Cold Acclimation-Induced WAP27 Localized in Endoplasmic Reticulum in Cortical Parenchyma Cells of Mulberry Tree Was Homologous to Group 3 Late-Embryogenesis Abundant Proteins

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We have shown that two 27-kD proteins, designated as WAP27A and WAP27B, were abundantly accumulated in endoplasmic reticulum-enriched fractions isolated from cortical parenchyma cells of mulberry tree (*Morus bombycis* Koidz.) during winter (N. Ukaji, C. Kuwabara, D. Takezawa, K. Arakawa, S. Yoshida, S. Fujikawa [1999] Plant Physiol 120: 480–489). In the present study, cDNA clones encoding WAP27A and WAP27B were isolated and characterized. The deduced amino acid sequences of WAP27A and WAP27B cDNAs had 12 repeats of an 11-mer amino acid motif that was the common feature of group 3 late-embryogenesis-abundant proteins. Under field conditions, transcripts of WAP27 genes were initially detected in mid-October, reached maximum level from mid-November to mid-December, and then gradually decreased. The transcript levels of WAP27 genes in cortical parenchyma cells harvested in October was drastically induced by cold treatment within a few days, whereas those in cortical parenchyma cells harvested in August were low even by cold treatment for 3 weeks. Immunocytochemical analysis by electron microscopy confirmed that WAP27 was localized specifically in vesicular-form ER and also localized in dehydration-induced multiplex lamellae-form ER. The role of WAP27 in the ER is discussed in relation to acquisition of freezing tolerance of cortical parenchyma cells in mulberry tree during winter.

Plants grown in the temperate zone acquire freezing tolerance as a result of cold acclimation (Levitt, 1980; Sakai and Larcher, 1987). During cold acclimation, diverse changes at cellular and molecular levels, including compositional changes in the plasma membranes (Śtepnikus, 1984; Yoshida, 1984; Uemura et al., 1995), intracellular accumulation of compatible osmolytes, such as soluble sugars, prolines, and betaines (Hare et al., 1998), heat shock proteins (Neven et al., 1992; Ukaji et al., 1999), cold-regulated (COR) proteins (Guy et al., 1985; Thomashow, 1999), extracellular accumulation of antifreeze proteins (Griffith and Antikainen, 1996), and changes of the property of cell walls (Rajashekar and Lafta, 1996; Fujikawa and Kuroda, 2000) occur in a wide variety of plant cells. These changes were found to be associated with increased freezing tolerance (Guy, 1990; Fujikawa et al., 1999; Thomashow, 1999).

Recent studies have focused on cold acclimation-induced accumulation of COR proteins. The majority of these proteins have structural similarity with late-embryogenesis abundant (LEA) proteins (Thomashow, 1994, 1999). LEA proteins were first identified during the maturation and a desiccation phase of seed development in cotton embryo (Dure et al., 1981), and the expression at high levels during embryo maturation is now known to occur with all angiosperms. These proteins also accumulate in a variety of vegetative tissues in response to osmotic stress or in response to exogenous application of abscisic acid (ABA) (Ingram and Bartels, 1996; Bray, 1997). LEA proteins have a highly hydrophilic feature and remain soluble upon boiling (Baker et al., 1988; Lin et al., 1990; Ingram and Bartels, 1996; Bray, 1997). Many LEA proteins or their genes have been characterized and, based on their common amino acid sequences, have been classified into three major groups and two or more additional groups (Baker et al., 1988; Bray, 1993; Ingram and Bartels, 1996). These LEA proteins have been proposed to contribute in various ways to desiccation tolerance in embryos and vegetative organs (Ingram and Bartels, 1996; Xu et al., 1996; Bray, 1997).

During cold acclimation, homologs of LEA proteins also accumulate in many plant species, including both herbaceous and woody plants (Arora and Wisniewski, 1994; Thomashow, 1999). During extracellular freezing, liquid water is withdrawn out of the cells, resulting in cellular dehydration (Levitt, 1980; Śtepnikus, 1984; Guy, 1990). Therefore, it has
been suggested that LEA protein homologs may play a role in conferring tolerance in plant cells under freezing condition (Thomashow, 1998, 1999). Recent studies have indicated that constitutive overexpression of COR15a, a highly hydrophilic protein with similarity to LEA proteins, in Arabidopsis increased the freezing tolerance either in chloroplasts or in protoplasts isolated from transgenic Arabidopsis (Artus et al., 1996). It has also been shown that overexpression of CAP85, a group 2 LEA protein, or CAP160, a highly hydrophilic protein with similarity to LEA protein, from spinach resulted in reduction of freezing injury of transgenic tobacco plants (Kaye et al., 1998). Overexpression of LEA genes, LE25 from tomato (Imai et al., 1996) and hiC6 from Chlorella vulgaris (Honjoh et al., 1999), enhanced the freezing tolerance in transformed yeasts. The yeasts overexpressing LE25 genes from tomato also increased salt tolerance, suggesting that LE25 has a function as an ion scavenger (Imai et al., 1996).

Seasonal periodic temperature changes produce large seasonal differences in the freezing tolerance of cortical parenchyma cells of mulberry tree (Morus bombycis Koidz.). The freezing tolerance of cortical parenchyma cells of mulberry tree grown in Sapporo, Japan is above −5°C in summer, increases gradually in autumn, reaches a maximum below −80°C in winter, and then decreases gradually in spring (Niki and Sakai, 1981; Fujikawa, 1994). In extremely cold-hardy woody plant cells including cortical parenchyma cells of mulberry tree, cold acclimation induces physiological and biochemical changes similar to those in herbaceous plant cells (Yoshida, 1984; Sakai and Larcher, 1987). Seasonal distinct morphological changes of cellular organelles, such as vacuolation of vacuoles or waving of plasma membranes, have also been reported during winter (Levitt, 1980; Sakai and Larcher, 1987). Among these morphological changes, seasonal changes in the endoplasmic reticulum (ER) are the most prominent phenomenon only in extremely cold-hardy woody plants. The ER changes in the morphology from a cisternae-form, dispersed sparsely in the cytoplasm during summer, to a vesicular-form, dispersed densely in the cytoplasm during winter (Niki and Sakai, 1981; Fujikawa and Kuroda, 2000). Freezing and/or osmotic dehydration induce the fusion of the vesicular-form ER to each other and develop into multiplex lamellae (MPL)-form ER with an extensive distribution beneath the plasma membranes (Fujikawa and Takabe, 1996). The MPL-form ER was hypothesized to prevent the close approach between plasma membranes and other organelle membranes caused by freeze-induced dehydration (Fujikawa and Takabe, 1996).

In our previous study, we found the accumulation of WAP20, WAP27A and WAP27B in the ER-enriched fractions in association with increased freezing tolerance in cortical parenchyma cells of mulberry tree (Ukaji et al., 1999). Comparison of the N-terminal amino acid sequences with those of other known proteins, WAP20 was identified as an ER-localized small heat shock protein. In the present study, cDNA clones encoding WAP27A and WAP27B were isolated from cortical parenchyma cells of mulberry tree. Sequence analysis revealed that these proteins are homologs of group 3 LEA proteins. The putative role of ER-localized WAP27 in conferring freezing tolerance to cortical parenchyma cells of mulberry tree during winter is discussed.

RESULTS

cDNA Sequence Analysis of WAP27A and WAP27B

A cDNA library was constructed from poly(A+) RNA isolated from cortical parenchyma cells of mulberry tree harvested in December in the field. From this cDNA library, 11 clones were isolated by immunoscreening using the purified anti-WAP27 antibodies. Among these 11 clones, eight clones shared a common nucleotide sequence, and the deduced amino acid sequences of them had the same N-terminal amino acid sequence as that of WAP27B reported previously (Ukaji et al., 1999). The other three clones shared a common nucleotide sequence and had a very similar nucleotide sequence to that of WAP27B cDNA. Because each of the sequences of the three clones lacked a 5′ region, this region of the gene was isolated by PCR to determine the full-length sequence. The deduced amino acid sequence of the three clones showed the same N-terminal amino acid sequence as that of WAP27B reported previously (Ukaji et al., 1999). The sequences of WAP27A cDNA (GenBank accession no. AF326119), had a length of 986 bp that included an open reading frame encoding 227 amino acids. The WAP27B cDNA (GenBank accession no. AF326120) had 926 bp in length and encoded a protein with 224 amino acids. The deduced amino acid sequences of cDNAs in WAP27A and WAP27B are shown in Figure 1. Both of the deduced amino acid sequences had a putative signal sequence in the N-terminal region. Predicted cleavage sites of the signal peptides were located just before the sequences that were determined as N-terminal amino acids of WAP27A and WAP27B in our previous study (Ukaji et al., 1999). The calculated pI values of WAP27A and WAP27B were 4.88 and 4.92, respectively. These pI values were in agreement with the result of two-dimensional gel electrophoresis of ER-enriched fractions reported previously (Ukaji et al., 1999).

The molecular masses of both proteins estimated by SDS-PAGE analysis were approximately 27 kDa (Ukaji et al., 1999), whereas the molecular masses of deduced WAP27A and WAP27B polypeptides estimated by the nucleotide sequences were 22.5 and 22.3 kDa, respectively. Such differences in the estimated molecular masses have also been observed in other LEA proteins, such as WCS120 (Houde et al.,
1992) or WCOR410 in wheat (Danyluk et al., 1998) and COR47 in Arabidopsis (Gilmour et al., 1992). The peptide sequence RDEEL was located just before the stop codon (Fig. 1). The tetrapeptide DEEL or the pentapeptides RDEEL have not been demonstrated to be a retention signal to the ER, although they have similar amino acid sequences to those of the putative retention signal sequence KDEL. However, the results of immunocytochemical analysis using electron microscopy clearly indicated that WAP27 is specifically located in the ER (see Fig. 7), suggesting that these sequences play a role in the retention signal to the ER lumen.

The deduced amino acid sequence of WAP27A and WAP27B had 12-time tandem repeats of the 11-mer amino acid motifs (Fig. 1). The 11-mer amino acid tandem repeats are a common feature of D-7 family or group 3 LEA proteins (Baker et al., 1988; Dure, 1993; Ingram and Bartels, 1996). This suggests that WAP27A and WAP27B are members of group 3 LEA protein.

Southern-Blot Analysis of WAP27 Genes

The copy number of WAP27-related genes in a mulberry genome was estimated by Southern-blot analysis (Fig. 2). Genomic DNA isolated from cortical parenchyma cells mulberry tree was digested with BglII, EcoRI, HindIII, and XhoI, followed by hybridization with 32P-labeled full-length WAP27B cDNA. WAP27B cDNA probes were also hybridized to WAP27A cDNA (data not shown). One band by XhoI digestion, two strong bands by EcoRI digestion, and three bands by BglII and HindIII digestions were detected (Fig. 2). WAP27A and WAP27B genes contained one internal BglII and two internal HindIII restriction sites, suggesting that a few copies of WAP27-related genes were present in mulberry genomic DNA.

Seasonal Change in Transcripts of WAP27 Genes

The transcript levels of WAP27 genes in cortical parenchyma cells of mulberry tree were determined by northern-blot analysis. In field conditions, transcripts of WAP27 genes were initially detected in mid-October, reached the maximum level between mid-November and mid-December, gradually decreased to mid-March, and then disappeared in summer (Fig. 3). Transcripts of WAP27 genes were not detected during summer either in cortical parenchyma cells (Fig. 3) or leaves (data not shown). Because it was impossible to distinguish the levels of the transcripts of WAP27A and WAP27B genes by northern-blot analysis, reverse transcription PCR detection was performed using differentiated primers for WAP27A and WAP27B genes. The amplified DNA bands corresponding to WAP27A and WAP27B genes by PCR showed that the two genes exhibited similar seasonal expression patterns and expression levels (data not shown). These results suggested that the transcript levels of WAP27A and WAP27B genes in association with seasonal changes were similar.

Tissue-Specific Accumulation of Transcripts of WAP27 Genes

To examine the tissue-specific accumulation of transcripts of WAP27 genes, total RNA of cortical, xylem, bud, and leaf tissues were extracted from mulberry trees from mid-September to mid-December. Northern-blot analysis of the tissues in September showed low transcript levels of WAP27 genes only in bud tissues, and transcripts of the genes were not detected in cortical, xylem, and leaf tissues (Fig. 4A). In October, transcript levels of WAP27 genes were increased in cortical, xylem, and bud tissues but were not detectable in leaf tissues. In cortical, xylem, and bud tissues, transcript levels of
WAP27 genes were further increased from November to December. To understand the accumulation levels of WAP27 in cortical, xylem, and bud (or leaf) tissues, microsome fractions in these tissues were seasonally prepared and analyzed by immunoblotting using purified anti-WAP27 antibodies. In cortical and xylem tissues, immunoreactive bands were detected in December and May but not in June and July (Fig. 4B). In bud (or leaf) tissues, strong bands were detected in December and slightly reactive bands were detected in May but not in July and June. In May samples, buds with partially opened leaves were used. These results indicated that WAP27 were accumulated in cortical, xylem, and bud tissues only in winter.

Induction of WAP27 Genes by Low Temperature, ABA, or Dehydration

Northern-blot analysis also showed that transcript levels of WAP27 genes increased by low temperature in cortical parenchyma cells of mulberry twigs (Fig. 5). During cold treatment at 4°C of mulberry twigs harvested in mid-August from the field, transcripts of WAP27 genes were barely detected after 1 week and gradually increased by prolonged exposure at least until 4 weeks in dark (Fig. 5A). At 24-h light condition, cold treatment for 1 week also did not produce remarkable transcripts of WAP27. During cold treatment of the twigs harvested in mid-October, the transcript levels of WAP27 genes increased within 1 d and were strongly induced until 7 d in the cortical parenchyma cells (Fig. 5B). On the other hand, the transcript levels of WAP27 genes in the twigs harvested in mid-October rapidly decreased with treatment at 20°C for 1 d and disappeared completely with treatment for 2 d (Fig. 5B).

In a variety of plants, genes encoding LEA proteins are induced by exogenous application of ABA or dehydration (Hajela et al., 1990; Ingram and Bartels, 1996; Thomashow, 1999). To investigate the responses of WAP27 genes to these stresses, de-acclimated twigs in a greenhouse were subjected to treatment with ABA or dehydration (Fig. 6). Transcripts of WAP27 genes in de-acclimated twigs were not detected before treatments with ABA or dehydration. Transcript levels of WAP27 genes were detected within 2 d by exogenous application of ABA and increased to the maximum level after 5 d (Fig. 6). Transcript levels of WAP27 genes were also detected by dehydration for 6 h and gradually increased with prolonged dehydration (Fig. 6).

Localization of WAP27 Proteins in Vesicular-Form and MPL-Form ER

To determine the localization of WAP27 in cortical parenchyma cells of mulberry tree, immunocytochemical electron microscopy was performed using purified anti-WAP27 antibodies. The purified anti-
WAP27 antibodies only reacted with WAP27A and WAP27B, as shown in our previous study (Ukaji et al., 1999). In cortical parenchyma cells of mulberry tree, it has been shown that the morphology of the ER changes from cisternae-form in summer to a vesicular-form in winter during seasonal cold acclimation (Niki and Sakai, 1981). Immunocytochemical electron microscopy revealed a heavy deposition of gold-particles against anti-WAP27 antibodies in vesicular-form ER but not in other organelles, including cell walls, cytosol, Golgi complex, plasma membranes, mitochondria, chloroplasts and vacuoles in winter bud cells (Fig. 7A), and in cortical parenchyma cells (data not shown) harvested in late-January. In the cortical parenchyma cells, gold-particles against anti-WAP27 antibodies were not detected in cisternae-form ER in summer (data not shown). In addition, no gold-particles were detected in any sections treated with preimmune serum as a primary antibody (data not shown).

It has been shown that the vesicular-form ER in cortical parenchyma cells of mulberry tree during winter is morphologically converted into MPL-form by freezing below \(-5^\circ C\) as well as osmotic stress corresponding to the freezing-induced dehydration (Fujikawa and Takabe, 1996). At osmotic stress corresponding to freeze-induced dehydration of \(-10^\circ C\), MPL was formed in cortical parenchyma cells of mulberry tree only in winter (Fig. 7B). In these samples, gold-particles against anti-WAP27 antibodies were detected only in the MPL-form ER (Fig. 7B).

**DISCUSSION**

The present study showed that WAP27A and WAP27B, which accumulated abundantly in the ER of cortical parenchyma cells of mulberry tree during winter, are a homolog of group 3 LEA proteins, due to a simple amino acid composition (Ingram and Bartels, 1996), due to 12-time repeats of 11-amino acid sequence motif (Lin et al., 1990; Ingram and Bartels, 1996; Bray, 1997), and due to their induction by ABA and dehydration (Bray, 1997; Thomashow, 1999).

Northern-blot analysis revealed that transcript levels of WAP27 genes were altered in response to seasonal cold acclimation (Fig. 3), showing similarity, but with slight difference, to the seasonal accumulation of WAP27 (Ukaji et al., 1999). The transcript levels of WAP27 genes decreased from February to March and were scarcely detected in April and May in cortical parenchyma cells (Fig. 3). On the other hand, the levels of WAP27 were maintained at high levels until May (Fig. 4B). This result suggests low degradation of WAP27 until May. The levels of WAP27 decreased by June, suggesting rapid degradation of WAP27 at this time, although the degradation mechanism remains to be determined.

The transcript levels of WAP27 genes were increased by cold treatment of twigs. However, the transcript levels of WAP27 genes of twigs harvested in mid-October immediately and intensively increased by cold treatment compared with those harvested in mid-August (Fig. 5). The freezing tolerance
of cortical parenchyma cells of twigs harvested in October increased remarkably by cold treatment, whereas cold acclimation-induced increase of freezing tolerance was low in twigs harvested in August (Sakai and Yoshida, 1968). Our results suggest that transcript levels of WAP27 genes by cold treatment in August and October twigs may be related to the acquisition of freezing tolerance in these twigs. In birch tree, it has been shown that dehydrin-like proteins were accumulated under short-day conditions and that the accumulation was further enhanced by subsequent low temperature treatment (Rinne et al., 1999). In woody plant species grown in the temperate zone, it has been indicated that endodormancy obtained by short-day conditions is prerequisite for the acquisition of cold acclimation (Weiser, 1970; Olsen et al., 1997). It is thought that initiation of endodormancy may be prerequisite for full induction of WAP27 genes by cold treatment as well as the acquisition of freezing tolerance.

Although the exact role of WAP27 in relation to the acquisition of freezing tolerance was not determined, it is speculated that WAP27 may protect cells from freezing-induced dehydration in a similar manner with other LEA proteins. Based on predicted secondary structures, it has been also suggested that group 3 LEA proteins function as ion scavengers (Dure, 1993). Extracellular freezing results in dehydration of cells and consequently leads to a concentration of ions in and around the cells. The toxicity of concentrated ions during freezing has been indicated in many organisms (Mazer, 1969). It is thought that WAP27, as a homolog of group 3 LEA proteins, might function as an ion scavenger and contribute to the acquisition of freezing tolerance in cortical tissue cells of mulberry tree by reducing harmful effects of concentrated ions.

WAP27 was found to be specifically distributed in ER. It has been shown that COR and cold-induced LEA proteins are distributed in a variety of cell compartments. WCS120, a dehydrin, is localized not only in cytosol but also in nucleus in cold-acclimated wheat leaves (Houde et al., 1995). WCOR410, an acidic dehydrin, is distributed on the extracellular surface of plasma membranes in cold acclimated wheat leaves (Danyluk et al., 1998). HVA1, a group 3 LEA protein, is induced during cold acclimation and is distributed in protein bodies of barley seeds (Marttila et al., 1996). COR15am, with similar properties to those of LEA proteins, is distributed in stroma of chloroplasts in cold-acclimated leaves of Arabidopsis (Thomashow, 1994; Gilmour et al., 1996). ER-localized LEA proteins that are induced by low temperature, such as WAP27, however, have not been reported.

In a wide variety of plant cells, freezing injury is caused by plasma membrane destabilization due to interbilayer events that occur due to a close approach of membranes (Fujikawa and Miura, 1986; Steponkus and Lynch, 1989). Upon freezing, when interbilayer events occur in plasma membranes due to the shrink-
age of cells by dehydration and by the deformation of cells by growth of extracellular ice, membrane destabilization takes place with the formation of either lamellar-to-hexagonal II phase transitions or membrane fusions, which are revealed as "fracture jump lesions" (Gordon-Kamm and Steponkus, 1984; Steponkus et al., 1993; Fujikawa, 1994; Fujikawa et al., 1999). Recent studies have demonstrated that freezing tolerance increased in chloroplasts and/or protoplasts isolated from transgenic Arabidopsis plants in which COR15am was constitutively expressed in the stroma of chloroplasts (Artus et al., 1996). It is hypothesized that the presence of COR15am in the stroma alters the intrinsic curvature of lipids in the inner envelope membranes of the chloroplast and consequently reduces membrane destabilization in plasma membranes that have a close approach to chloroplast envelope membranes (Steponkus et al., 1998). However, it is not known how an alteration in the lipids in the inner chloroplast envelope membranes due to the presence of COR15am brings about a reduction in interbilayer events with plasma membranes through the existence of outer chloroplast envelope membranes between them.

The nature of WAP27 is similar to that of COR15am, which exhibits four-time repeats of a 13-amino acid sequence motif. WAP27 is rich in Ala, Lys, Glu, Thr, and Asp residues, which make up approximately 55% of the total amino acids. COR15am is also rich in Ala, Lys, Glu, and Asp residues, which make up approximately 64% of the protein (Thomashow, 1994, 1999). In cortical parenchyma cells of mulberry tree, WAP27 is localized in the ER, which produces MPL just beneath the plasma membranes by initiation of freezing (Fujikawa and Takabe, 1996). It is speculated that if WAP27 in the ER can stabilize the membrane in a manner similar to that of COR15am, it will be more efficient reducing interbilayer events with the plasma membranes because of the close distribution of MPL to the plasma membranes. We hypothesize that conversion of the ER to MPL and accumulation of WAP27 in the ER during winter have the specific effects of inhibiting or minimizing plasma membrane destabilization due to the close approach of membranes and consequently confer extremely high freezing tolerance to cortical parenchyma cells of mulberry tree. To clarify the exact role of ER-localized WAP27 in the acquisition of freezing tolerance, we are currently examining freezing tolerance of transgenic Arabidopsis, which constitutively expresses WAP27.

MATERIALS AND METHODS

Plant Material

One-year-old twigs were collected from mulberry tree (Morus bombycis Koidz.) grown in field conditions on the campus of Hokkaido University, Sapporo, Japan. For determination of transcript levels of WAP27 genes under field conditions, twigs were collected monthly from mid-January to mid-December, 1998.

Treatment of Twigs

Twigs harvested on August 13 and October 11, 1999, were used for cold treatment. Both cross-sectional ends of the twigs were sealed with Parafilm (American National Can, Menasha, WI), and each of the twigs was wrapped in a wet paper towel to avoid desiccation during treatment. For cold treatment, the twigs were kept at 4°C in the dark or 24-h-light condition (twigs harvested on August 13) or at 4°C in a growth chamber with an 11-h-light/13-h-dark cycle (twigs harvested on October 11). As a control, twigs harvested on October 11 were kept at 20°C in a growth chamber with an 11-h-light/13-h-dark cycle, similar with natural photoperiodic cycle of October 11, in Sapporo, Japan.

For treatment with exogenous application of ABA or dehydration stress, twigs harvested on February 18, 2000 were put in a pot of water and de-acclimated at 20°C in a greenhouse. After the leaves were opened by 3 weeks of incubation, the twigs were subjected to each of the stress treatments. ABA treatment was performed by transferring the twigs into an aqueous solution containing 500 μM ABA and 0.02% (v/v) Tween 20 (Nacalai Tesque, Kyoto) at room temperature. Dehydration stress was performed by incubation of the twigs in a desiccator at room temperature.

RNA Extractions and Construction of a cDNA Library

Total RNA was isolated from plant tissues by a mortar and pestle in liquid nitrogen and then homogenized with 10 volume of extraction buffer containing 2% (w/v) cetyltrimethylammonium bromide, 0.1 M Tris-HCl (pH 8.0), 0.02 M EDTA, 1.4 M NaCl, and 1% (w/v) β-mercaptoethanol. After incubation at 65°C for 15 min followed by chloroform extraction, the upper phase was precipitated by isopropanol. The precipitates were dissolved in Tris-EDTA (pH 8.0), and LiCl was added to make a final concentration of 2 M. Precipitates of RNA were collected by centrifugation, dissolved in Tris-EDTA, and purified with extraction by phenol and chloroform.

To construct the cDNA library, the poly(A)+ RNA was isolated from cortical parenchyma cells of mulberry tree harvested in December 1998 using an oligo(dT)-cellulose column (Sambrook et al., 1989). The cDNA library was constructed in Uni-ZAP vector (Stratagene, La Jolla, CA) according to the instructions provided by the manufacturer.

Immunoscreening of a cDNA Library

Phages from the library were plated at a density of approximately 10^8 plaque-forming units on 10 × 14 cm NZCYM plates using Escherichia coli XL1-Blue host cells. After cultivating phages for 3 to 4 h at 42°C, phages were overlaid with a Hybond-C membrane (Amersham-Pharmacia Biotech, London) presoaked in 10 mM...
isopropyl-1-thio-β-d-galactopyranoside and incubated at 37°C for 4 h. Filters were removed from the plates and incubated in a blocking buffer containing 20 mM Tris-buffered saline and 5% (w/v) skim milk for 1 h. Immunoreaction was performed with purified anti-WAP27 antibodies (1:1,000) and with anti-rabbit IgG alkaline phosphatase conjugate (Sigma, St. Louis). The immunoreactive spots were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in Tris-HCl (pH 9.2). After two rounds of screening steps, positive clones were subcloned into pBluescript SK(-) by in vivo excision. The clones encoding WAP27A gene lacked 5' region. To obtain the full-length cDNA clones, PCR reaction was performed using the cDNA library with WAP27A gene-specific oligonucleotide primer, 5' CGAAGTACACGGCCTTCTTC 3', and T3 vector primer. The amplified DNA fragments were subcloned into pGEM-T Easy Vector (Promega, Madison, WI).

Sequence Analysis

DNA sequencing was performed by the chain termination method using an ABI 377 automated sequencer and the Forward and T3 Dye Primer Sequencing Kit (Applied Biosystems, Foster City, CA).

DNA Extraction and Southern-Blot Analysis

Genomic DNA was extracted from cortical parenchyma cells of mulberry tree using ISOPLANT II DNA extraction kit (NIPPON GENE, Toyama, Japan). DNA (10 μg) was digested with BglII, EcoRI, HindIII, and XhoI, separated on 0.8% (w/v) agarose gel, and transferred onto a Hybond-N+ membrane (Amersham-Pharmacia Biotech) with 0.4 N NaOH solution. Hybridization was performed at 60°C with 32P-labeled WAP27B cDNA as a probe in Church phosphate buffer containing 0.5 mM Na-phosphate buffer (pH 7.2), 1 mM EDTA, and 7% (w/v) SDS (Church and Gilbert, 1984). The membrane was washed in 1× SSC and 0.1% (w/v) SDS at room temperature for 5 min and at 60°C for 20 min two times. The washed membrane was exposed to x-ray film at −80°C.

Preparation of Crude Microsome Fractions from Cortical, Xylem, Leaf, and Winter Bud Tissues

Cortical, xylem, leaf, and winter bud tissues were extracted from twigs of mulberry tree harvested in February, May, June, and July, 1999. Each tissue (5 g in fresh weight) was cut into small pieces and homogenized in 40 mL of homogenizing medium using a Polytron T-20 (Kinematica, Lucerne, Switzerland) as described in Ukaji et al. (2000). Before homogenization, only xylem tissues were frozen-crushed using a mortar and a pestle in liquid nitrogen. Precipitates of the crude microsome were suspended in a resuspending medium and used for the crude microsome fractions.

Immunoelectron Microscopy

The cortical and winter bud tissues of mulberry twigs harvested on January 30, 1999 were cut into 1 × 2 × 2 mm blocks with a razor blade and placed in a fixing medium containing 4% (w/v) paraformaldehyde, 2% (v/v) glutaraldehyde, and 60 mM Suc in 50 mM sodium phosphate buffer (pH 7.4) for 12 h at 4°C after evacuation for a few minutes. To convert the morphology of ER from vesicular form to MPL-form, sliced cortical tissues were immersed in 46.2% (w/v) sorbitol solution (5.4 osmol, corresponding to freezing-induced osmotic stress at −10°C) at 4°C for 30 min and then fixed with a medium containing 46.2% (w/v) sorbitol (Fujikawa and Takabe, 1996).

After chemical fixation, the samples were dehydrated in a graded ethanol series and embedded in London Resin White medium grade (Polysciences, Warrington, PA). Polymerization was carried out at 50°C for 24 h in a gelatin capsule. Ultra-thin sections (50 nm in thickness) were cut with a diamond knife on an ultramicrotome (Reichert, Vienna) and mounted on uncoated nickel grids.

The sections on grids were treated with a blocking solution containing 1% (w/v) bovine serum albumin in Tris-buffered saline at room temperature for 1 h and then incubated in a blocking solution containing purified anti-WAP27 antibodies as a primary antibodies diluted to 1:1,000 at 4°C overnight. For reference, sections were treated with preimmune serum as a primary antibody. After washing the sections with Tris-buffered saline several times, sections were incubated with a 1:20 diluted blocking solution containing anti-rabbit IgG gold conjugates (British BioCell International, Golden Gate, UK) at room temperature for 30 min. The sections were washed with distilled water several times and then stained with 2% (w/v) uranyl acetate for 10 min and 2% (w/v) lead citrate for 3 min. All sections were observed under a JEM 1200 EX transmission electron microscope (JEOL, Tokyo) accelerated at 100 kV.
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LITERATURE CITED


