Two Tomato Expansin Genes Show Divergent Expression and Localization in Embryos during Seed Development and Germination[1]

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Expansins are plant proteins that can induce extension of isolated cell walls and are proposed to mediate cell expansion. Three expansin genes were expressed in germinating tomato (*Lycopersicon esculentum* Mill.) seeds, one of which (*LeEXP4*) was expressed specifically in the endosperm cap tissue enclosing the radicle tip. The other two genes (*LeEXP8* and *LeEXP10*) were expressed in the embryo and are further characterized here. *LeEXP8* mRNA was not detected in developing or mature seeds but accumulated specifically in the radicle cortex during and after germination. In contrast, *LeEXP10* mRNA was abundant at an early stage of seed development corresponding to the period of rapid embryo expansion; it then decreased during seed maturation and increased again during germination. When gibberellin-deficient (*gib-1*) mutant seeds were imbibed in water, *LeEXP8* mRNA was not detected, but a low level of *LeEXP10* mRNA was present. Expression of both genes increased when *gib-1* seeds were imbibed in gibberellin. Abscisic acid did not prevent the initial expression of *LeEXP8* and *LeEXP10*, but mRNA abundance of both genes subsequently decreased during extended incubation. The initial increase in *LeEXP8*, but not *LeEXP10*, mRNA accumulation was blocked by low water potential, but *LeEXP10* mRNA amounts fell after longer incubation. When seeds were transferred from abscisic acid or low water potential solutions to water, abundance of both *LeEXP8* and *LeEXP10* mRNAs increased in association with germination. The tissue localization and expression patterns of both *LeEXP8* and *LeEXP10* suggest developmentally specific roles during embryo and seedling growth.

Plant cells are encased in a complex wall that is composed of structurally diverse polysaccharides, proteins, and other materials (Carpita and Gibeaut, 1993). The cell wall serves many functions, including structural support and cell shape, protection against pathogens and other environmental assaults, storage and release of signaling molecules, and storage of carbohydrates, ions, and other materials (Cosgrove, 1999). As a fundamental determinant of cell size and shape, plant cell walls undergo dramatic changes during the plant life cycle. Precise spatial and temporal patterns of wall growth occur as cells expand 10 to 1,000 times in volume after differentiation (Cosgrove, 2000).

Intriguing questions remain about the mechanism of wall expansion and the integration of newly synthesized materials into existing walls. Several types of polymer rearrangements could plausibly lead to turgor-driven wall expansion. These include cleavage of the backbone of the major matrix polymers, weakening of the non-covalent bonds between polysaccharides, and breakage of cross-links between matrix polymers (Cosgrove, 1998). Many candidates have been proposed to be involved in wall relaxation. Although cell wall hydrolases that can cleave the major matrix polymers are almost certainly involved in cell expansion, hydrolytic enzymes alone, including β-1,4-endoglucanases and xyloglucan endotransglycosylase, are unable to cause wall extension in in vitro assays (McQueen-Mason et al., 1992). However, using such a reconstitution assay, proteins termed "expansins" were identified based on their ability to cause extension of killed cucumber (*Cucumis sativus*) hypocotyl segments held under tension (McQueen-Mason et al., 1992). Because purified expansin protein had little or no hydrolytic activity, it was proposed to function by disrupting the hydrogen bonds between cellulose and hemicellulose polymers (McQueen-Mason and Cosgrove, 1994).

Expansin genes subsequently have been identified from many species and are highly conserved in gymnosperms and in both monocots and dicots among the angiosperms (Cosgrove, 1998; Hutchison et al., 1999). The occurrence of multigene families of expansins suggests that different expansins play unique developmental or tissue-specific roles (Cho and Kende, 1997; Cosgrove, 1997; Harrison et al., 2001; Wu et al., 2001b). Most of the expansin genes characterized are proposed to be involved in cell expansion during tissue growth (Cho and Kende, 1997; Fleming et al., 1997; Reinhardt et al., 1998; Brummell et al., 1999b; Hutchison et al., 1999), and

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this has been confirmed by manipulating the expression of a specific expansin gene in Arabidopsis (Cho and Cosgrove, 2000). Expansins are also expressed in tissues where cell wall disassembly rather than cell growth occurs (Rose et al., 1997; Civello et al., 1999; Chen and Bradford, 2000). For example, a tomato (*Lycopersicon esculentum* Mill.) expansin gene *LeEXP1* was expressed in ripening fruits at a time when fruit softening was occurring (Rose et al., 1997, 2000; Brummell et al., 1999a, 1999b). Extensive cell wall degradation and solubilization of wall components occurs during ripening (Fischer and Bennett, 1991), resulting in tissue softening and cell separation without cell enlargement. When the expression of *LeEXP1* was modified in antisense transgenic tomato fruits, softening and cell wall polymer metabolism were altered during ripening, demonstrating a physiological role for *LeEXP1* in fruit ripening (Brummell et al., 1999a). Thus, expansins appear to be involved in diverse gene-specific roles in developmental processes related to cell wall expansion, disassembly, or separation (Cosgrove, 1997; Cho and Cosgrove, 2000).

Multiple roles of specific expansins can be illustrated by fruit development and seed germination in tomato. A tomato expansin gene, *LeEXP4*, was expressed early in fruit development (Brummell et al., 1999b), and its mRNA also was localized specifically to the endosperm cap tissue enclosing the radicle tip of imbibed seeds (Chen and Bradford, 2000). Expression of *LeEXP4* mRNA in the endosperm cap was correlated with physical weakening of this tissue, which is required to allow radicle emergence during germination. Two additional expansin genes, *LeEXP8* and *LeEXP10*, were expressed during tomato seed germination and showed different tissue localization from that of *LeEXP4* (Chen and Bradford, 2000). *LeEXP8* mRNA was detected only in the radicle tip tissue, whereas *LeEXP10* mRNA was present in both the radicle tip and the rest of seed (comprising the embryo and lateral endosperm). Here, we report the tissue localization and regulation of expression of *LeEXP8* and *LeEXP10* during tomato seed development, germination, and early seedling growth. The results support distinct roles for these two expansins in embryo development and growth.

RESULTS

Sequence Analysis of Tomato Expansins

LeEXP8 and *LeEXP10* were isolated by reverse transcription-PCR and screening of a germinating tomato seed cDNA library (Chen and Bradford, 2000). A phylogenetic tree was generated from deduced amino acid sequences of α-expansins from several species, together with the sequence of a pollen allergen (Phlp1) that belongs to the β-expansins (Shcherban et al., 1995; Fig. 1). The sequences align

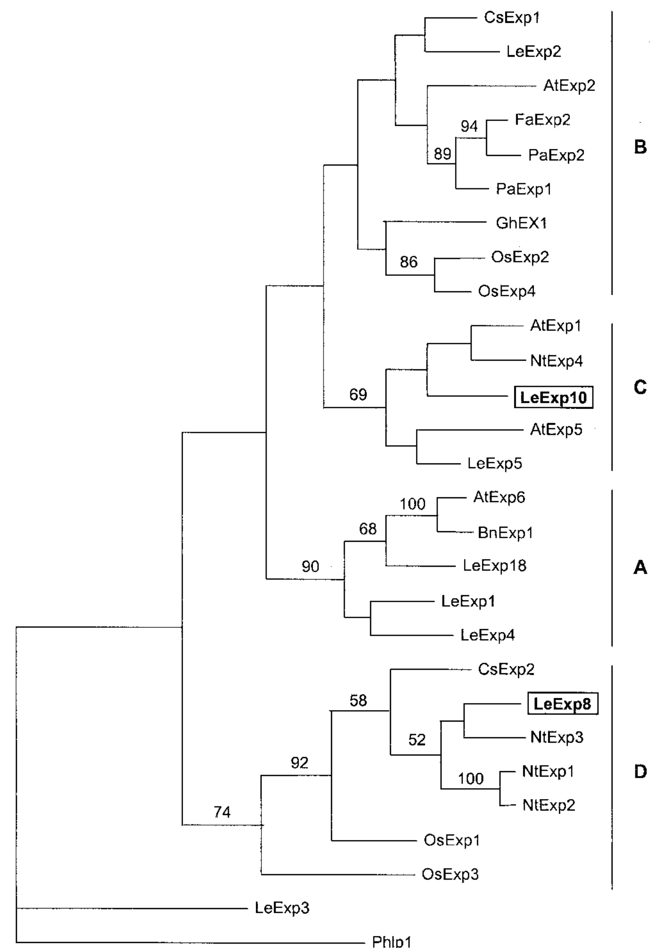

Figure 1. Phylogenetic analysis of expansin genes. The phylogenetic tree was generated based on an alignment of the deduced amino acid sequences of 27 α-expansins together with a pollen allergen (Phlp1), which belongs to β-expansins. Alignments were made using the MEGALIGN software (DNASTAR Inc., Madison, WI) based on the CLUSTAL algorithm. The two expansin genes expressed in tomato seeds that are characterized in this paper are boxed. Vertical lines indicate subgroups A, B, C, and D. The bootstrap values, which correspond to match percentage of branching orders, are indicated at each branch point. The GenBank accession numbers of the expansins included are as follows: Arabidopsis, AtEXP1 (U30476), AtEXP2 (U30481), AtEXP5 (U30487), and AtEXP6 (U30480); *Brassica napus*, BnEXP (AJ000885); cucumber, CsEXP1 (U30382) and CsEXP2 (U30460); strawberry (*Fragaria ananassa*), FaEXP2 (AF159563); *Gossypium hirsutum*, GhEXP (AF043284); tomato, LeEXP1 (U82123), LeEXP3 (AF059487), LeEXP4 (AF059488), LeEXP5 (AF059489), LeEXP8 (AF184232), LeEXP10 (AF184233), and LeEXP18 (AJ004997); tobacco (*Nicotiana tabacum*), NtEXP1 (AF049353), NtEXP2 (AF049351), NtEXP3 (AF049352), and NtEXP4 (AF049353); deepwater rice (*Oryza sativa*), OsEXP1 (Y07782), OsEXP2 (U30477), OsEXP3 (U30479), and OsEXP4 (U85246); and *Prunus armeniaca*, PaEXP1 (U93167) and PaEXP2 (AF038815).

within four major groups: A, B, C, and D (after Link and Cosgrove, 1998; Rose et al., 2000). *LeEXP8* and *LeEXP10* are included in subgroups D and C, respectively (Fig. 1), whereas subgroup A includes *LeEXP4*, the expansin expressed during tomato fruit expan-

sion and seed germination (Brummell et al., 1999b; Chen and Bradford, 2000). Among the expansins most closely related to *LeEXP8* and *LeEXP10* are *NtEXP1*, *NtEXP2*, *NtEXP3*, and *NtEXP4* isolated from tobacco cell cultures (Link and Cosgrove, 1998) and *AtEXP1* isolated from growing Arabidopsis leaves (Shcherban et al., 1995).

LeEXP8 and *LeEXP10* Show Seed-Specific Expression

To determine whether *LeEXP8* and *LeEXP10* are expressed in tissues other than germinating seeds, RNA gel-blot analyses were carried out using gene-specific probes hybridized with total RNA isolated from tomato roots, stems, leaves, flowers, and dry and germinating seeds. Among these tissues, *LeEXP8* mRNA was abundant in germinating seeds and was present at a much lower level in roots (Fig. 2). *LeEXP10* mRNA was abundant in dry and germinating seeds, but not in any other tissues (Fig. 2). Because neither of these genes was identified among the expansins amplified from developing or ripening fruits (Brummell et al., 1999b), these genes apparently are not expressed significantly in those tissues either.

Tissue Localization of *LeEXP8* and *LeEXP10* Expression

To determine the tissue localization of *LeEXP8* and *LeEXP10* gene expression in seeds, total RNA was extracted separately from the endosperms and the embryos of seeds imbibed for 24 h (before radicle emergence, which would begin at about 40 h). Both *LeEXP8* and *LeEXP10* mRNAs were present exclusively in the embryo (Fig. 3, A and B). Localization of expression was further characterized by tissue printing of 24-h imbibed embryos (before radicle emergence). *LeEXP8* mRNA was localized specifically to the cortical tissue of the radicle, whereas *LeEXP10* mRNA was present throughout the entire embryo (Fig. 3, C and D). The localization of expression of both genes was also characterized in embryos 24 h after radicle emergence. *LeEXP8* mRNA remained localized to the cortical tissue of the elongated root (Fig. 3E), whereas *LeEXP10* mRNA was present mainly in the cotyledons, which were still enclosed within the endosperm (Fig. 3F). The elongation zone of the radicle and emerging embryo was determined by a marking experiment, which showed that the majority of elongation occurred in the tissue adjacent to the radicle tip (Fig. 3, G and H). *LeEXP8* mRNA was most abundant in the cortical tissue of this elongation zone (compare Fig. 3, E with H).

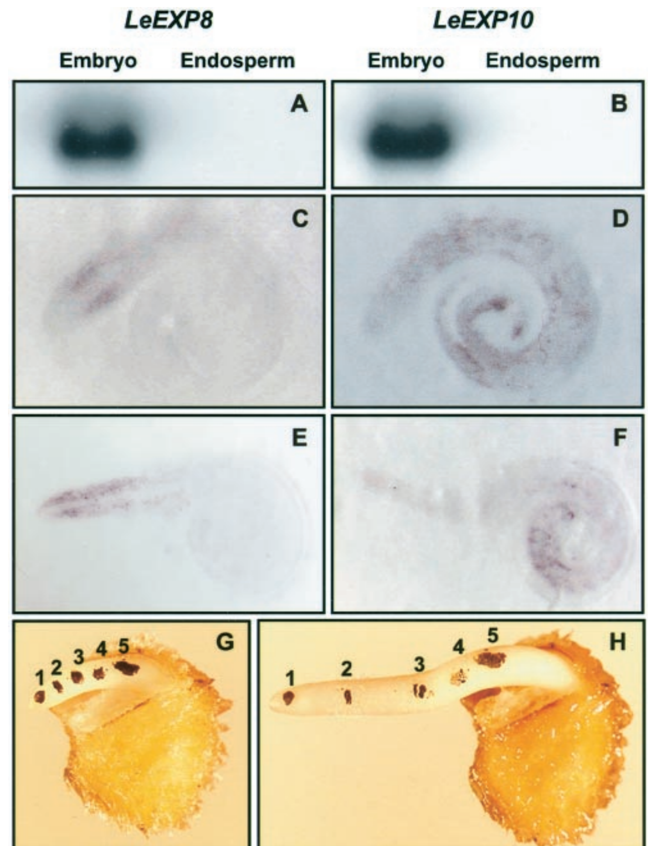

Figure 3. Tissue localization of *LeEXP8* and *LeEXP10* in imbibed and germinated seeds. Total RNAs from embryo or endosperm tissues were hybridized with *LeEXP8* (A) or *LeEXP10* (B) cDNA probes. Tissue prints show localization of expression of *LeEXP8* (C and E) and *LeEXP10* (D and F) in imbibed but ungerminated embryos (C and D) and in embryos 24 h after radicle emergence (E and F). Tomato seeds imbibed for 24 h (before radicle emergence; C and D) or for 72 h (24 h after radicle emergence; E and F) were bisected, and the cut surfaces of the embryos were printed onto membranes. The membranes were then hybridized with gene-specific antisense RNA probes, and hybridization was detected by colorimetry. *LeEXP8* mRNA was detected only in the cortical tissue of the radicle (C and E), whereas *LeEXP10* mRNA was present throughout the embryo, particularly in the cotyledons (D and F). No hybridization was detected using sense probes (not shown). To determine where elongation is most rapid in germinating embryos, marks were made on the exposed radicle of an ungerminated seed that was exposed by removing the surrounding endosperm tissue (G). After 24 h of growth, the same seed was photographed again (H). The greatest growth occurred between marks 1 and 3, corresponding to the tissue where *LeEXP8* is expressed (E).

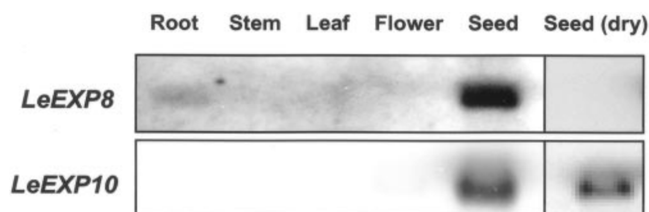

Figure 2. RNA gel-blot analysis showing *LeEXP8* and *LeEXP10* mRNA abundance in different tissues. Total RNAs were extracted from root, stem, leaf, and flower tissues of tomato plants and from 24-h imbibed or dry tomato seeds. Total RNA (10 μg) from each sample was separated by electrophoresis and hybridized with *LeEXP8*- and *LeEXP10*-specific cDNA probes.

Expression of *LeEXP8* and *LeEXP10* during Seed Development and Germination

Developing seeds were collected from fruits staged according to size and color from field-grown plants. Eight maturity categories were established based upon fruit and seed characteristics. The first four categories included seeds from immature green fruits in which the seeds were increasing in size and changing from green to brown in color. The last four categories were seeds from mature green, breaker, ripe, and overripe fruits where seed fresh and dry weight accumulation had ceased (Berry and Bewley, 1991). Total RNA was extracted from these developing seeds, and RNA gel-blot analyses showed that *LeEXP8* mRNA could not be detected during seed development (Fig. 4A), but it appeared in germinating seeds after 12 h of imbibition and remained relatively constant thereafter (Fig. 4B). In contrast, expression of *LeEXP10* mRNA was highest at early stages of seed development and remained present at a low level thereafter (Fig. 4A). After imbibition, *LeEXP10* mRNA abundance increased and remained relatively constant during germination (Fig. 4B).

Hormonal and Environmental Regulation of *LeEXP8* and *LeEXP10* Expression

Because seed germination is subject to control by hormonal and environmental factors, expression of *LeEXP8* and *LeEXP10* was examined in relation to these factors. Gibberellin (GA)-deficient *gib-1* mutant seeds do not complete germination in the absence of exogenous GA (e.g. Ni and Bradford, 1993). When *gib-1* seeds were imbibed in water for 24 h, no expression of *LeEXP8* mRNA was detected and *LeEXP10* mRNA abundance was low, whereas both genes were abundant in wild-type (Moneymaker [MM]) seeds at this time (Fig. 5A). However, expression of both genes was induced in *gib-1* seeds within 24 h when imbibed in GA (Fig. 5A), which also stimulated radicle emergence to begin at around 48 h. Abscisic acid (ABA), which is required for seed dormancy (Hilhorst and Karssen, 1992) and is a seed germination inhibitor (e.g. Ni and Bradford, 1993), did not block expression of either *LeEXP8* or *LeEXP10* after 24 h of imbibition (Fig. 5A). However, when seeds were imbibed in ABA for a longer time (48 and 96 h), the mRNA abundance of both genes decreased and no seeds germinated (Fig. 5B), compared with $<$95% germination of seeds imbibed in water for 96 h. Transfer of these seeds to water resulted in increased accumulation of *LeEXP8* and *LeEXP10* mRNAs within 12 h and subsequent initiation of germination (Fig. 5B). Imbibition in low water potential PEG solution ($-$1.0 MPa), which prevented radicle emergence up to 96 h, completely blocked the expression of *LeEXP8* but had no effect on the accumulation of *LeEXP10* mRNA at 24 h of imbibition (Fig. 5A). However, after 96 h of incubation at $-$1.0 MPa, *LeEXP10* mRNA had declined to low levels (Fig. 5A). Transfer of the seeds to water resulted in expression of *LeEXP8* and reaccumulation of *LeEXP10* mRNA along with the initiation of radicle emergence (Fig. 5A).

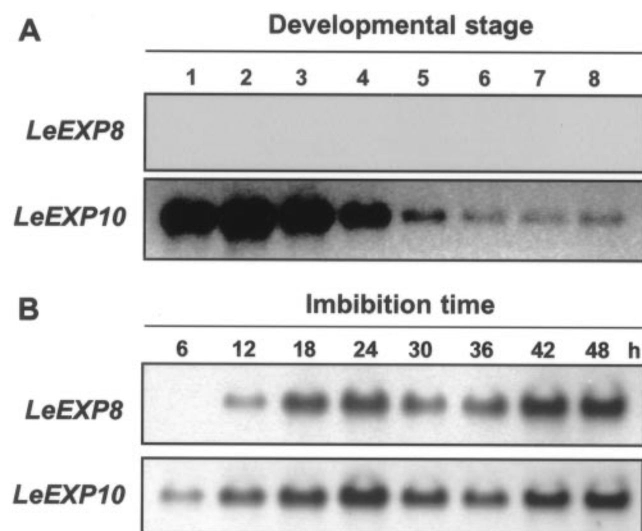

Figure 4. Abundance of *LeEXP8* and *LeEXP10* mRNA in developing (A) and germinating (B) tomato seeds. Total RNA was extracted from eight categories of developing seeds (A) or from germinating seeds at various times after imbibition (B), separated by electrophoresis, and hybridized with gene-specific probes. Developing seeds were collected from fruits grouped based on fruit and seed development characteristics (1–4 are developing seeds from immature fruits, 5 from mature green fruit, 6 from breaker fruits, 7 from ripe fruits, and 8 from overripe fruits).

DISCUSSION

Expansins comprise large gene families in most species that have been well studied (Shcherban et al., 1995; Cosgrove, 2001). The phylogenetically divergent subgroups of expansins may reflect isoforms with different biochemical properties such as substrate affinities or pH optima and may fulfill unique and diverse functions in plant development (Cosgrove, 1997; Rose et al., 2000; Wu et al., 2001b). Characterization of expression of multiple expansins suggests that individual gene family members are involved in distinct physiological processes (Cho and Kende, 1997; Brummell et al., 1999b; Cho and Cosgrove, 2000; Harrison et al., 2001). In some cases, expansins proposed to be involved in the same physiological process appeared in the same phylogenetic subgroup (Rose et al., 1997), whereas in other cases there was little phylogenetic relationship among expansins having similar expression patterns (Harrison et al., 2001). Here, the three expansin genes expressed in germinating tomato seeds (*LeEXP4*, *LeEXP8*, and *LeEXP10*) fell into three different subgroups by phylogenetic analysis (Fig. 1), and they also exhibited distinct tissue localization and developmental pat-

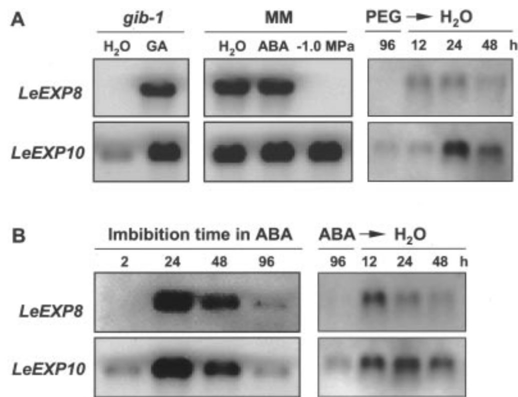

Figure 5. Hormonal and environmental regulation of *LeEXP8* and *LeEXP10* gene expression. A, GA-deficient (*gib-1*) mutant seeds were imbibed for 24 h in water (H_2O) or in 100 μM GA_{4+7} (GA), and wild-type MM seeds were imbibed for 24 h in water, in 100 μM ABA, or in −1.0 MPa polyethylene glycol (PEG) 8000 solutions before total RNAs were extracted. In a separate experiment, MM seeds were imbibed for 96 h in −1.0 MPa PEG 8000 solution, which prevented germination, and the seeds were then rinsed and transferred to water. Samples for RNA extraction were taken at the time of transfer (96 h) and after 12, 24, and 48 h of further incubation in water, by which time 11% of the seeds had completed germination. If initially imbibed on water, >95% of seeds would have completed germination within the first 96 h. B, Wild-type MM seeds were sampled after imbibition in 100 μM ABA for 2, 24, 48, and 96 h, which prevented germination. In a separate experiment, MM seeds were imbibed in 100 μM ABA for 96 h, and the seeds were then rinsed and transferred to water. Samples were taken at 96 h and after 12, 24, and 48 h of further incubation in water, by which time 27% of the seeds had completed germination. In both A and B, total RNA was separated by electrophoresis, blotted to membranes, and hybridized with gene-specific probes for *LeEXP8* and *LeEXP10*.

terns of expression (Figs. 3–5; Chen and Bradford, 2000). Whereas both *LeEXP8* and *LeEXP10* are primarily expressed in seeds (Fig. 2), *LeEXP4*, whose expression in seeds is endosperm cap specific (Chen and Bradford, 2000), is also expressed early in fruit development (Brummell et al., 1999b). Other individual expansin genes are also expressed at multiple sites during plant development (Cho and Cosgrove, 2000; Wu et al., 2001b). Thus, phylogenetic similarity of the protein sequences does not as yet reveal any obvious expansin functional groupings, and an individual expansin gene can apparently play multiple roles in different tissues or at different stages of development.

Although both *LeEXP8* and *LeEXP10* were expressed only in the embryos of seeds (Figs. 2 and 3, A and B), they exhibited distinct tissue expression patterns. *LeEXP10* mRNA was detected throughout the embryo of imbibed seeds (Fig. 3D), whereas *LeEXP8* expression was restricted to the radicle cortex (Fig. 3C). This distinction was maintained after radicle emergence because *LeEXP8* mRNA expression remained confined to the elongation zone of the radicle (Fig. 3, E and H), whereas *LeEXP10* mRNA was localized mainly to the cotyledons (Fig. 3F). This tissue-specific expression supports the hypothesis that individual expansins play different roles in cell expansion and differentiation. *LeEXP8* appears to be involved in initial (and perhaps continued) elongation of the radicle, whereas *LeEXP10* may play a more general role in embryo growth. The control of tissue specificity of gene expression would be expected to lie in the promoter regions of the two genes. When the promoter region of the *LeEXP8* gene was isolated and fused with a glucuronidase reporter sequence and transformed into Arabidopsis plants, glucuronidase activity was mainly expressed in root tissues of transgenic seedlings (Chen, 2000), consistent with the expression pattern of this gene in germinating and germinated tomato seeds (Fig. 3, C and E).

Divergent roles for *LeEXP8* and *LeEXP10* are also indicated by the temporal expression patterns and hormonal regulation of these two genes. Expression of *LeEXP8* was detected only in germinating seeds, whereas *LeEXP10* mRNA was present in both dry and imbibed seeds (Fig. 2). The presence of *LeEXP10* mRNA in dry seeds implied that it was synthesized during seed development. In fact, *LeEXP10* mRNA amounts peaked during the early stages of seed development, followed by maintenance of a lower level of mRNA abundance throughout seed maturation (Fig. 4A). Tomato seed development can be divided into three major phases: (a) phase I, histodifferentiation and expansion; (b) phase II, reserve accumulation and maturation; and (c) phase III, dehydration, which occurs after removal from the fruit (Berry and Bewley, 1991). In phase I, seeds gain fresh weight due to cell division and early expansion. In phase II, seeds increase in both fresh and dry weight because of cell enlargement and reserve deposition. In phase III, seeds maintain constant fresh and dry weights until they dehydrate after removal from the fruit (Berry and Bewley, 1991). The peak of *LeEXP10* expression occurred at early stages of seed development corresponding to phases I and II when rapid embryo expansion is occurring. This suggests a role for *LeEXP10* in cell wall expansion in this growing tissue. However, a lower level of *LeEXP10* mRNA was maintained in later stages of seed development when elongation of the embryo had ceased. A study with tomato hypocotyls found a relationship between growth rate and expression of some expansin genes, but the correlation was not absolute (Caderas et al., 2000). The conclusion was drawn that elongation growth is likely to be controlled by expansins acting in concert with other factors that may limit growth under some physiological conditions, which is likely to be the case also for *LeEXP10* in developing seeds.

During imbibition of tomato seeds, *LeEXP8* mRNA could be detected after 12 h, and the abundance of *LeEXP10* mRNA also increased at that time (Fig. 4B). These are among the earliest germination-associated genes known to be expressed in tomato seeds after imbibition and the first whose expression is localized

solely in the embryo (Bradford et al., 2000; Chen and Bradford, 2000; Nonogaki et al., 2000; Feurtado et al., 2001; Wu et al., 2001a). Germination of GA-deficient *gib-1* mutant tomato seeds is dependent upon exogenous GA (Groot and Karssen, 1987), and a number of germination-associated genes are expressed in response to GA in these seeds, including expansin *LeEXP4* in the endosperm cap (Bradford et al., 2000; Chen and Bradford, 2000). This was also the case for both *LeEXP8* and *LeEXP10* in *gib-1* mutant seeds (Fig. 5A). A close correlation between the induction of the expansin gene *OsEXP4* by GA and the initiation of cell growth was also documented in deepwater rice (Cho and Kende, 1997). It appears that in tomato seeds, distinct expansins under the regulation of GA may contribute to both weakening of the endosperm cap tissue and the early expansion of the embryo associated with germination.

ABA inhibited seed germination, but it did not prevent the expression of *LeEXP4* or of several cell wall hydrolases in the endosperm cap nor the majority of physical weakening of this tissue (Bradford et al., 2000; Chen and Bradford, 2000; Nonogaki et al., 2000; Toorop et al., 2000; Wu et al., 2001a). Similarly, ABA did not reduce the initial expression of either *LeEXP8* or *LeEXP10*, but mRNA abundance of these genes subsequently decreased to low levels during further incubation (Fig. 5, A and B). Inhibition of expansin expression may be associated with the reduced growth potential of the embryo in the presence of ABA (Schopfer and Plachy, 1985; Ni and Bradford, 1992). Furthermore, when seeds were subsequently transferred from ABA to water, which allowed germination to proceed, the expression of both *LeEXP8* and *LeEXP10* increased within 12 h (Fig. 5B). Thus, the relatively late action of ABA in inhibiting seed germination (Toorop et al., 2000) may be due to down-regulation of embryo expansins required for growth, even though the restraint offered by the enclosing endosperm cap has been reduced.

Low water potential also inhibits germination, but in contrast to ABA, it decreased expression of *LeEXP4* and prevented weakening of the endosperm cap (Chen and Bradford, 2000). Expression of *LeEXP8* was also inhibited by low water potential after 24 h of imbibition, whereas the expression of *LeEXP10* was not affected at this time (Fig. 5A). However, after longer incubation at low water potential, abundance of *LeEXP10* mRNA declined to low levels (Fig. 5A). Upon transfer to water, expression of both genes increased as germination proceeded (Fig. 5A). How water potential regulates gene expression remains unknown, but it exerts an effect on the rate of progress toward completion of germination that is proportional to the water potential reduction (Bradford, 1995). Water stress often acts via stimulation of ABA synthesis, but this is not the case in tomato seeds (Ni and Bradford, 1992), and the different effects of ABA and of low water potential on the expression of expansin genes also indicate that their mechanisms of action are distinct (Fig. 5). Nonetheless, there was a strong correlation between the effects of GA, ABA, and low water potential on the expression of embryo expansins, particularly *LeEXP8*, and the effects of these factors on germination, although a direct causal connection remains to be demonstrated.

The distinct spatial and temporal expression patterns of *LeEXP4*, *LeEXP8*, and *LeEXP10* and their differential regulation by developmental, hormonal, and environmental signals suggest multiple roles for expansins in tomato seed development and germination. This complexity is reflected in the similarly diverse expression patterns found in deepwater rice seedlings, in which four expansin genes were differentially expressed in coleoptile, root, and internode tissues and in response to GA and submergence (Cho and Kende, 1997), in tomato hypocotyls, where *LeEXP2* and *LeEXP18* showed tissue-specific, hormonal, and light-regulated expression (Caderas et al., 2000), in tomato and strawberry fruits, in which five to six expansin genes exhibited unique expression patterns during fruit development (Brummell et al., 1999b; Harrison et al., 2001), and in maize (*Zea mays*) tissues at different developmental stages, where numerous expansins were represented (Wu et al., 2001b). The requirement for multiple expansins might be related to the differences in cell wall composition among tissues. For example, endosperm cap cell walls of tomato seeds contain >60% Man, whereas embryo cell walls contain only 30% Man (Dahal et al., 1997). The different expansin proteins might interact with distinct cell wall substrates or cooperate with similarly diverse and specific hydrolase isoforms to contribute to cell expansion in the early stages of embryo development, to cell wall disassembly in the endosperm cap, to embryo elongation during germination, and to root expansion after radicle emergence. Additional information on the proteins coded by these genes and their activities on different types of cell walls are needed to confirm this hypothesis.

MATERIALS AND METHODS

Plant Materials

Tomato (*Lycopersicon esculentum* Mill.) seeds from either wild-type cv MM or homozygous GA-deficient (*gib-1*) mutant plants were harvested from field-grown plants in 1998. The *gib-1* mutant and its isogenic parent line were obtained originally from Dr. Cees Karssen (Wageningen Agricultural University, The Netherlands). Mutant plants were sprayed three times per week with 100 μM GA_{4+7} to revert the dwarf habit and allow more vigorous growth and fertility. After fruits were harvested, seeds were extracted, treated with 0.25 M HCl, dried to 6% moisture content (fresh basis), and stored at −20°C until used (Ni and Bradford, 1993). For germination, seeds were incubated at 25°C

in the dark in 9-cm diameter petri dishes on top of two layers of blotter paper moistened with 12 mL of deionized water, 100 μM GA_{4+7}, 100 μM ABA, or PEG 8000 solutions having a water potential of -1.0 MPa. In experiments involving extended incubation (seed transfer experiment in Fig. 5), 2 mg L^{-1} of benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate; DuPont, Wilmington, DE] was added to the solutions to prevent fungal contamination.

Cloning of Full-Length cDNAs Encoding Expansins

Isolation of RNA from germinating tomato seeds, reverse transcription-PCR amplification, and screening of a cDNA library prepared from germinating tomato seeds were described in Chen and Bradford (2000). The two novel tomato expansin genes described here were named *LeEXP8* and *LeEXP10* (Cosgrove, 2001).

Phylogenetic Alignments of Expansin Genes

The deduced amino acid sequences of a selection of α-expansin genes were used to generate a phylogenetic tree. Alignments were made using the default parameters of personal computer-based MEGALIGN software (DNASTAR Inc.), using the CLUSTAL algorithm. The phylogenetic tree was generated using PAUP*4.0b software (Sinaner Associates, Inc., Sunderland, MA) by selecting pollen allergen (Phlp1), a β-expansin, as outgroup. Phylogenetic relationships were defined by PAUP software using a heuristic search with 100 replicates. Bootstrap values are indicated above the branches.

RNA Gel-Blot Analyses

Total RNA was isolated from seeds, seed parts, or different tissues of tomato plants as described in Chen and Bradford (2000). For embryo or endosperm RNA extraction, imbibed seeds were bisected and the embryo halves were removed from the surrounding endosperm and pooled. Total RNA from each sample (5 μg) was subjected to electrophoresis on 1% (w/v) agarose/10% (v/v) formaldehyde denaturing gels, transferred to Hybond-N^{+} (Amersham Pharmacia Biotech, Piscataway, NJ) membrane, and UV cross-linked. Gene-specific probes (Chen and Bradford, 2000) were generated from the 3′ regions of the genes by PCR amplification incorporating digoxigenin (DIG)-labeled nucleotides. The labeling efficiency was estimated according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis). The DNA probes were included in hybridization buffer at a final concentration of 25 ng mL^{-1}. High SDS buffer (7% [w/v] SDS) was used for hybridization at 42°C. Washing (60°C) and detection followed the recommended method using the chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (Boehringer Mannheim). Exposure time was from 10 min to 2 h depending on the strength of the signal. For some experiments (seed transfer experiments of Fig. 5), DIG-labeled antisense RNA probes were generated by digesting pBKCMV-LeEXP8 and pBKCMV-LeEXP10 DNA with *Eco*RI and transcribing using T7 RNA polymerase. After hybridization in standard 50% (v/v) formamide buffer, stringent washing was carried out twice at 75°C in 0.2× SSC. The chemiluminescent signal was detected using anti-DIG-alkaline phosphate conjugate (Roche Molecular Biochemicals, Indianapolis) in Lumihos-530 (Lumigen Inc., Southfield, MI; Nonogaki et al., 2000).

Tissue Printing

Tomato seeds were imbibed as described for germination. After 24 h of imbibition, the seeds were bisected using a razor blade, and the cut surfaces of the embryos were pressed onto a positively charged membrane (Hybond N^{+}) for 10 to 15 s before the tissue was removed. Alternatively, seeds were imbibed for 72 h (approximately 24 h after radicle emergence), and the entire seed and emerged tissues were bisected and pressed onto a membrane as above. The membranes were cross-linked using UV light and hybridized with gene-specific RNA probes generated by in vitro transcription from the T7 (antisense) and T3 (sense) promoters and incorporating DIG-labeled nucleotides (Chen and Bradford, 2000). Colorimetry was used for signal detection (detection reagent: 0.18 M Tris-HCl buffer, pH 8.8, containing 0.025 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-phosphate, 0.1 mg mL^{-1} nitroblue tetrazolium, and 2 mM $MgCl_2$). Reaction time varied depending on the development of target signal.

Elongation Zone Identification

To identify the radicle elongation zone, seeds were imbibed in water for 24 h before removing the endosperm caps and some lateral endosperm tissues to expose the radicles. The radicles were marked with India ink at five equally spaced locations. The seed samples were then incubated at 25°C with the radicles oriented downward. After 24 h, the distances between neighboring spots indicated where expansion had been most rapid.

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LITERATURE CITED

Berry T, Bewley JD (1991) Seeds of tomato (*Lycopersicon esculentum* Mill.) which develop in a fully hydrated environment in the fruit switch from a developmental to a germinative mode without a requirement for desiccation. Planta **186:** 27–34

Bradford KJ (1995) Water relations in seed germination. *In* J Kigel, G Galili, eds, Seed Development and Germination. Marcel Dekker, New York, pp 351–396

Bradford KJ, Chen F, Cooley MB, Dahal P, Downie B, Fukunaga KK, Gee OH, Gurusinghe S, Mella RA, Nonogaki H et al. (2000) Gene expression prior to radicle emergence in imbibed tomato seeds. *In* M Black, KJ Bradford, J Vazquez-Ramos, eds, Seed Biology: Advances and Applications. CAB International, Wallingford, UK, pp 231–251

Brummell DA, Harpster MH, Civello PM, Palys JM, Bennett AB, Dunsmuir P (1999a) Modification of expansin protein abundance in tomato fruit alters softening and cell wall polymer metabolism during ripening. Plant Cell **11:** 2203–2216

Brummell DA, Harpster MH, Dunsmuir P (1999b) Differential expression of expansin gene family members during growth and ripening of tomato fruit. Plant Mol Biol **39:** 161–169

Caderas D, Muster M, Vogler H, Mandel T, Rose JKC, McQueen-Mason S, Kuhlemeier C (2000) Limited correlation between expansin gene expression and elongation growth rate. Plant Physiol **123:** 1399–1413

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J **3:** 1–30

Chen F (2000) Molecular characterization of expansin and xyloglucan endotransglycosylase gene expression during tomato seed germination. PhD thesis. University of California, Davis

Chen F, Bradford KJ (2000) Expression of an expansin is associated with endosperm weakening during tomato seed germination. Plant Physiol **124:** 1265–1274

Cho HT, Cosgrove DJ (2000) Altered expression of expansin modulates leaf growth and pedicel abscission in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **97:** 9783–9788

Cho HT, Kende H (1997) Expression of expansin genes is correlated with growth in deepwater rice. Plant Cell **9:** 1661–1671

Civello PM, Powell ALT, Sabehat A, Bennett AB (1999) An expansin gene expressed in ripening strawberry fruit. Plant Physiol **121:** 1273–1279

Cosgrove DJ (1997) Creeping walls, softening fruit, and penetrating pollen tubes: the growing roles of expansins. Proc Natl Acad Sci USA **94:** 5504–5505

Cosgrove DJ (1998) Cell wall loosening by expansins. Plant Physiol **118:** 333–339

Cosgrove DJ (1999) Enzymes and other agents that enhance cell wall extensibility. Annu Rev Plant Physiol Plant Mol Biol **50:** 391–417

Cosgrove DJ (2000) Expansive growth of plant cell walls. Plant Physiol Biochem **38:** 109–124

Cosgrove DJ (2001) Other species. Expansin Central. http://www.bio.psu.edu/expansins/other_species.htm (May 1, 2001)

Dahal P, Nevins DJ, Bradford KJ (1997) Relationship of endo-β-mannanase activity and cell wall hydrolysis in tomato endosperm to germination rates. Plant Physiol **113:** 1243–1252

Feurtado JA, Banik M, Bewley JD (2001) The cloning and characterization of α-galactosidase present during and following germination of tomato (*Lycopersicon esculentum* Mill.) seed. J Exp Bot **52:** 1239–1249

Fischer RL, Bennett AB (1991) Role of cell wall hydrolases in fruit ripening. Annu Rev Plant Physiol Plant Mol Biol **42:** 675–703

Fleming AJ, McQueen-Mason S, Mandel T, Kuhlemeier C (1997) Induction of leaf primordia by the cell wall protein expansin. Science **276:** 1415–1418

Groot SPC, Karssen CM (1987) Gibberellins regulate seed germination in tomato by endosperm weakening: a study with gibberellin-deficient mutants. Planta **171:** 525–531

Harrison EP, McQueen-Mason SJ, Manning K (2001) Expression of six expansin genes in relation to extension activity in developing strawberry fruit. J Exp Bot **52:** 1437–1446

Hilhorst HWM, Karssen CM (1992) Seed dormancy and germination: the role of abscisic acid and gibberellins and the importance of hormone mutants. Plant Growth Regul **11:** 225–238

Hutchison KW, Singer PB, McInnis S, Diaz-Sala C, Greenwood MS (1999) Expansins are conserved in conifers and expressed in hypocotyls in response to exogenous auxin. Plant Physiol **120:** 827–831

Link BM, Cosgrove DJ (1998) Acid-growth response and α-expansins in suspension cultures of Bright Yellow 2 tobacco. Plant Physiol **118:** 907–916

McQueen-Mason S, Cosgrove DJ (1994) Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension. Proc Natl Acad Sci USA **91:** 6574–6578

McQueen-Mason S, Durachko DM, Cosgrove DJ (1992) Two endogenous proteins that induce cell wall expansion in plants. Plant Cell **4:** 1425–1433

Ni B-R, Bradford KJ (1992) Quantitative models characterizing seed germination responses to abscisic acid and osmoticum. Plant Physiol **98:** 1057–1068

Ni B-R, Bradford KJ (1993) Germination and dormancy of abscisic acid- and gibberellin-deficient mutant tomato (*Lycopersicon esculentum*) seeds: sensitivity of germination to abscisic acid, gibberellin and water potential. Plant Physiol **101:** 607–617

Nonogaki H, Gee OH, Bradford KJ (2000) A germination-specific endo-β-mannanase gene is expressed in the micropylar endosperm cap of tomato seeds. Plant Physiol **123:** 1235–1245

Reinhardt D, Wittwer F, Mandel T, Kuhlemeier C (1998) Localized upregulation of a new expansin gene predicts the site of leaf formation in the tomato meristem. Plant Cell **10:** 1427–1438

Rose JKC, Cosgrove DJ, Albersheim P, Darvill AG, Bennett AB (2000) Detection of expansin proteins and activity during tomato fruit ontogeny. Plant Physiol **123:** 1583–1592

Rose JKC, Lee HH, Bennett AB (1997) Expression of a divergent expansin gene is fruit-specific and ripening-regulated. Proc Natl Acad Sci USA **94:** 5955–5960

Schopfer P, Plachy C (1985) Control of seed germination by abscisic acid: III. Effect on embryo growth potential (minimum turgor pressure) and growth coefficient (cell wall extensibility) in *Brassica napus* L. Plant Physiol **77:** 676–686

Shcherban TY, Shi J, Durachko DM, Guiltinan MJ, McQueen-Mason SJ, Shieh M, Cosgrove DJ (1995) Molecular cloning and sequence analysis of expansins: a highly conserved, multigene family of proteins that mediate cell wall extension in plants. Proc Natl Acad Sci USA **92:** 9245–924

Toorop PE, van Aelst AC, Hilhorst HWM (2000) The second step of the biphasic endosperm cap weakening that mediates tomato (*Lycopersicon esculentum*) seed germination is under control of ABA. J Exp Bot **51:** 1371–1379

Wu C-T, Leubner-Metzger G, Meins F Jr, Bradford KJ (2001a) Class I β-1,3-glucanase and chitinase are expressed specifically in the micropylar endosperm of tomato seeds prior to radicle emergence. Plant Physiol **126:** 1299–1313

Wu Y, Meeley RB, Cosgrove DJ (2001b) Analysis and expression of the α-expansin and β-expansin gene families in maize. Plant Physiol **126:** 222–232