFP fusion protein was analyzed by immunoblotting with anti-GFP antibody. Crude extracts were prepared from tobacco BY-2 cells or E. coli transformed by pSA701 (35S-SGFP) or pSA702 (35S-A5sGFP). In this experiment, E. coli expressing A5sGFP was used as a control. As a result, two signals were detected from the E. coli protein fraction (Fig. 7a, lane 1). The larger one was estimated to have a molecular mass of 31.0 kDa coinciding with the deduced molecular mass of A5sGFP. The smaller one was estimated to have a molecular mass of 29.5 kDa and was speculated to be the processed product by signal peptidase in E. coli. The size of the polypeptide detected in the extracts from transformed BY-2 cells was almost the same as the processed form in E. coli (Fig. 7a, lane 2). These results indicate that the precursor of ATCYP5 is processed in the cells.

We also examined the secretion of A5sGFP in the medium. When the BY-2 cells transformed with pSA702 were liquid-cultured, the fluorescence thought to be derived from A5sGFP was observed in the medium (data not shown). Subsequently, to ascertain whether the fluorescence was actually derived from A5sGFP, BY-2 cells transformed by pSA701 (35S-sGFP) or pSA702 (35S-A5sGFP) were liquid-cultured for 4 d and proteins were prepared from each medium. The signal with 26 kDa was detected in the protein fraction prepared from culture medium of BY-2 cells expressing A5sGFP (Fig. 7b, c, lane 7), but not in that of the cells expressing sGFP (Fig. 7b, c, lane 6). As described above, the precursor and processed forms of A5sGFP were estimated to be 31 kDa and 29.5 kDa, respectively (Fig. 7a, lane 1, 2). The secreted form of the A5sGFP seems to be degraded into the 26 kDa form. These results indicate that the N-terminal extension of A5sGFP may function as an ER-targeting signal and some of the processed form is secreted into the medium.

**Northern blot analysis**—To examine the expression of ATCYP5, total RNAs were prepared from roots, stems, leaves and flowers of day 28 Arabidopsis plants and analyzed by Northern hybridization with ATCYP5 cDNA as a probe. We checked the cross-hybridization of ATCYP5 probe to other CyP genes by genomic Southern hybridization. No signals other than from the ATCYP5 gene were detected under the conditions described in Materials and Methods (data not shown). ATCYP5 mRNA was detected in almost equal amounts in all samples (Fig. 8a).

Several CyP genes are induced by various stresses. We examined whether the expression of ATCYP5 is induced by cold, heat or salt stress. Northern hybridization indicated that mRNA of ATCYP5 increased only slightly by cold stress and salt stress, but not by heat stress (Fig. 8b).

**Expression of ATCYP5 promoter-GUS fusion gene in Arabidopsis**—To identify the tissue specificity of ATCYP5 expression, Arabidopsis (ecotype WS) was stably transformed with pSA601 by vacuum infiltration (Bechtold et al. 1993) (Fig. 9). Then three independent lines that were resistant to hygromycin were obtained and T2 plants were used for experiments. GUS activity was not detected in the day 3 seedlings (Fig. 10a), but was detected in the shoot apex, upper root region and petioles of true leaves at a later stage (Fig. 10b). In the bolting stage, GUS activity was detected in stems especially in the apical region (Fig. 10c). In day 21 plants, GUS activity was strongly detected in peduncles, base of peduncles, but not in flowers (Fig. 10d–f). In stems and peduncles, the expression of GUS in the apical region continued to be detected. No visible changes were observed in the expression of the fusion gene in the transgenic plants after salt or cold stress compared to the untreated plants (data not shown) as expected from the results of Northern blot analysis (Fig. 8b). These results indicate that the expression of ATCYP5 is specific, and ATCYP5 was expressed first in both apical and basal regions in stems and peduncles, then later became
Fig. 6 Subcellular distribution of sGFP in tobacco BY-2 cells. Images of BY-2 cells transformed with pSA701 (a) and pSA702 (b) by fluorescent microscopy. Sequential confocal images of BY-2 cells transformed with pSA701 (c–f) and pSA702 (g–j). The frames are ordered from the upper left to the lower right, with increase of confocal depth. Scale bars indicate 30 μm.
Discussion

In this study, we isolated ATCYP5, a CyP gene the product of which contains hydrophobic N-terminal extension predicted as ER-targeting signal, from Arabidopsis thaliana. Interestingly, ATCYP5 included insertion of seven amino acids previously thought to be characteristic of plant cytosolic CyPs (Fig. 2, 4). Despite the high degree of conservation, the role of this region has not been established. This region was not found in either ROC4 isolated from Arabidopsis or pCyPB isolated from fava bean, both chloroplast-localized CyPs, or CyP from cyanobacteria (Hassidim et al. 1992). In addition, considering that ATCYP5 showed a high degree of identity with plant cytosolic CyPs rather than ER-localized CyPs from other sources, we hypothesized the origins of organelle CyPs of Arabidopsis as follows. ATCYP5 might evolve from cytosolic CyP which already had insertion of seven amino acids by obtaining an ER-targeting signal, and ROC4 might have been originally encoded in the chloroplast genome, then transferred to the nuclear genome. Thus it is very interesting to examine whether or not plant mitochondrial CyPs are encoded in nuclear genome and have the insertion of seven amino acids.

To investigate the intracellular localization of ATCYP5, we employed synthetic green fluorescent protein (sGFP; S65T) as a reporter and expressed the fusion protein in tobacco BY-2 cells. When the control construct 35S-sGFP was expressed, intense green fluorescence was mainly observed in the nucleus (Fig. 6a, c–f). There may be potential sequences promoting translocation to the nucleus in sGFP (Baulcombe et al. 1995). The fluorescence in the cells was not evenly distributed, probably because the cytosol was narrowed by the developed vacuoles. In contrast, when 35S-A5sGFP was expressed, intense green fluorescence was observed mainly around the nucleus (Fig. 6b, g–j), indicating that fusion proteins were accumulated mainly in the nuclear envelope and possibly in ER. A similar image was obtained from BY-2 cells visualized by immunofluorescence using antibodies against HDEL ER-retention signal (Gomord et al. 1997), also supporting our interpretation. By immunoblot analysis, we ascertained that A5sGFP was processed into 29.5 kDa form in the

![Figure 7](https://academic.oup.com/pcp/article-abstract/40/1/77/1901186) Processing and secretion of A5sGFP. (a) Immunoblot with protein from E. coli (70 μg) (lane 1), tobacco BY-2 cells (3 μg) (lane 2), both transformed with pSA702. (b) Immunoblot with protein from cell (lane 4 and 5) or culture medium (lane 6 and 7) of BY-2 cells, which were transformed with pSA701 (lane 4 and 6) or pSA702 (lane 5 and 7), respectively. Three μg of crude proteins were loaded in each lane (lane 4–7). Protein from E. coli (70 μg) is also shown in lane 3. Blots were probed with anti-GFP antibody. (c) Same filter shown in (b) was overexposed to X-ray film. Secreted protein is detected clearly (lane 7').

![Figure 8](https://academic.oup.com/pcp/article-abstract/40/1/77/1901186) Northern blot analysis of ATCYP5. RNA gel blots with equal amounts (10 μg) of total RNA except for 4°C treatment were probed with DIG-labeled ATCYP5 cDNA. (a) RNA was isolated from roots (lane 1), leaves (lane 2), stems (lane 3) and flowers (lane 4) of day 28 Arabidopsis plants. (b) Day 14 Arabidopsis plants were kept at 4°C or 37°C or on Whatman 3MM paper immersed with 200 mM NaCl. These plants were treated for various periods as indicated and total RNAs were isolated from the whole plant. Only 7 μg of total RNA was loaded in the lane of 4°C for 0.5 h treatment because of poor recovery of RNA from plant sample.

![Figure 9](https://academic.oup.com/pcp/article-abstract/40/1/77/1901186) Structure of GUS expression plasmid. Construction scheme of plasmid was as described in Materials and Methods. NOS, nopaline synthase promoter; NPTII, gene for neomycin phosphotransferase II; Tnos, nopaline synthase terminator; 35S, cauliflower mosaic virus 35S promoter; GUS, β-glucuronidase; HPT, gene for hygromycin phosphotransferase. Region between right and left borders (black arrowheads) are indicated.
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Fig. 10 Histochemical GUS analysis. GUS staining of transgenic Arabidopsis plants carrying ATCYP5 promoter-GUS gene. Each photograph shows day 3 seedling (a), day 9 plant (b), inflorescence of day 16 plant (c), and shoot apical region (d), lateral branch (e) and secondary inflorescence (f) of day 21 plant.

cells, and the difference of molecular mass between precursor and the processed form coincided to that of the predicted signal peptide (Fig. 7a), suggesting that cleavage occurs probably between Ala-23 and Lys-24 estimated from the results of the PSORT program and N-terminal sequence of maize microsomal CyP (Sheldon et al. 1996). N-terminal amino acid sequence analysis is required to clarify the exact cleavage site in the ATCYP5. We also ascertained that part of the processed form of the A5sGFP fusion protein was secreted to medium probably due to the absence of an ER-retention signal (Fig. 7b). These results support our speculation that ATCYP5 is an ER form of CyP.

Proteins localized in the ER lumen include a KDEL or HDEL ER-retention signal sequence in its C-terminal and one of the CyP isoforms from Saccharomyces cerevisiae (CyPD; Frigerio and Pelham 1993) actually has a HDEL sequence. However, in some CyP isoforms localized in the ER lumen, the typical ER-retention signal was not found, suggesting the presence of other mechanisms for retention of ER proteins without a typical ER-retention signal sequence. Bram et al. (1994) showed that human CyPB localized in ER by interaction with ER membrane bound protein termed calcium-signal modulating cyclophilin ligand (CAML), and implicated the role of CyPB in the calcium-signal transduction pathway. In some conditions, CyPB was also secreted into milk or blood as a truncated form (Mariller et al. 1996). In this case, C-terminal five
that ATCYP5 might also play a role in the folding of some proteins, probably in the ER, of roots, stems, peduncles in normal development. To elucidate the precise physiological functions of ATCYP5, further efforts are necessary to identify the natural substrates of ATCYP5.

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