Chapter 12
The Role of Expansins A in Petunia Development

Sara Zenoni, Anita Zamboni, Andrea Porceddu, and Mario Pezzotti

Abstract Expansins, a diverse set of proteins found in plants and some other organisms, appear to play a key regulatory role in cell expansion, thereby serving critical functions in plant morphogenesis, development, and adaptation to stress. We have isolated a number of expansin genes from Petunia. Their ongoing functional analysis provides evidence for their involvement in cell wall functions, including cellulose metabolism, disruption of noncovalent cellulose/glycan bonds, and separation of the cell wall matrix during cell expansion.

12.1 Introduction

Morphogenesis in development refers to the formation of intricate shapes and structures in developing tissues. In animals, the formation of cell and tissue structures is strongly dependent on cell–cell interactions, particularly the gain and loss of cell adhesion. This allows cells to move in relation to one another either as whole tissues or as individual migrating cells. In contrast, the rigid plant cell wall and the way cells are tightly cemented together means that such mechanisms can hardly be used in plants (Twyman 2003). Changes of shape and structure in the developing plant are therefore mediated by alternative mechanisms, such as control of the location, rate, plane and symmetry of cell division, and the regulation of cell expansion (Meyerowitz 1997). A complex surveillance system monitors the correct and productive integration of these two processes by regulating the activity and availability of key factors (Gutierrez 2005).

The extent of cell enlargement is a critical factor in the ultimate determination of cell shape and size, and this in turn depends predominantly on the extensibility of the primary cell wall (Kotilainen et al. 1999; Martin, Bhatt, and Baumann 2001;

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Smith 2003). Several cell wall proteins have been implicated in the control of extension but much of the evidence now indicates that expansins are the key regulators of this process (Brummell, Harpster, and Dunsmuir 1999; Cosgrove 1999; Choi, Lee, Cho, and Kende 2003). Their mechanism of action is not fully understood, but it appears to involve the disruption of hydrogen bonds between cellulose microfibrils and cross-linking glycans, thus allowing cellulose fibers to slide relative to one another and thereby facilitate cell expansion (McQueen and Cosgrove 2000). To better understand the role of cell expansion in Petunia development we have focused on the identification, characterization, and functional analysis of expansin genes in Petunia (*Petunia hybrida*).

12.2 Plant Cell Growth: Loosen and Slide

Plant cells enlarge via the longitudinal expansion of their cell walls, a process involving the careful regulation of "polymer creep" (Cosgrove 2005). The growing cell wall has a fiberglass-like structure with crystalline cellulose microfibrils embedded in a matrix of hemicelluloses and pectins. Hemicelluloses are cellulose-binding polysaccharides, which together with cellulose form a network that is strong yet resilient. Pectins are complex polysaccharides that have several functions: they form hydrated gels that push microfibrils apart, thus allowing them to slip over one another during cell growth; they are important determinants of cell wall porosity and thickness; and they provide the adhesive that binds plant cells together. Pectins are also the primary targets for attack by invading microbes.

Cellulose is synthesized by a large membrane-bound complex that extrudes microfibrils from the cell surface. In contrast, matrix polysaccharides are synthesized in the Golgi apparatus and are packaged into tiny vesicles that fuse with the plasma membrane and thereby deliver their cargo to the wall. Matrix polysaccharides then become integrated into the wall network by noncovalent physical interactions as well as enzymatic ligation and cross-linking reactions. The detailed structure of the cell wall is not precisely understood, and this remains a key issue for research into how the cell wall expands. Xyloglucan is believed to crosslink microfibrils either through the formation of direct molecular tethers or through some form of indirect linkage. The integration of newly secreted matrix polysaccharides into the existing network could also be mediated by endotransglycosylases/hydrolases, which cleave glycans and ligate them together. Molecular modeling data suggest that some members of the xyloglucan endotransglycosylase/hydrolase (XTH) family could target arabinoxylan and (1,3;1,4)-β-D-glucan, hemicelluloses that are notably abundant in grass cell walls (Cosgrove 2005). The complexity of the cell wall network provides many potential sites where loosening and expansion could be initiated.

Polymers in the cell wall are placed under stress and therefore undergo stretching as they resist turgor pressure, and this provides the mechanical energy required for expansion. The wall can stretch without bursting if polymer creep is carefully

controlled through the selective loosening and shifting of load-bearing linkages between cellulose microfibrils. In this way the matrix yields, allowing the cellulose microfibrils to move apart. To prevent fatal thinning, cell wall expansion must be balanced by the synthesis and integration of new cellulose and matrix polysaccharides. However, these two processes appear to be only loosely coupled in most plant cells.

"Wall loosening" refers to a molecular modification of the wall network that reduces wall stress without substantially changing its thickness. Candidate wall-loosening agents include XTH, endo-(1,4)-β-D-glucanase, hydroxyl radicals, and expansin. It is useful to distinguish between so-called primary and secondary wall-loosening agents. Primary agents catalyze stress-relaxation reactions directly in isolated cell walls, whereas secondary agents modify wall extension indirectly, by amplifying the physical effects of primary agents.

12.3 Are Expansins the Key Factors Controlling Cell Expansion?

At least four lines of evidence support a key role for expansins in the regulation of cell wall enlargement in growing cells. First, when the acid-growth behavior of isolated cell walls is eliminated by heat or protease treatment, it can be almost fully restored by the addition of purified expansin proteins (McQueen-Mason, Durachko, and Cosgrove 1992). This indicates that expansins alone are sufficient to restore extensibility to isolated cell walls. Second, the addition of exogenous expansins to growing tissues can stimulate their growth (Fleming, McQueen-Mason, Mandel, and Kuhlemeier 1997). Third, the modification of expansin gene expression also affects cell wall growth – ectopic expression stimulates growth, whereas suppression by gene silencing inhibits it (Cho and Cosgrove 2000; Pien, Wyzykowska, McQueen-Mason, Smart, and Fleming 2001; Choi et al. 2003; Zenoni et al. 2004). Finally, endogenous expansin gene expression correlates with the onset, increase, and cessation of cell growth, that is, the genes are expressed at the right time and in the right place to cause the observed morphological processes (Brummel et al. 1999; Wu, Thorne, Sharp, and Cosgrove 2001; Cho and Cosgrove 2002).

Although genetic studies provide compelling evidence that expansin plays an important role in cell expansion, little is known about the precise mechanism of action. Expansins appear to increase polymer mobility in the cell wall, allowing microfibrils to slide apart (Darley, Forrester, and McQueen-Mason 2001). However, studies of expansin activity have generally involved the use of quite crude extracts, which reveal little in the way of molecular detail. It has proved difficult to produce active recombinant expansins.

Most of the available information about expansin activity reflects studies carried out with cucumber CsEXP1A, which appears to induce cell wall extension by disrupting hydrogen bonds between cellulose fibrils and cross-linking glycans (McQueen-Mason and Cosgrove 1995, 2000). Sequence analysis of the expansin family reveals the presence of a conserved N-terminal domain (∼15 kDa) with

distant homology to the catalytic domain of GH45 endoglucanases, and a C-terminal domain (∼10 kDa) homologous to a family of grass-pollen allergens of unknown function. The crystal structure of a native EXPB1 purified from maize pollen was solved, revealing two domains (Yennawar, Li, Dudzinski, Tabuchi, and Cosgrove 2006). Domain 1 resembles a GH45 glycoside hydrolase, while domain 2 has an Ig-like beta sandwich with aromatic and polar residues potentially forming a polysaccharide-binding surface. Despite the presence of an endoglucanase-like domain, no catalytic activity has been detected that accounts for the action of expansins on the cell wall.

Expansins can weaken paper, which is a hydrogen-bonded network of cellulose fibrils, but this does not involve cellulose hydrolysis. Expansins also act very quickly. They induce extension within seconds of addition to wall preparations, but they do not affect the plasticity or elasticity of the cell wall (Yuan, Wu, and Cosgrove 2001). Furthermore, expansins synergistically enhance the hydrolysis of crystalline cellulose by cellulase, perhaps indicating that expansins promote the release of glucans on the surface of the cellulose microfibrils, making them available for enzymatic attack. Finally, expansins stimulate extension immediately after entering the wall, and subsequent removal restores the wall to an inextensible state. This indicates that expansin does not alter the gross wall structure or the degree of cross-linking, but may dissociate the polysaccharide complexes that link microfibrils together.

12.4 The Expansin Gene Family in Plants

Genome-wide searches in *Arabidopsis thaliana* and rice have revealed large families of expansin genes that can be organized into four subfamilies. The expansin A subfamily (*EXPA)* has the most members, with 26 genes in Arabidopsis and 34 in rice. The expansin B subfamily (*EXPB)*, originally known as the group 1 pollen allergens (Cosgrove, Bedinger, and Durachko 1997), has 6 members in Arabidopsis and 19 in rice. The mature EXPB proteins show much greater sequence divergence than the EXPA proteins. The remaining subfamilies lack some of the canonical features of the true expansins and are defined as expansin-like. The expansin-like A subfamily (*EXLA*) has three members in Arabidopsis and four in rice, whereas each species has only a single expansin-like B (*EXLB*) gene (Choi, Cho, and Lee 2006). All of the expansin proteins predicted from these sequences contain N-terminal signal peptides of about 20 amino acids that show no significant sequence conservation, but imply that all of the proteins are targeted for secretion into the cell wall.

Following the signal peptide, the EXPA and EXPB proteins share a series of conserved cysteine residues, suggesting that expansins may share a tertiary structure based on the formation of disulfide bonds. Another notable feature is the His-Phe-Asp (HFD) box in the central region, which may be a catalytic motif based on similarity to the catalytic core of microbial endoglucanases. The distal sequences of the EXPA and EXPB proteins share a series of conserved tryptophan residues, whose

position and spacing resemble those in the cellulose-binding domain of some cellulases. The EXLA proteins are highly conserved (84% sequence similarity among the Arabidopsis proteins, 73% in rice) but they lack the central HFD motif found in the EXPA and EXPB subfamilies. The EXLB proteins have conserved cysteine and tryptophan residues but also lack the central HFD domain.

Genomic DNA alignments have confirmed the presence of seven introns at conserved positions within the expansin gene sequences (Choi et al. 2006). Phylogenetic analysis indicates that the *EXPA* and *EXPB* subfamilies had already split by the time vascular plants and mosses diverged, and that they have continued to grow and diversify in different plant lineages (Sampedro, Lee, Carey, dePamphilis, and Cosgrove 2005). The authors also estimated the number of expansin genes in the last common ancestor of eudicots (including Arabidopsis) and monocots (including rice), and on this basis proposed that the four angiosperm expansin families should be divided into 17 clades (Sampedro et al. 2005).

12.5 Nonplant Expansins

Expansin proteins have been identified in several nonplant species. The N-terminal GH45-like domain is found in diverse expansin-like proteins from many organisms, such as the slime mold *Dictyostelium discoideum*, fungi, mussels (Xu, Janson, and Sellos 2001), and nematodes (Kudla et al. 2005). This polyphyletic group of nonplant expansins can be termed expansin-like family X (EXLX). In *Dictyostelium*, there are at least five EXLX genes and these sequences show by far the closest similarity to plant expansins. *Dictyostelium* expansins may lubricate the movement of cellulose microfibrils during individual cell growth, wall extension, and extracellular cellulosic matrix production, and/or they may serve to maintain the fluid state of the multicellular slug cell wall. The existence of expansin-like sequences in nonplant species is intriguing and appears to be restricted to organisms that infect plants and need to digest plant cell walls.

There are many differences in gene structure between plant and nonplant expansins, indicating separate evolutionary lineages. Their divergence might predate the origin of land plants, or alternatively the nonplant expansins could have been acquired from plants more recently by horizontal gene transfer.

12.6 Expansins in Development and Adaptation to Stress

A growing number of reports provide evidence that expansins play diverse roles in plant growth, development, and adaptation to stress. For example, expansins have been shown to play key roles in the early development of leaf primordia, fruit softening, internodal growth, sexual reproduction, and cell wall disassembly during fruit abscission (Cosgrove et al. 1997; Cho and Cosgrove 2000). Expansins are also involved in adaptations to drought and flooding, and in the extensive structural

remodeling that occurs during symbiotic microbial colonization. Some of these roles are discussed in more detail below.

12.6.1 Production of Leaf Primordia

Fleming et al. (1997) showed that topical application of expansins to discrete regions on the flanks of tomato vegetative meristems led to induction of ectopic leaf primordia, although these did not ultimately produce normal leaves. They proposed that expansins induced changes in the cell wall that modified the physical stress pattern in the meristem, causing aberrant tissue bulging, which led to the acquisition of primordium identity in cells at ectopic sites. A role for endogenous expansin in leaf initiation was indicated by in situ hybridization analysis. Expansin genes are not only expressed in the apical meristem but mRNA levels are highest in the bulging cells of the leaf primordia. More recently, Pien et al. (2001) showed that the localized induction of expansin transgene expression on the flanks of tobacco vegetative meristems not only induced the appearance of leaf primordia but reiterated the entire process of leaf development and produced phenotypically normal leaves.

12.6.2 Fruit Softening

Several reports have shown that expansins are expressed abundantly in softening fruit, for example, strawberry (Civello, Powell, Sabehat, and Bennett 1999), tomato (Rose, Cosgrove, Albersheim, Darvill, and Bennett 2000), pear (Hiwasa, Rose, Nakano, Inaba, and Kubo 2003), and banana (Trivedi and Nath 2004). Overexpression or downregulation of fruit-specific expansins was reported to influence fruit texture and juice viscosity in tomato (Brummel et al. 1999; Powell, Kalamaki, Kurien, Gurrieri, and Bennett 2003). Expansin genes are differentially regulated during fruit development, with the expression of some genes increasing in concert with fruit size, and the expression of others increasing specifically during the fruit-ripening stage. It is likely that different expansins with distinct expression patterns participate at different stages of fruit development. The role of expansin in fruit softening might be to facilitate the breakdown of glucans in the cell walls.

12.6.3 Abscission and Tissue Differentiation

In addition to cell elongation, expansins may also be involved in organ abscission and wall disassembly. Cho and Cosgrove (2000) showed that β-glucuronidase accumulated at the base of petioles in transgenic Arabidopsis plants when expressed under the control of the *AtEXP10* promoter, and the alteration of *AtEXP10* expression resulted in pedicel abscission as well as leaf growth. Increased expansin activity was observed in tissue undergoing cell separation during leaflet abscission in blue

elderberry (*Sambucus nigra*), indicating that expansins may be involved in abscission (Belfield, Ruperti, Roberts, and McQueen-Mason 2005). Chen and Bradford (2000) showed that an expansin is closely associated with endosperm weakening during the germination of tomato seeds. This process involves wall breakdown and weakening rather than growth. At least six different expansin genes are expressed in differentiating tracheary elements in *Zinnia* cell cultures. The expansin described by Im, Cosgrove, and Jones (2000) appears to be associated with the intrusive growth of protoxylem elements in *Zinnia* stems.

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. Expression analysis suggests that *GhEXP1,* which is abundantly expressed specifically in the fiber, plays an important role in cell wall loosening during fiber elongation (Harmer, Orford, and Timmis 2002).

12.6.4 Sexual Reproduction

Expansin activity has also been linked to important roles in pollination and the development of floral organs. Seven *EXPA* and three *EXPB* genes were analyzed during the development of *Mirabilis jalapa* flowers, and were shown to be regulated differentially during cell expansion and senescence (Gookin, Hunter, and Reid 2003). Proteomic analysis of a maize gametophytic male-sterile mutant *gaMS-2,* comparing the profiles of anthers and immature pollen grains in heterozygous mutants and wild-type plants, showed that Zea m 1, a group I pollen allergen, was greatly depleted in sterile pollen from the heterozygous mutant (Wang et al. 2004). The association of Zea m 1 with the sterile phenotype suggested that Zea m 1 could have two distinct roles: binding to pectins in order to facilitate cell wall deposition in pollen grains, or softening stigmatic tissues in order to facilitate penetration by the pollen tube. A tobacco *EXPB* gene is strongly expressed at the stigmatic surface during pollination, suggesting that expansins may facilitate pollination in dicots and grasses (Pezzotti, Feron, and Mariani 2002).

12.6.5 Response to Abiotic and Biotic Stress

Drought imposes a major abiotic stress that severely limits plant growth. *Craterostigma plantagineum*, the resurrection plant, is able to survive periods of drought by extensive folding of the cell wall, which maintains the integrity of connections between the plasma membrane and cell wall even when water loss results in cell shrinkage (Jones and McQueen-Mason 2004). Expansin activity increases in the leaves of this plant during the early stages of both dehydration and rehydration, providing evidence that expansins are associated with cell wall folding and desiccation tolerance. Expansins also appear to be involved in the response to flooding-induced stress. In the semi-aquatic species *Rumex palustris*, differential expansin expression

was detected in the roots in response to low O_2 levels (Colmer et al. 2004). This is consistent with the observation that expansin expression in deepwater rice correlates with internodal growth (Lee and Kende 2001, 2002).

Interactions between plants and microbes can involve extensive tissue remodeling, for example, during the formation of root nodules and mycorrhizae, and expansins have been shown to feature prominently in these processes. For example, Balestrini, Cosgrove, and Bonfante (2005) investigated the expression profiles of two cucumber *EXPA* genes, *CsEXPA1* and *CsEXPA2*, in uncolonized roots and in roots colonized with a mycorrhizal fungus. Immunoblot analysis using antibodies against CsEXPA1 and CsEXPA2 showed the presence of expansin specifically in the colonized roots, with CsEXPA1 localized at the interface zone, which is characteristic of endomycorrhiza, and CsEXPA2 localized within the cell wall. Therefore it appears that some expansins participate in the formation and maintenance of the interface, whereas others function as cell wall loosening agents to achieve the fungus-induced enlargement of cortical cells.

Giordano and Hirsch (2004) looked at the expression of *MaEXP1* (an *EXPA* gene) in developing root nodules of sweet clover during its interaction with the nitrogen-fixing bacterium *Sinorhizobium meliloti*. In situ hybridization showed that following inoculation *MaEXP1* was induced in both roots and nodules, suggesting an integral role for the gene product in the structural modifications that accompany nodule formation.

12.7 Isolation and Characterization of Petunia Expansin A Genes

Petunia hybrida is a useful model system for the analysis of expansins due to its well-characterized morphology and the availability of a large number of insertional mutants (see Chapter 17). The first Petunia expansin cDNA was isolated by screening a *P. hybrida* ovary cDNA library, yielding a 1250-bp clone with a 780-bp open reading frame that predicted a 25 kDa protein of 260 amino acids (Zenoni et al. 2004). Comparison with other expansin sequences placed this protein in the EXPA subfamily, and the gene was duly named *PhEXP1A* (Zenoni et al. 2004). Southern blot analysis with a probe hybridizing to the unique 3′-untranslated region of the sequence showed that *PhEXP1A* is a single-copy gene in the Petunia genome. A subsequent Southern blot was carried out using a probe hybridizing to a region of the *PhEXP1A* cDNA fragment that is highly conserved in *EXPA* genes (Cosgrove 2000). After high-stringency washing, five bands were identified by this probe, suggesting the presence of multiple *EXPA* genes in Petunia.

A semi-permanent *dTph1* insertional library (Gerats et al. 1990) was screened by PCR, combining a transposon-specific primer with two *EXPA*-specific primers, the latter based on the sequences of the two highly conserved expansin boxes. A total of 4096 plants from the Juliet library were screened, sampled in 3D array (see Chapter 17; Vandenbussche et al. 2003). Three genomic DNA fragments and the

corresponding insertional mutants were identified. In a parallel experiment, an ovary cDNA library was screened to extend the sequences identified in the transposon library and to isolate specific sequences for which primers were available (Kuhlemeier, personal communication). Using these combined approaches, we identified four additional Petunia expansin A cDNA sequences, which were named *PhEXP2A* (1198 bp), *PhEXP3A* (1171 bp), *PhEXP4A* (1151 bp), and *PhEXP5A* (1099 bp).

All the genomic expansin sequences contained the I-1 and I-3 introns characteristic of *EXPA* genes (Lee, Choi, and Kende 2001) at the correct sites, with lengths of the introns varying from 107 to 1300 bp. *PhEXP1A* and *PhEXP4A* had the longest introns – 1143 bp for I-1 and 1300 bp for I-3. Such large introns, comparatively rare in plants, are considered likely sites for enhancers and other regulatory elements.

Multiple alignment of amino acid sequences predicted from the five expansin cDNA sequences confirmed that they are all members of the *EXPA* gene family (Fig. 12.1). All the deduced amino acid sequences contain key EXPA features such as the two N-terminal GACGYG motifs and a C-terminal NWGQNWG motif. The predicted N-terminal signal peptides are diverse in sequence but the N-termini of the mature polypeptides are highly conserved. The amino acid sequences of all five expansins contain four conserved tryptophan residues (+) that may facilitate interaction with cellulose, eight cysteine residues (∗) that may participate in disulfide bonds, and the HFD domain (boxed), deduced from its homology to the catalytic site of GH45 glycosyl hydrolases (Cosgrove 2000), which may carry out the catalytic function of the EXPA proteins. The central region of each protein also contains a so-called a-insertion, a motif of about 14 residues that contains the highly conserved sequence GWCN at its 3′ end.

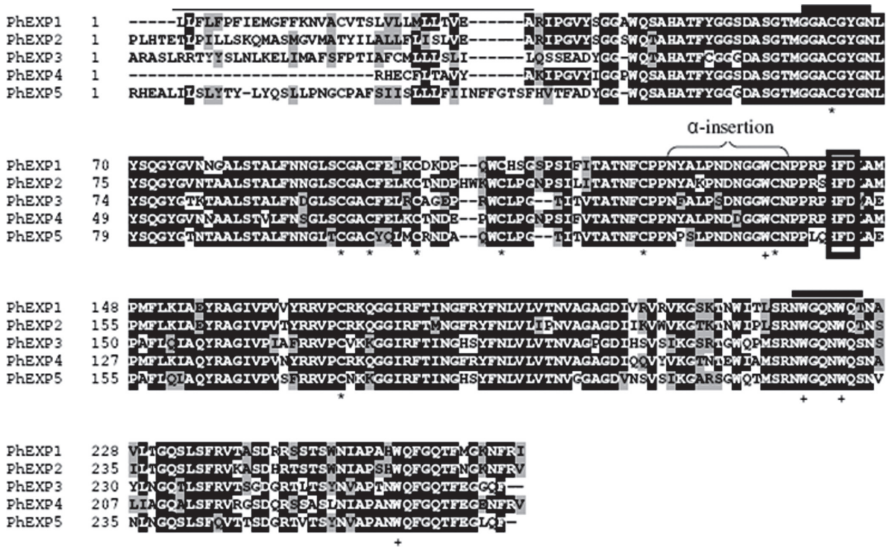

Fig. 12.1 Alignment of the predicted sequences for five *P. hybrida* expansins

Phylogenetic analysis was carried out to place the five new Petunia expansin A genes among the sequences already considered by Sampedro et al. (2005). *PhEXP3A* and *PhEXP5A* were placed within EXPA clade III, whereas *PhEXP1A*, *PhEXP2A*, and *PhEXP4A* clustered in clade XIII (Fig. 12.2). The new sequences were also classified according to an earlier phylogenetic study that divided the EXPA subfamily into four further subgroups (A, B, C, and D; Link and Cosgrove 1998). Within this system *PhEXP1A*, *PhEXP2A*, and *PhEXP4A* aligned with subgroup A, members of which are characterized by the presence of a conserved methionine residue near the HFD motif, a conserved RIPGV sequence immediately after the predicted signal peptide, and a conserved C-terminal sequence KNFRV. Subgroup A contains clades EXPA-IV and EXPA-XIII, and includes genes from diverse plant species such as corn (*ZmEXP4*) and pine (*pinusEXP2*). It has been suggested that conservation of EXPA sequence motifs within each clade may reflect conserved functions and/or the ability to respond to similar upstream signals (Link and Cosgrove 1998). The two characteristic motifs in this subgroup could be important for post-translational modification, targeting to specific compartments of the cell wall, or for interaction with particular substrates. There is some evidence for a functional association of subgroup A expansins with cell wall disassembly during germination and fruit ripening (Harmer et al. 2002).

The remaining Petunia expansins, *PhEXP3A* and *PhEXP5A*, cluster within subgroup B (clade EXPA-III). This contains many EXPA proteins that are expressed in

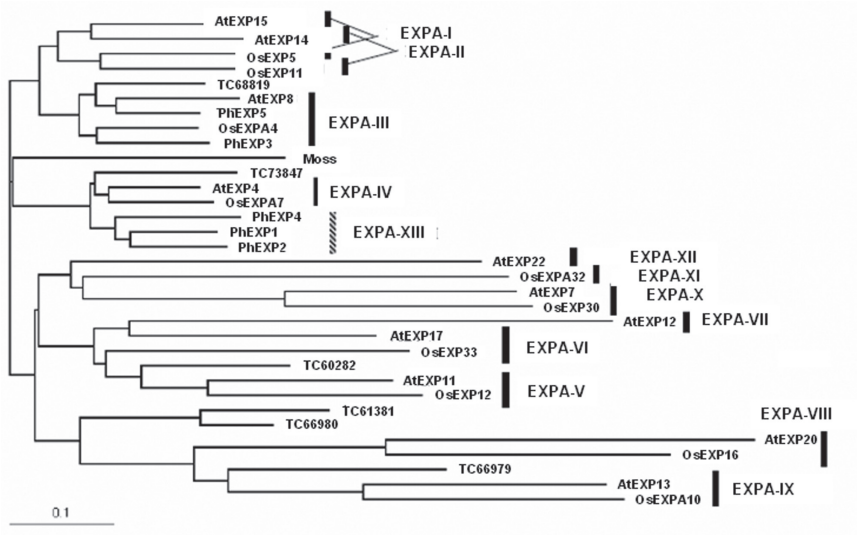

Fig. 12.2 Phylogenetic tree showing positions of cloned *P. hybrida* expansins (Ph) relative to expansin A protein sequences from *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Pinus* species (TC), and *Physcomitrella patens* (moss). The tree was calculated by the neighbor-joining method and bootstrap analysis (1000 replications) using PHYLIP. Proposed clades (Sampedro et al. 2005) are indicated at *right*

rapidly growing tissues, like elongating cotton fibers and elongating hypocotyls in a variety of species (e.g., *CsEXP1*, *CsEXP2*, *LeEXP2*, and *OsEXP4).* We have yet to isolate Petunia expansin genes from subgroup C (clade EXPA-I), whose members have heterogeneous expression patterns, and subgroup D (clade EXPA-V), whose members are also expressed in rapidly growing tissues.

The expression profiles of *PhEXP1A*, *PhEXP2A*, *PhEXP3A*, *PhEXP4A*, and *PhEXP5A* were analyzed by semi-quantitative real-time reverse transcriptase (RT) PCR, using gene-specific primers annealing to the unique 3′-untranslated regions of each gene (Fig. 12.3). *PhEXP1A* is strongly expressed in petals, ovaries, and elongating stems; there are intermediate levels in sepals, roots, styles, and stigmata, and low levels in leaves and anthers. *PhEXP2A* is strongly expressed in leaves but only weakly expressed in sepals, ovaries, stems, petals, and roots. The *PhEXP2A* transcript is barely detectable in anthers, styles, and stigmata. *PhEXP3A* is expressed strongly and specifically in styles and stigmata, whereas low expression levels are

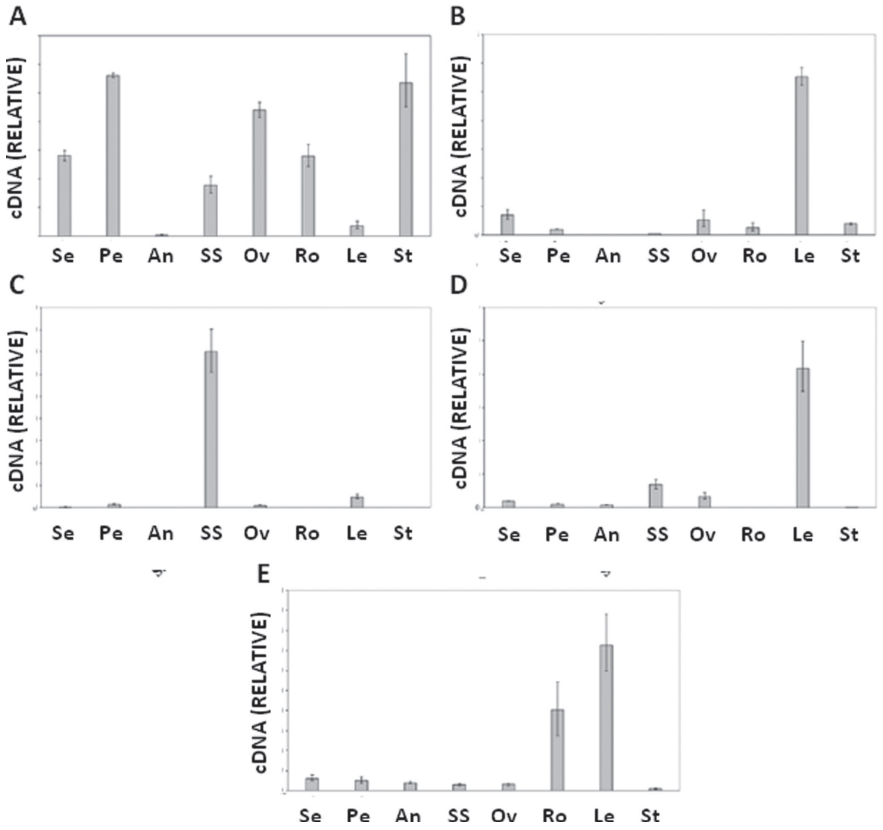

Fig. 12.3 Organ-specific expression patterns for (**A**) *PhEXP1A*; (**B**) *PhEXP2A*; (**C**) *PhEXP3A*; (**D**) *PhEXP4A*; and (**E**) *PhEXP5A.* Data were derived from analysis of RT-PCR products based on amplification from gene-specific primers. Se, Sepal; Pe, Petal; An, Anther; SS, Stigma and Style; Ov, Ovary; Ro, Root; Le, Leaf; and St, Stamen

found in other tissues. *PhEXP4A* is expressed strongly in leaves, and at lower levels in sepals, styles, stigmata, and ovaries, whereas no *PhEXP4A* can be detected in anthers, roots, and stems. *PhEXP5A* is expressed at high levels in leaves and roots, but at low levels in all other organs.

To assess whether EXPA proteins in the same clade may be functionally related, we compared the phylogenetic classifications of the five new Petunia *EXPA* genes with the expression profiles. *PhEXP1A*, *PhEXP2A*, and *PhEXP4A*, which group together (Fig. 12.2), were strongly expressed in petals and leaves, tissues in which cell enlargement is characterized by anisotropic growth. *PhEXP3A*, expressed specifically in the style and stigma, and *PhEXP5A*, expressed in leaves and roots, also group together phylogenetically (Fig. 12.2); in both style and root, isotropic cell growth contributes to the determination of organ morphology. On this basis, there does seem to be evidence for some functional significance in the phylogenetic profile of the Petunia EXPA subfamily.

12.7.1 Functional Analysis

The functions of individual genes can be established by the creation of mutants, but in many systems including Petunia it is easier to create phenocopies by interfering with target gene expression. The expression of antisense RNA is one such approach, and this was used to knockdown *PhEXP1A* gene expression in order to observe the impact on petal morphogenesis (Reale et al. 2002). Semi-quantitative RT-PCR showed that *PhEXP1A* is expressed throughout petal development, with peaks at stages 7 and 10.

Stage 7 marks the onset of cell expansion in the mesophyll of petal limbs, while stage 10 involves the expansion of epidermal cells that control flower unfolding. This suggested a correlation between *PhEXP1A* transcript levels and petal cell enlargement during petal development. We therefore focused our analysis on *PhEXP1A* expression in the petal tube and limb from stages 7 to 13. Because *PhEXP1A* expression in the limb is 3–5-fold higher than in the tube, we proposed that *PhEXP1A* is required for limb cell growth.

To test this hypothesis we produced transgenic Petunia plants in which *PhEXP1A* mRNA levels were reduced by antisense RNA expression (Zenoni et al. 2004). Four independent transgenic lines were recovered showing aberrant petal morphology (Fig. 12.4A), which correlated with the downregulation of *PhEXP1A* expression. Homozygous plants showed a significant reduction in petal length, corresponding to an average 75% reduction in the levels of *PhEXP1A* mRNA in petals. The length of the petal limb, but not that of the tube, was reduced, correlating with a significant reduction in *PhEXP1A* levels in the same flower part during development (Fig. 12.4B). The total limb surface area was reduced to approximately one-third that of the wild type, and this was attributable to reduced cell surface areas in the adaxial and abaxial epidermal limb. There were no significant differences in cell number between transgenic and wild-type plants.

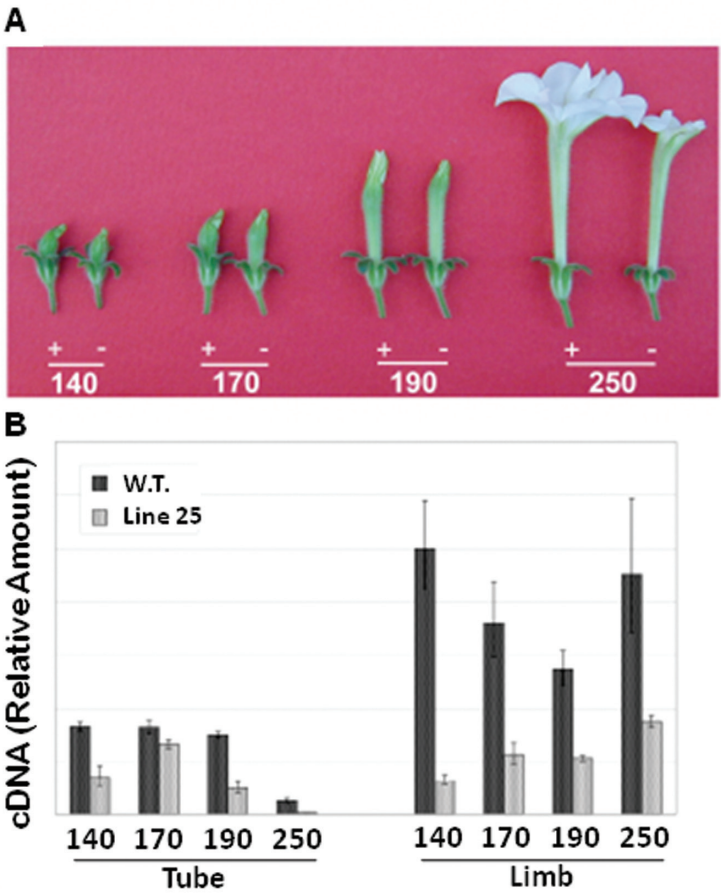

Fig. 12.4 Effect of antisense suppression of *PhEXP1A*. (**A**) Morphological changes during petal development at 140, 170, 190, and 250 h AFBA (after flower bud appearance). (**B**) *PhEXP1A* expression in tube and limb of wild-type and mutant petals at 140, 170, 190, and 250 h AFBA

Morphological analysis of epidermal limb cells (Fig. 12.5) revealed that cells in transgenic plants were smaller and had a less pronounced conical tip – the overall shape was irregular and the characteristic lobes were reduced or absent. Tangential and radial cell walls appeared thinner in the transgenic lines. Fourier transform infrared (FTIR) spectroscopy showed that the reduction in cell wall thickness in the transgenic plants could be attributed to a deficiency in crystalline cellulose. Indeed, the walls of petal epidermal cells in the transgenic lines had a higher protein content and were depleted in cellulose compared to wild-type cells.

On the basis of these results we propose that expansins may fulfill two functions in Petunia petal development: first, they may help to disrupt noncovalent bonds between cellulose microfibrils and cross-linking glycans, thereby promoting

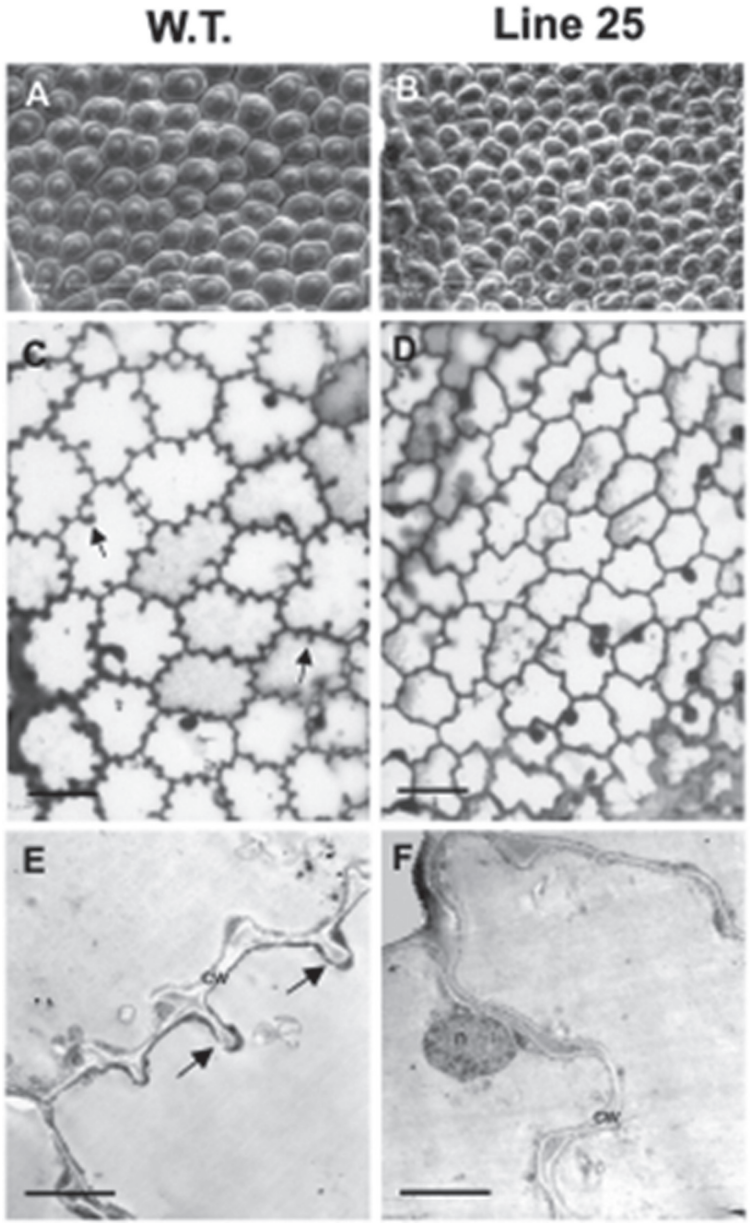

Fig. 12.5 Morphological comparison of epidermal limb cells in wild-type and *PhEXP1A*-downregulated Petunia. (**A, B**) scanning electron micrographs; (**C, D**) tangential semi-thin sections; (**E, F**) transmission electron micrographs

polymer creep as in the model proposed by Cosgrove (2000); and second, they may help to prepare the cell wall for new cellulose deposition during creep by separating the wall matrix during expansion, although a mechanism for the latter has yet to be established.

12.7.2 Identification and Analysis of Insertional Mutants

It can be difficult to create specific knockout mutants in many plant species; an alternative strategy is to first generate random libraries of mutants by saturation insertional mutagenesis and then isolate mutants in interesting genes by PCR, using primers specific for the insertional DNA construct and the target gene. A Petunia *dTph1* insertional library was screened using specific primers matching the 5′ and 3′ ends of the *PhEXP1A* cDNA sequence together with a primer specific for the *dTph1* transposon, resulting in the isolation of four *PhEXP1A* insertional mutants, one with the insertion in the first exon and three with insertions in the first intron (Fig. 12.6).

Insertional mutants for the other four expansin genes were sought by screening the same library with two family-specific primers (designed around conserved motifs in EXPA genes) together with the *dTph1* primer. No insertions were found in *PhEXP2A*, and single insertions were identified for the remaining three genes – in the third exon of *PhEXP3A*, the second intron of *PhEXP4A*, and the second exon of *PhEXP5A* (Fig. 12.6).

Lines with exon insertions were chosen for further analysis, as these were deemed most likely to show loss-of-function phenotypes. However, no morphological anomalies were observed in homozygous insertional mutants for *PhEXP1A*, *PhEXP3A*, or *PhEXP5A*. This is consistent with results obtained with equivalent

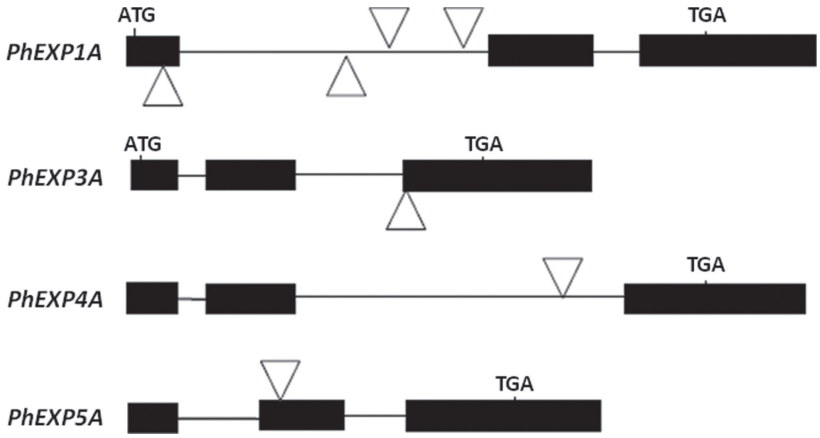

Fig. 12.6 Positions of transposable element insertions in *P. hybrida* expansin genes recovered from a *dTph1* insertional library

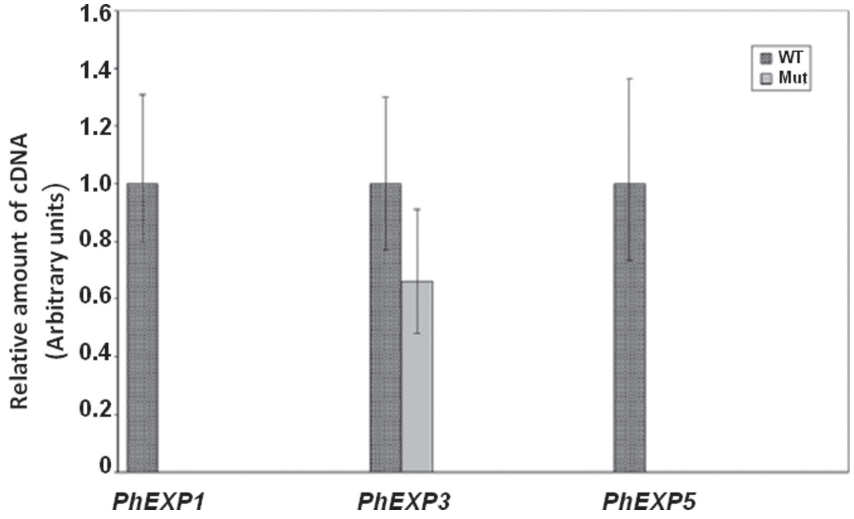

Fig. 12.7 RT-PCR-based analysis of gene expression in tissues normally expressing *PHEXP1A* (*petal*), *PhEXP3A* (*style and stigma*), and *PhEXP5A* (*leaf*), comparing levels of cDNA recovered from wild-type plants and the triple mutant

T-DNA insertion lines in Arabidopsis, perhaps indicating functional redundancy among the related genes (Cosgrove et al. 2002). To verify this hypothesis, we created three double mutants (*PhEXP1A-PhEXP3A*, *PhEXP3A-PhEXP5A*, and *PhEXP1A-PhEXP5A*) and one triple mutant (*PhEXP1A-PhEXP3A-PhEXP5A*). These plants, too, showed no sign of morphological changes compared to wild-type plants.

To confirm that the triple mutants lacked the capability to produce functional transcripts, real-time RT-PCR analysis was carried out in organs where the wild-type transcript should be expressed strongly. Thus, *PhEXP1A* was analyzed in petals, *PhEXP3A* in styles and stigma, and *PhEXP5A* in leaves. *PhEXP1A* and *PhEXP5A* transcripts were undetectable in homozygous plants but *PhEXP3A* expression persisted, albeit reduced by about 40% compared to that of the wild type (Fig. 12.7). This residual *PhEXP3A* expression in mutant plants presumably reflects the position of the *dTph1* insertion (Fig. 12.6), which might interfere with splicing of the second intron yet allow the production of some still-functional transcripts.

12.8 The Future: Expansion Beyond Expansin

Our functional analysis of *PhEXP1A* suggests a novel biological role for the expansin A subfamily. The reduction of crystalline cellulose in *PhEXP1A* mutants suggests PhEXP1A involvement in cellulose metabolism, which is a rate-limiting step in cell expansion. The enlargement of cells in development and in other processes could be blocked until this step is completed. Alternatively, plant cells could

overcome the limited availability of particular expansins by relying on the expression of genes with related functions.

In order to monitor global transcriptional changes occurring during the development of mutant petals with defective limb cell expansion, we used a cDNA-AFLP approach to compare transcript profiles in antisense *PhEXP1A* lines and wild-type plants. Messenger RNA was isolated from petal limbs at the same four developmental stages used in the *PhEXP1*A expression profiling experiments described above, and a transcriptional profile was created using 32 primer combinations, surveying about 6100 mRNAs. This analysis revealed 370 cDNA fragments (expressed sequence tags, ESTs) ranging from 50 to 500 bp, whose expression patterns differed between antisense and wild-type plants. The ESTs were isolated, re-amplified, and cloned into a TOPO® vector (Invitrogen, Carlsbad, CA) and sequenced, resulting in 190 sequences that could be used in BLAST searches against sequence databases (Fig. 12.8). Further data analysis and clustering showed that a majority of the ESTs encode proteins with metabolic functions (39%) or roles in signal transduction (17%).

One attractive theory is that the reduction of crystalline cellulose in the *PhEXP1A* antisense line causes a compensatory shift in carbohydrate metabolism to enhance cellulose synthesis. In addition, about 8% of the ESTs encode proteins known to be involved in the assembly and development of cell walls, while 10% are nucleic acid binding proteins, 5% are chloroplast proteins, 4% are defence related, and 2% are components of the cell cycle. The differential expression of these sequences was confirmed by real-time RT-PCR (data not shown).

The global transcript profile in the antisense line was also analyzed in the context of sequence ontology. The genes involved in cell wall metabolism generally

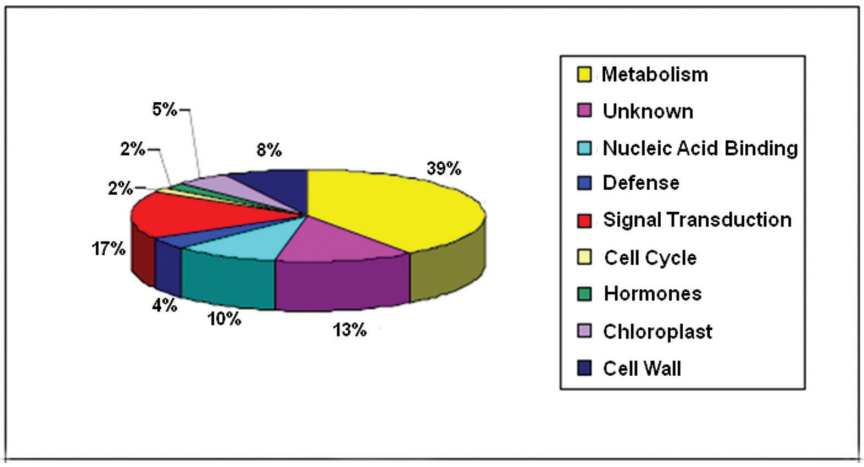

Fig. 12.8 Categorization of genes with altered expression in the *Ph1EXP1A* mutant. The analysis was based on ESTs recovered from transcript profiling of RNA from wild-type and *Ph1EXP1A* mutant petals harvested at four developmental stages

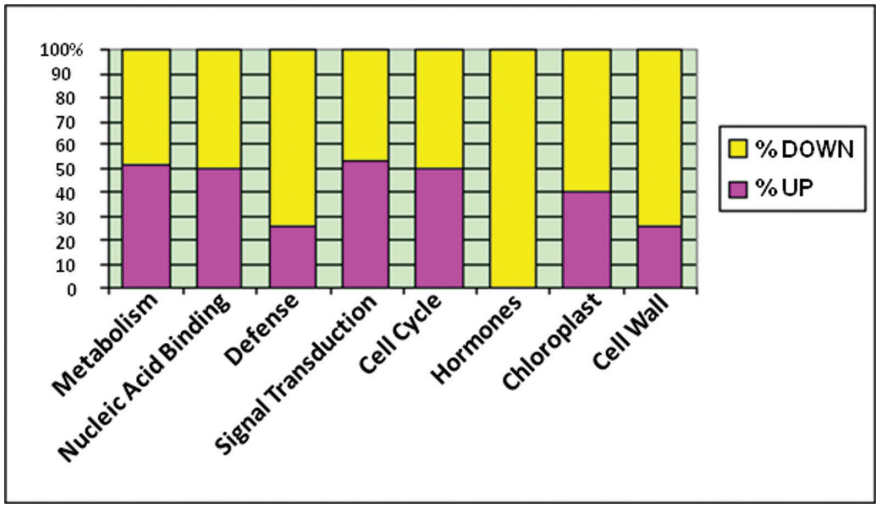

Fig. 12.9 Global modification of gene expression in petals of the *PhEXP1A* mutant, illustrating patterns of upregulation and downregulation

showed a strong reduction in expression, an average of 75% below wild-type levels (Fig. 12.9). This group contained endoglucanases, pectin methylesterase, and one additional expansin A gene. These data indicate that *PhEXP1A* may act upstream of the cell-enlargement process, regulating the expression or activity of other cell wall loosening agents. This is consistent with the idea that the expansins play an important regulatory role during cell wall extension, hence their description as primary rather than secondary wall-loosening agents. Expansins could be responsible for the critical modifications in the cell wall network, making the cell wall molecules available as substrates for other enzymes, such as endoglucanases. The additional expansin identified in this group is very similar to *LeEXP11A*, and is strongly inhibited. This suggests that some expansins can influence the activity and expression of others in a complex regulatory hierarchy that is still largely a black box despite many years of diligent research.

12.9 Conclusions

We identified five members of the *EXPA* gene family in *P. hybrida* and carried out detailed expression profiling in different tissues, revealing that *PhEXP1A* is temporally regulated during development. The single, double, and triple insertional mutants for *PhEXP1A*, *PhEXP3A*, and *PhEXP5A* had normal morphologies, which suggests that multiple knockouts will be required to avoid the effects of functional redundancy in order to establish gene function unambiguously. Antisense knockdown of *PhEXP1A* supported the involvement of this expansin in the regulation of cell wall extension. We also propose a new role for PhEXP1A: preparing the

growing cell wall for new cellulose deposition. Expansins could be also involved in regulating the expression of secondary cell wall loosening agents. Major questions remain regarding the specific wall polysaccharides targeted by expansin and the molecular mechanisms underlying cell wall loosening.

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