

REVIEW

Expansins: expanding importance in plant growth and development

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Expansins were originally identified as cell wall-loosening proteins. The existence and various roles of expansins have been discovered in many plants. Expansins are encoded by a superfamily of genes comprised of subfamilies that evolved from a common ancestor and encode the α -expansins (EXPA), the β -expansins (EXPB), the expansin-like A (EXLA), and expansin-like B (EXLB) proteins. Several expansin-like genes have also been identified in non-plant organisms (e.g. a slime mold, fungi, nematodes, and a mollusk). Localization of EXPA and EXPB in the cell wall was confirmed by immunogold electron microscopy. Studies using transgenic plants provided evidence for a broad range of biological roles of expansins in diverse aspects of plant growth and development, such as cell wall extension, fruit softening, abscission, floral organ development, symbiosis, and the response to environmental stresses.

Introduction

Expansins are proteins that promote cell wall loosening and extension and are encoded by a superfamily of genes that are organized into four families on a phylogenetic tree (for reviews, see Cosgrove, 1999, 2000; Lee et al., 2001; Li et al., 2002, 2003b). Recent studies have documented the functional importance of expansins in cell enlargement, fruit tissue softening, abscission, germination, stress response, parasitism, and others. The role of expansins as mediators of hormone action has been summarized recently (Cho and Cosgrove, 2004). There are a number of proteins with structural similarities to expansins, and some of them are considered to have an evolutionary relationship to expansins. However, the biochemical properties of expansins have not yet been fully characterized.

Here, we review recent studies on the roles of expansin proteins in plant growth and development and

suggest biochemical and transgenic approaches to elucidating the function of expansins in plant cells.

The expansin superfamily

The availability of the full-genome sequence of rice (<http://rgp.dna.affrc.go.jp/IRGSP/>) and Arabidopsis (<http://www.arabidopsis.org>) permitted the genome-wide search of expansin gene sequences. The results of genome-wide searches have revealed that expansins form a large gene superfamily composed of four subfamilies (Fig. 1).

As many researchers have studied expansins and named the genes independently, the names of expansin genes have become confusing to readers. To alleviate this problem, researchers agreed on a standardization of expansin nomenclature (Kende et al., 2004), and the web site <http://www.bio.psu.edu/expansins/>, currently

Abbreviations – EXLA, expansin-like A; EXLB, expansin-like B; EXPA, α -expansin; EXPB, β -expansin.

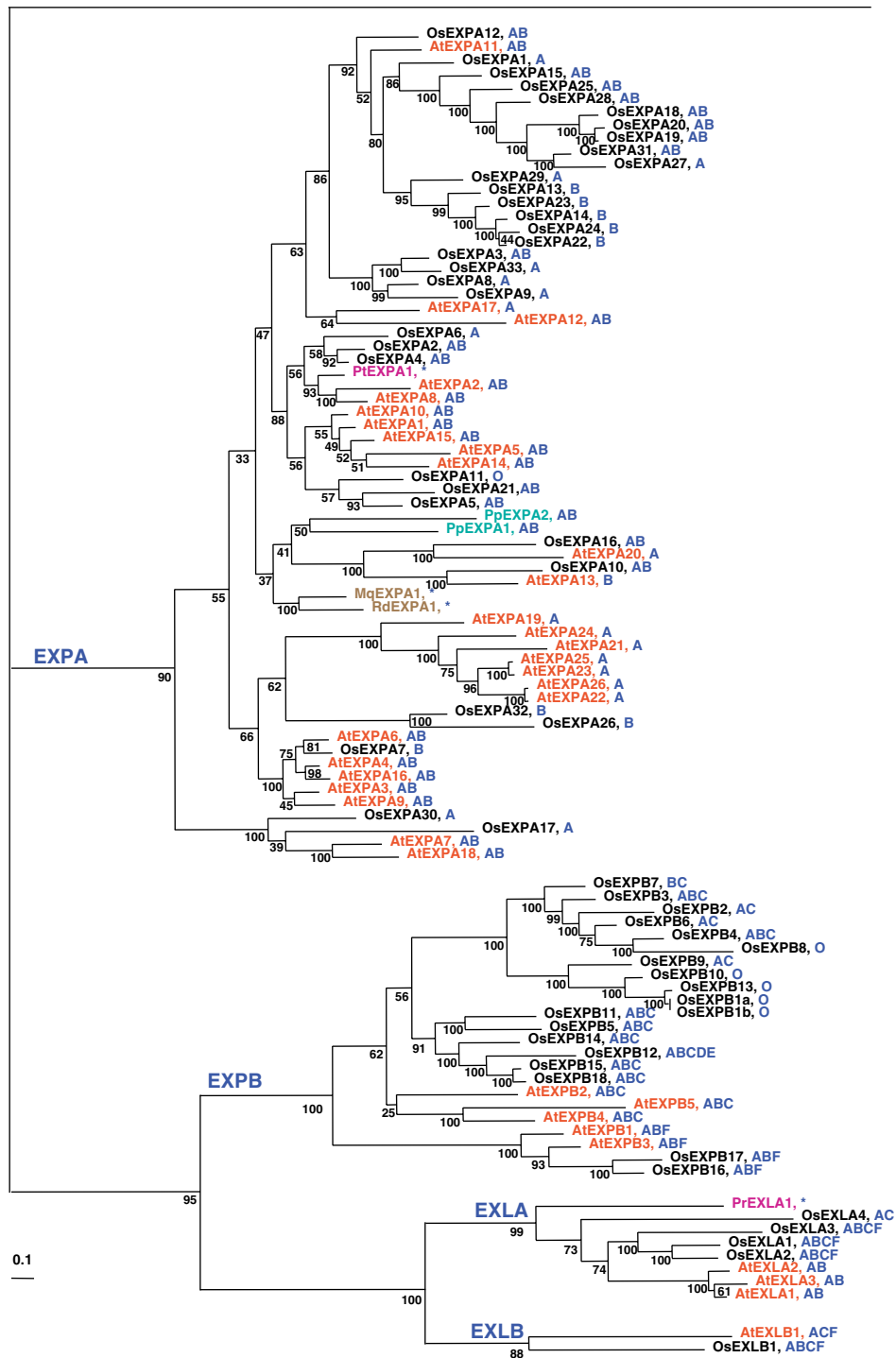


Fig. 1. Phylogenetic tree of the expansin superfamily of Arabidopsis, rice, and selected other species. Amino acid sequences (accessions, <http://www.bio.psu.edu/expansins/>) of full-protein regions, including the signal peptide, were aligned using the CLUSTAL X (1.81). The tree was constructed using Bayesian inference with MRBAYES (2.01) program (Jones amino acid model, gamma estimation, 750 000 generations, four channels) (Huelsenbeck and Ronquist, 2001). The topology is drawn with the amino acid sequence of *Dictyostelium* expansin-like protein (accession, XP_647352) rooting the tree. Genes of Arabidopsis are indicated in red, rice in black, pines in purple, ferns in brown, and of *Physcomitrella* in green. Intron types are indicated right side of each gene in blue color. The genes whose genomic sequences are not available are indicated by an asterisk (*). Posterior probabilities are noted below the branches. Branch lengths are mean values and are proportional to the number of substitutions per site (bar, 0.1 substitutions/site).

maintained by Daniel Cosgrove (Pennsylvania State University, USA), provides recent and archival information on the naming of expansins.

The α -expansin (*EXPA*, formerly *EXP* or *EXP α 1*) subfamily is composed of 26 genes in Arabidopsis and 34 genes in rice. The second subfamily of expansins, called β -expansin (*EXPB*, formerly *EXPB* or *EXP β 1*), was originally recognized as group I pollen allergens and was later identified as a group of expansins on the basis of sequence similarity and an expansin-like cell wall-loosening activity in grass tissues (Cosgrove et al., 1997). This family consists of six genes in Arabidopsis and 19 genes in rice. The Arabidopsis and rice genomes also have a third group of expansin-like genes, represented by expansin-like A (*EXLA*, formerly *EXPL* or *EXP β 2*) and expansin-like B (*EXLB*, formerly *EXPR* or *EXP β 3*). There are three and four *EXLA* genes in Arabidopsis and rice, respectively. Only a single *EXLB* gene was found each in Arabidopsis and rice (<http://www.bio.psu.edu/expansins/>).

Expansin proteins have a signal peptide containing about 20 amino acids at their N-terminus. For most expansins, the *PSORT* program (Nakai and Kanehisa, 1992) predicts the secretion of the protein into the cell wall (Fig. 2). EXPAs and EXPBs have conserved Cys residues in the N-terminal putative catalytic domain a His-Phe-Asp (HFD) motif in the center region and conserved Trp residues in the C-terminal putative cellulose-binding domain (Fig. 2). Mature EXPB proteins are more divergent from each other (average 44% amino acid identity in Arabidopsis and 51% in rice) than EXPAs (average 55% amino acid identity in both Arabidopsis and rice).

EXLA proteins have a very high amino acid identity among one another (84% in Arabidopsis and 73% in rice). They have conserved Cys residues in the N-terminus and conserved Trp residues in the C-terminal regions. However, unlike the EXPAs and EXPBs, they

lack the HFD motif in the center region, and the positions of the Trp residues are different from those in EXPAs and EXPBs. EXLA proteins contain additional Trp residues in their C-terminal regions (Fig. 2). The EXLB protein has conserved Cys and Trp residues in the C-terminal region. It lacks the HFD domain in its center part, and one conserved Trp residue is missing in the C-terminal region (Fig. 2).

Evolution of expansin genes

The sequence analysis of expansin genes led to the identification of seven introns named A, B, C, D, E, F, and G in the order in which they were identified (Lee et al., 2001; Sampedro et al., 2005). Li et al. (2002) designated the introns I-1, I-2, I-3, and I-4, corresponding to A, C, B, and F, respectively, in Lee et al. (2001). Introns A, B, C, and F are widely distributed among *EXPA*, *EXPB*, *EXLA*, and *EXLB* genes (Fig. 1). Each type of intron interrupts the nucleotide sequence at a position corresponding to the equivalent amino acid in aligned protein sequences and at the same intron phase (the intron phase represents the position of the intron within the codon). Intron A is inserted in phase 1, intron B in phase 2, intron C in phase 0, intron D in the 5' untranslated region, intron E in phase 2, intron F in phase 0, and intron G in phase 2 (Fig. 2). Sampedro et al. (2005) studied the genomic history of expansins and showed that the history of the gene family is very helpful for the understanding of correct phylogeny and births and deaths in a gene family.

The GH45-like domain, which is located in the N-terminal region of expansins, is found in diverse expansin-related proteins from many organisms, such as *Dictyostelium* (Li et al., 2002), fungi (Li et al., 2002), nematodes (Kudla et al., 2005), and mussels (Xu et al., 2001). But, the genes encoding these proteins do not share the gene structure with plant expansins

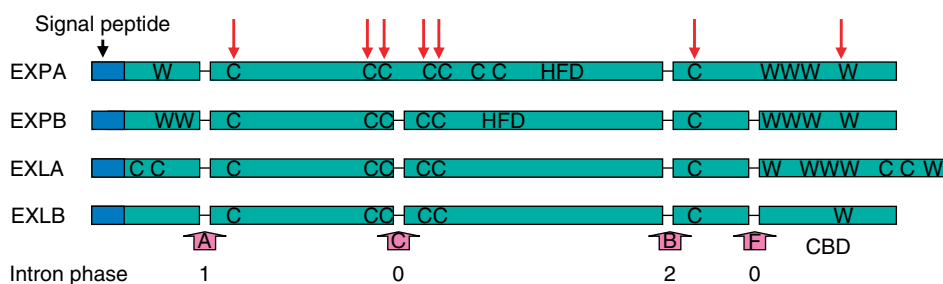


Fig. 2. Schematic gene structures of each expansin gene family. Positions of the highly conserved sequences, cysteines, His-Phe-Asp domain, and tryptophans are indicated. Signal peptide region and the amino acid residues conserved in all families are indicated by downward arrows. The position and name of each intron are indicated by upward arrows. Intron phases are indicated by number. Putative cellulose-binding domains (CBDs) are indicated.

(Y Lee and H Kende, unpublished data). This difference in gene structure indicates that the plant expansin genes evolved separately from these genes in non-plant organisms.

The expansin gene family has many members compared with other gene families, and many expansin genes are tandemly repeated in the genome. The tandemly repeated genes are grouped together in the phylogenetic tree (Sampedro et al., 2005), indicating that those genes were duplicated relatively recently. In rice, most expansin genes were found to be expressed. Twenty-eight *EXPA* cDNAs corresponding to all 34 rice genes were found (Lee and Kende, 2002; Shin et al., 2005). Eight genes of 19 rice *EXPBs* (Lee and Kende, 2001) and three of four rice *EXLAs* (Lee and Kende, 2002) were expressed. However, no expression of *EXLB* has been reported (Lee and Kende, 2002). From the searches of GenBank and rice full-length cDNA collections (Kikuchi et al., 2003), we also found EST or full-length cDNA clones for 20 *OsEXPA*, 17 *OsEXPB*, and four *OsEXLA* genes. But no one for *OsEXLB* gene was found.

Although it is clear that expansin-like genes evolved from the common ancestor with *EXPA* and *EXPB*, we do not know the actual biological function of expansin-like genes. Most *EXPA* and *EXPB* genes were upregulated by the induction of stem elongation following GA treatment. On the contrary, *OsEXLA2* and *OsEXLA3* of rice showed a downregulation by GA treatment (Lee and Kende, 2002).

Biochemical and biophysical properties of expansins

Transgenic approaches have provided strong evidence that endogenous EXPAs are involved in regulating vegetative growth (Cho and Cosgrove, 2000; Choi et al., 2003; Pien et al., 2001). Yet, the role of EXPBs in vegetative growth has not been established.

The PSORT program predicts that most expansin proteins of rice are secreted into the cell wall (Lee and Kende, 2001; Lee et al., 2001). Localization of EXPAs in rice and maize cell walls was confirmed by immunogold labelling with anti-cucumber EXPA antibodies and electron microscopy (Balestrini et al., 2005; Cosgrove et al., 2002). Immunohistochemistry and immunogold labelling with the OsEXPB3-specific antibody also demonstrated the tissue-specific localization of OsEXPB3 proteins and their tight binding to the primary cell wall (Lee and Choi, 2005).

OsEXPB3 was tightly bound to the cell wall and could not be removed without SDS treatment (Lee and Choi, 2005). An experiment using purified recombinant

OsEXPB3 and immunoblot analysis showed that the recombinant OsEXPB3 protein binds to native cell walls, SDS-washed cell walls, and pure celluloses. Once the recombinant OsEXPB3 protein was bound to cellulosic material, the protein was poorly removable from it (Y Lee and H Kende, unpublished data). *Zea m 1*, an EXPB protein, also appeared to bind tightly to the coleoptile wall because wall extension activity was not removed by exchanging the protein solution with fresh buffer lacking *Zea m 1d* (Li et al., 2003a).

It has been proposed that the expansin action is not based on hydrolytic activities (McQueen-Mason and Cosgrove, 1995). However, there has been some controversy concerning the action mechanism of EXPB. Grobe et al. (1999, 2002) reported that EXPB has proteolytic activity and that the papain-like properties of EXPB resemble those of cathepsin B, a member of the papain (C1) family of cysteine proteinases. However, Li and Cosgrove (2001) could not observe any protease activities from purified *Lol p 1*, *Phl p 1*, *Zea m 1* (hydrolysis of BAPNA and Chromozyme PL, digestion of bovine serum albumin and ovalbumin in solution, SDS-PAGE zymogram, Native PAGE zymogram, and detection of proteolytic activity from agarose gel), nor did they find any cell wall-loosening activity from various proteases. Furthermore, they found that protease inhibitors did not inhibit the EXPB-mediated cell wall creep activity.

The recombinant OsEXPB3 proteins from tobacco BY2 cells showed cell wall-binding activity, but no expansin activity was detectable in the reconstitution experiment using rice internodes or coleoptiles (Y Lee and H Kende, unpublished data). Until now, no biologically active recombinant plant expansin has been reported. To understand the molecular mechanism of expansin action, it is crucial to establish an efficient expression system to acquire biologically active recombinant expansins. The only active recombinant expansin was produced from GrEXPB1, an expansin-like protein from the potato cyst nematode *Globodera rostochiensis*. Recombinant GrEXPB1 was expressed in transgenic tobacco plants and showed expansin activity on wheat coleoptiles (Kudla et al., 2005).

Expansins in plant growth and development

A growing number of reports have provided additional evidence that expansins play more diverse roles in plant growth and development than do most other cell wall proteins and cell wall-loosening factors. In deepwater rice, the expression of expansin genes was shown to be correlated with internodal growth, which supports the hypothesis that expansins mediate plant growth (Lee

and Kende, 2001, 2002). Expansins are also involved in root growth and development. The expression of two root-specific Arabidopsis expansin genes, *AtEXPA7* and *AtEXPA18*, has a tight spatial and temporal correlation with root hair formation (Cho and Cosgrove, 2002). In an experiment using soybean roots, a root-specific expansin gene was expressed exclusively in growing regions of root (Lee et al., 2003).

Despite accumulating evidence for the role of expansins in plant growth, it appears that expansins are not always related to plant growth. A few studies have shown a lack of correlation between growth and expansin expression (Caderas et al., 2000; Rochange et al., 2001). In *Festuca pratensis*, a member of the grass family, expansins were expressed along elongating leaves, but the expression level of expansin genes did not show a direct correlation with leaf elongation rates. Instead, these expansins were more likely involved in the differentiation of tissues and organs such as vasculature and initiating tillers (Reidy et al., 2001). In addition to organ elongation, a possible role of expansins has been studied in organ abscission, fruit development, and cotton fiber development. Cho and Cosgrove (2000) reported that the alteration of gene expression of an expansin (*AtEXPA10*) resulted in pedicel abscission as well as leaf growth. Increased expansin activity was observed in tissues undergoing cell separation during leaflet abscission in blue elderberry (*Sambucus nigra*), which indicates that expansins are involved in abscission (Belfield et al., 2005).

Expansins may act as mediators of cell wall softening or increase in fruit size during fruit development in various species such as tomato, pear, and banana (Hiwasa et al., 2003; Kitagawa et al., 2005; Rose et al., 1997; Trivedi and Nath, 2004). The ripening inhibition (*rin*) mutant tomato with mutated *LeMADS-RIN* gene produces non-ripening fruits. A mutant and F1 hybrid between mutant and wild-type tomatoes showed altered mRNA accumulation of ripening-related genes along with an *EXPA* gene, *LeEXPA1*, whose expression was greatly reduced (Kitagawa et al., 2005). Overexpression or downregulation of fruit-specific expansins was reported to influence fruit texture and juice viscosity in tomato (Brummell et al., 1999; Powell et al., 2003). Expansin genes were differentially regulated during fruit development; the expression of some expansin genes was correlated with increase in fruit size, whereas that of other expansin genes increased only in the ripening stage. Given these findings, it is probable that different expansins participate in fruit development with distinct expression patterns.

Development of floral organs also requires expansin activity. Gene expression of seven *EXPA*s and three

EXPBs was analyzed and shown to be differentially regulated during the expansion and senescence of flowers of *Mirabilis jalapa* (Gookin et al. 2003). A maize gametophytic male-sterile mutant *gaMS-2* was characterized by proteomic analysis comparing protein profiles of anthers and immature pollens between heterozygous mutants and wild-type plants (Wang et al., 2004). Zea m 1, a group I pollen allergen, was present at a greatly reduced level in sterile pollen grains of the heterozygous mutant, which indicates that reduced Zea m 1 is associated with the sterile phenotype of *gaMS-2*. This experiment suggests that Zea m 1 has two distinct roles: (1) the protein may bind to pectins and facilitate cell wall deposition in pollen grains or (2) because of its hydrophilicity and localization on the surface of pollen grains, it may act as a wick, drawing water from the stigma for pollen germination.

Cotton fiber has been used as a model experimental system because fiber elongation requires extensive cell wall loosening. Six *EXPA* genes have been identified and characterized in upland cotton. Gene expression study revealed that *GhEXP1* was abundantly expressed only in fiber, suggesting that GhEXP1 plays an important role in cell wall loosening during fiber elongation (Harmer et al., 2002).

The role of expansins in plant response to environmental change

Plants respond to changes in the biotic and abiotic environment by modifying their metabolism. Deepwater rice is well known to grow rapidly upon submergence. Rapid internodal elongation is initiated by reduced O₂ level in the internodes resulting from submergence, and presumably expansins take part in rapid elongation (Kende et al., 1998). Likewise, *Rumex palustris*, a semi-aquatic species, undergoes submergence-induced hyponastic growth. Colmer et al. (2004) analyzed the expression pattern of *Rumex EXPA* genes during root acclimation to O₂ deficiency. Expansins showed differential expression in various root types and tissues within root types and in response to low levels of O₂. This differential expression pattern is consistent with the observation in deepwater rice (Lee and Kende, 2001) that the expression of expansin genes is complex and flexible in normal plant growth and development as well as in response to changes in the abiotic environment.

Drought is a major source of abiotic stress to plants, severely limiting plant growth. *Craterostigma plantagineum*, a resurrection plant, is capable of surviving drought by extensive cell wall folding, which maintains the integrity of the plasma membrane-to-cell wall

connections when cells shrink as a result of water loss (Jones and McQueen-Mason, 2004). Expansin activity in leaves increases during the early stages of both dehydration and rehydration, which indicates that expansins are associated with cell wall folding and desiccation tolerance.

Interaction between plants and mycorrhizal fungi or nitrogen-fixing bacteria is very useful in studying plant responses to biotic environmental changes. Because of their involvement in root structure modification, the formation of mycorrhizae and root nodules may require extensive expansin activity. The expression level and localization of proteins and mRNAs of two cucumber *EXPA* genes, *CsEXPA1* and *CsEXPA2*, were investigated in cucumber roots with or without a mycorrhizal fungus (Balestrini et al., 2005). Immunoblot analysis using antibodies against *CsEXPA1* and *CsEXPA2* showed the presence of mycorrhizal root-specific expansin. Interestingly, immunolabelling experiments revealed that an expansin recognized by anti-*CsEXPA1* antibody was mainly localized to the interface zone, which is characteristic of endomycorrhiza, whereas another expansin labelled by anti-*CsEXPA2* was localized only to the cell wall. This result suggests that some expansins participate in the formation and maintenance of the interface, whereas other expansins may act as major cell wall-loosening agents required during the intracellular colonization. Consequently, the presence of *EXPA* proteins in mycorrhizal cells suggests that fungus-induced enlargement of cortical cells is achieved by the activity of *EXPA*s (Balestrini et al., 2005).

Legumes develop root nodules as a response to the inoculation of *Rhizobium* spp. An *EXPA* gene, *MaEXP1*, was obtained and characterized in the process of nodule formation during the sweet clover–*Sinorhizobium* interaction (Giordano and Hirsch, 2004). A whole-mount *in situ* hybridization experiment showed that the expression of *MaEXP1* gene increased following the inoculation of *Sinorhizobium* in both roots and nodules. Likewise, *LjEXP*, an expansin gene from *Lotus japonicus*, was expressed in infected roots and upregulated in the early stage of nodule development (Flemetakis et al., 2004). These reports support the notion that expansins in legume roots may contribute to nodule formation by enlargement of root cells.

Transgenic approach and mutant analysis to investigate biological functions of expansins

Studies on the biological roles of expansin have focused on plant growth and development by overexpression or downregulation of target expansin genes. Choi et al. (2003) demonstrated that the overexpression of

OsEXPA4 enhanced seedling growth and leaf formation, whereas downregulation resulted in reduced growth in rice. However, transgenic plants with strong expression of *OsEXPA4* showed stunted growth concomitant with the formation of additional leaves. This bushy phenotype might have been caused by the presence of expansins beyond an optimal concentration for stem growth. Stunted growth in expansin overexpressors was also observed in transgenic tomato plants (Rochange et al., 2001). In Arabidopsis, the regulation of *AtEXPA10* resulted in the alteration of leaf growth and pedicel abscission (Cho and Cosgrove, 2000). Transgenic tobacco plants harboring *GmEXP1*, a soybean root-specific expansin, showed accelerated root growth (Lee et al., 2003). A transgenic approach using *PhEXP1*, a petunia *EXPA* gene, suggested a new possible biological role of expansins. Downregulation of *PhEXP1* caused decreased petal limb size, alteration in cell wall morphology, and reduction in crystalline cellulose (Zenoni et al., 2004). The reduction of crystalline cellulose by downregulation of *PhEXP1* indicates that the activity of *PhEXP1* protein is associated with cellulose metabolism.

The knockout strategy has been recognized as an efficient methodology to determine the biological function of a protein. However, to study a gene family like expansins with overlapping gene expression patterns, knockout mutation has limited value. Four null mutants for expansins were generated by homologous recombination in the bryophyte *Physcomitrella patens*, but none of the mutants showed observable phenotypes (Schipper et al., 2002). Likewise, a number of single-gene T-DNA knockout mutants of expansins have been identified and characterized in Arabidopsis, but no obvious phenotype was observed (Cosgrove et al., 2002). To avoid the interference of functional redundancy in the expansin gene family, the establishment of multiple knockouts is required.

Future prospects

Expansins are involved in various biological processes associated with wall loosening and cell expansion. The regulation of expansins occurs in a complex manner, and little is known about the importance of differential expression. To determine the role of expansins, it is necessary to establish transgenic plants and multiple knockout mutants. Preparation of specific antibodies is also essential to explain differential expression of expansins because the level of gene expression does not conclusively represent the actual involvement of expansins in diverse biological processes. The exact biochemical mechanism of expansin action and the identification of the active site have not been established (Cosgrove

et al., 2002). To study the mode of expansin action, an efficient expression system to produce biologically active recombinant expansin is needed.

Expansins also have the potential to be utilized as targets for crop improvement. Future research needs to emphasize the genetic modification of expansin expression in crop plants.

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