Cloning of an Arabidopsis Patatin-Like Gene, STURDY, by Activation T-DNA Tagging

Shihshieh Huang*, R. Eric Cerny, Deepti S. Bhat, and Sherri M. Brown

Mystic Research, Monsanto Company, 62 Maritime Drive, Mystic, Connecticut 06355 (S.H.); and Agricultural Sector, Monsanto Company, 700 Chesterfield Parkway North, St. Louis, Missouri 63198 (R.E.C., D.S.B., S.M.B.)

Activation T-DNA tagging can generate dominant gain-of-function mutants by overexpression of a particular endogenous gene. We identified an activation-tagged mutant, sturdy, exhibiting a stiff inflorescence stem, thicker leaves, shorter siliques, larger seeds, round-shaped flowers, and delayed growth. It is most important that unlike its wild-type counterpart, this mutant is less prone to lodging. Cloning of STURDY revealed that in sturdy, there is an open reading frame containing a single intron encoding a patatin-like homolog. The T-DNA is inserted into the 3′ region of the second exon. The mutant phenotype was shown to be the result of overexpression of STURDY by mRNA analysis and transgenic studies. Preliminary histological studies have revealed an increase in cell number in the inflorescence stem of mutant plants; however, additional studies are needed to better understand the overexpression phenotype.

Lodging occurs when crop plants fail to withstand environmental challenges, resulting in extensive financial losses in agriculture each year throughout the world. Crop plants fail to the ground prematurely, making them recalcitrant to mechanical harvest. Even in areas where manual harvesting is practiced, lodged plants can absorb moisture from the ground surface, interfering with the desiccation process and compromising the quality of the grain. In 1992, it was reported that lodging in winter wheat cost ≤£130 million in loss of yield alone (Berry et al., 1998) and reduced the grain quality standard (Hagberg falling number) from a U.K. 5-year average of 287 to 254 (Home Grown Cereal Authority, 1993). Through conventional breeding methods, dwarf traits have been identified and introgressed into various crop species, resulting in shorter plants with stiffer stems less prone to lodging. For example, the gibberellin-insensitive semidwarf Rht genes in wheat and the dw genes in sorghum have been used to reduce plant height (Quinby and Karper, 1954; Gale and Youssuffian, 1985). However, these genetic alterations in plant stature are limited to certain crops or varieties and are often accompanied with negative pleiotropic effects including reduced yield and lower grain protein levels. The progress in recombinant DNA technologies recently has provided alternative approaches for improvement of agronomic crops. We are interested in studying plant stem structure at the molecular level and identifying target genes to genetically modify crops with improved lodging resistance.

In principle, there are at least three aspects in which genetic engineering can be applied to improve lodging resistance. Many genes implicated in cell wall biosynthesis, composition, and lignification have been cloned (for review, see Boudet, 1998; Reiter, 1998). By altering the composition of the stem cell walls, it may be possible to increase stem strength. A change in stem strength alternatively could come from altering the organization of the stem structure (e.g. rearrangement of vascular bundles). Several homeobox genes were shown to preferentially express in the vascular tissues of Arabidopsis (Baima et al., 1995; Tornero et al., 1996). An Arabidopsis mutant displaying the transformation of collateral vascular bundles into amphivasal vascular bundles recently was identified (Zhong et al., 1999). An increase in mass could also result in a stronger stem structure. Studies of transgenic plants demonstrated the possibility of modifying the stem mass by varying the level of auxins or cytokinins (Medford et al., 1989; Romano et al., 1991). However, no mutant or transgenic plant showing a strengthened stem phenotype with the normal plant height has been reported.

We initiated an activation T-DNA screen to isolate mutants displaying unusual stem phenotypes. First described by Hayashi et al. (1992), activation T-DNA tagging produces dominant mutations caused by increased or ectopic expression of endogenous genes. This approach allows discovery of novel genes in which recessive, loss-of-function mutations have no obvious phenotype. In this study, we report the recovery of a dominant gain-of-function mutation affecting stem, leaf surface, silique length, seed size, flower shape, and growth rate. The mutant allele, named sturdy, was cloned and shown to be related to the patatin family of proteins. All phenotypes observed in sturdy mutants are due to overexpression of the gene. A preliminary microscopic examination of
sturdy inflorescence stems revealing their unusual structure is presented.

RESULTS

Identification of a Dominant, Stiff-Stem Mutant, Sturdy, in an Activation T-DNA-Tagging Population

We transformed Arabidopsis plants (Columbia ecotype) with a binary vector, pMON29963, to generate an activation T-DNA-tagging population. The T-DNA region of pMON29963 contains four enhancers of the cauliflower mosaic virus (CaMV)-35S promoter stacked near the right border, and the selection marker neomycin phosphotransferase II gene (NPTII) driven by the nopaline synthase promoter close to the left border. The transformants were first germinated on medium containing kanamycin and resistant seedlings were transferred to soil for phenotypic evaluation. Under our growth conditions, Arabidopsis plants of the Columbia ecotype can reach an average height of 40 cm (Huang et al., 1998) at the global proliferative arrest stage (Hensel et al., 1994). The stems typically cannot physically sustain growth and the plants bend toward the soil surface or lodge. In screening 4,200 transformants, we identified a single lodge-resistant plant. It appeared to have stiffer and thicker inflorescence stems and branches than wild-type plants, so it was named sturdy. This phenotype was observed in 83 of 114 T2 progeny, suggesting that sturdy is a dominant mutation caused by a T-DNA insertion at a single locus ($\chi^2 = 0.3$).

As shown in Figure 1, B and C, 2-week-old sturdy plants are small and dark green in color, similar to gibberellin-deficient mutants. However, sturdy did not respond significantly different than wild-type plants when active gibberellin analogs were applied. Moreover, unlike gibberellin-insensitive mutants, sturdy plants grow to a height comparable with wild-type plants with only a slight (15%) reduction in height. However, it takes about 55 d for sturdy plants to reach global proliferative arrest stage compared with 47 d for wild-type plants grown under the same conditions.

Figure 1. The phenotype of the sturdy mutant. A, Mature mutant plant. B and C, Two-week-old mutant and wild-type plants, respectively. D and E, Mutant and wild-type flowers, respectively. F and G, Mutant siliques and wild-type siliques, respectively. H, Topical view of a mutant flower cluster.
conditions. The growth rate was monitored through the plants’ life cycle by measuring leaf expansion and plant height. The most significant difference is that sturdy mutants’ stems are an average of 40% thicker than those of the wild-type plants. These results are summarized in Table I and Figure 2. It appears that sturdy develops greater stiffness in its inflorescence stems and branches through an increase in diameter and at the expense of plant height and growth rate. The sturdy mutation affects other parts of the plant as well. Its leaves have a rougher surface, and its flowers and siliques are shorter (Fig. 1, D–G). The shape of mutant flowers develops as round instead of oval. Also, the mutant flower clusters appear to be more compact with the stigmas protruding out of the flower buds (Fig. 1H). When the length of the siliques and number of seeds in each siliqua were measured (Table II), we found that mutant siliques also produced fewer seeds; however, total seed weight remained about the same. On average, individual mutant seeds weighed 30% more than wild type. We noticed that leaves and stems of the sturdy mutant are brittle and prone to fracture.

Cloning of the Genomic DNA of Sturdy

To clone sturdy, we first analyzed the mutant with a genomic DNA gel blot. Three different restriction enzymes (HindIII, BglII, and SpeI) were used to digest the genomic DNA of wild-type and mutant plants and the CaMV-35S enhancer was used as the probe for hybridization (Fig. 3A). DNA gel-blot analysis with three enzyme digests displayed a single band for hybridization (Fig. 3A). DNA gel-blot analysis using equal amounts of RNA (2 μg) isolated from leaves of wild-type and mutant plants and probed with the ORF sequence. The RNA blot shown in Figure 3C clearly indicated that the ORF was highly expressed in the mutant leaves but was undetectable in the leaves of wild-type plants and in a second activation-tagging line grown under the same condition. This suggests that the putative gene identified by the screen is the STURDY gene.

Overexpression of the Putative STURDY cDNA in Wild-Type Plants Conferring the Sturdy Phenotype

To demonstrate that overexpression of STURDY is solely responsible for the mutant phenotypes, we isolated the sturdy cDNA by RT-PCR and constructed a transformation vector, pMON42154, containing the cDNA driven by the enhanced CaMV-35S promoter. The sequence of the cDNA fragment amplified by the primer designed according to the genomic DNA also confirmed the location of the intron. Among 49 transgenic plants transformed with pMON42154, 26 of them clearly exhibited a sturdy-like phenotype. We selected five transgenic plants from each group for further analysis. As shown in Figure 5, plants numbered 2, 3, 6, 7, and 8 had the typical sturdy floral cluster, round and compact, whereas 1, 4, 5, 9, and 10 appeared to be normal. When the RNA samples prepared from leaves were analyzed on an RNA gel blot, only those sturdy-like transgenic plants showed strong expression of STURDY. These results confirm that the sturdy phenotype is caused by the overexpression of the STURDY gene identified in the T-DNA activation-tagging screen alone.

Molecular Characterization of the STURDY Gene

When the STURDY gene was used to search the GenBank sequence database by BLAST programs

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Flowering Timea</th>
<th>Terminal Heightb</th>
<th>Time-to-Terminal Height</th>
<th>Stem Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sturdy</td>
<td>30 ± 0.3</td>
<td>35.4 ± 0.63</td>
<td>55 ± 0.2</td>
<td>2.10 ± 0.04</td>
</tr>
<tr>
<td>Wild type</td>
<td>25 ± 0.2</td>
<td>41.8 ± 0.64</td>
<td>47 ± 0.3</td>
<td>1.50 ± 0.03</td>
</tr>
</tbody>
</table>

a Number of days for the primary bolt to reach a height of 1 cm.  b Height at which main stem produced at global proliferative arrest (Hensel et al., 1994).
(Altschul et al., 1997), it matched a putative gene in an Arabidopsis chromosome 3 bacteria artificial chromosome clone, F16M2 (accession no. AL138648). No other significant homology was identified at the nucleotide level. The predicted protein sequence showed similarity to a group of patatin or patatin-like proteins. STURDY was compared with three other predicted patatin-like proteins from the Arabidopsis genome sequences along with patatin and patatin homologs from potato, tobacco, and rubber by protein sequence alignment using the MegAlign program (DNASTAR Inc., Madison, WI). As illustrated in Figure 6, striking resemblance exists across the entire sequence between STURDY and these patatin proteins. Patatin, a 40-kD glycoprotein, has been found in all Solanum species tested (Prat et al., 1990) and accounts for up to 40% of the total water soluble protein present in the tubers (Racusen and Foote, 1980). In potato, patatin is a multigene family with 10 to 18 members per haploid genome (Mignery et al., 1988; Twell and Ooms, 1988), and its expression often associates with tuber formation (Perl et al., 1991). However, patatin-like proteins have been found in other plant organs (Paiva et al., 1983; Van Canneyt et al., 1989) and other species as well (Van Canneyt et al., 1989; Ganal et al., 1991). We examined the genomic composition and expression pattern of the STURDY gene. Even under less stringent wash conditions (0.5x SSC, 50°C for the high stringent washing), no other homologous sequence of the STURDY gene was identified (Fig. 7A). This is consistent with sequence search results that failed to

**Table II. Physical characterization of the silique and seed produced by sturdy plants**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Silique Length</th>
<th>Total Seed Wt</th>
<th>Total No. of Seeds</th>
<th>Average Seed Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm</td>
<td>mg</td>
<td>mg</td>
<td></td>
</tr>
<tr>
<td>sturdy</td>
<td>8.0 ± 0.15</td>
<td>47.1 ± 1.35</td>
<td>25.3</td>
<td>0.027</td>
</tr>
<tr>
<td>Wild type</td>
<td>15.0 ± 0.33</td>
<td>60.9 ± 2.78</td>
<td>24.5</td>
<td>1,217</td>
</tr>
</tbody>
</table>

**Figure 2.** Growth rate comparison between sturdy and wild-type plants. A, Chart of length (cm) of the sixth leaf by days. Under our growth conditions, the fifth and sixth leaf are the initial adult leaves in most Arabidopsis plants and they continue to expand throughout their life cycle. We recorded the length of the sixth leaf every other day and used these data as one of the growth rate indicators. B, Chart of plant height (cm) by days. The height of the plants was recorded every other day and was also used as a growth rate indicator.
identify homologous Arabidopsis sequences. Also, the STURDY gene was found to be expressed in all tissues tested with highest expression levels in roots (Fig. 7B).

**Microscopic Analysis of Sturdy Mutant Stems**

To investigate the stem structure of the sturdy mutant, we sectioned and analyzed the inflorescence stems from several individual mutant and wild-type plants. Due to the differences in their growth rate, the plant material was collected at a similar developmental stage based on height (14–15 cm) rather than days after planting. As shown in Figure 8, which represents the stem transverse sections of typical sturdy (A and C) and wild-type (B and D) plants, the diameter is significantly greater in sturdy plants compared with wild type at the same magnifications. In general, sturdy appears to increase in cell number rather than cell size. At this developmental stage, there are eight equally spaced vascular bundles in the wild-type plants. In sturdy plants, the number of vascular bundles remains the same, but many of them appear to be expanding and branching out at the tip toward the pith (indicated by the arrows in Fig. 8, A and C) as if it is undergoing the formation of extra vascular bundles. When stained with toluidine blue, xylem and fiber cells become blue due to the presence of lignin in cell walls. The sturdy stem has at least one more layer of blue-staining cells than wild type (Fig. 8). Therefore, the stiffness of sturdy stems could be explained by the presence of additional layers of lignin-containing cells.

**DISCUSSION**

We described the isolation and characterization of a novel dominant Arabidopsis mutant, sturdy, generated by activation T-DNA tagging. The sturdy mutant was identified by its stiff inflorescence stem that supported its vertical growth and prevented lodging. The phenotype also includes thicker leaves, shorter siliques, larger seeds, round flowers, and delayed growth. By screening the genomic library prepared from sturdy genomic DNA using the CaMV-35S enhancer sequence as the probe, we discovered a putative gene of 1,234 bp linked to the CaMV-35S enhancer tetramer. This putative gene, containing a
Figure 4. The nucleotide sequence of the STURDY gene and its flanking regions. The STURDY ORF is shown in uppercase letters and lowercase letters refer to the flanking and intron sequences of the STURDY gene. The CAAT and TATA sequences (positions 21–60) and the putative polyadenylation signal (positions 2,185–2,190) are in boldfaced font. The single-letter abbreviations for amino acids were used and an asterisk denotes a stop codon. The sequences used as primers for reverse transcriptase (RT)-PCR to isolate the STURDY cDNA are underlined.

Huang et al.

Patatin is a major soluble glycoprotein found in potato tubers (Racusen and Foote, 1980) and is encoded by a multigene family (Mignery et al., 1988; Twell and Ooms, 1988). Because of the diversity and complexity of the gene family, most of the molecular characterization of patatin has focused on cloning and expression of different classes of patatin genes (Bevan, et al., 1986; Rosahl et al., 1986; Pikaard et al., 1987; Köster-Toepfer et al., 1989; Rocha-Sosa, et al., 1989). The expression of patatin and patatin-like genes subsequently were detected in other plant or-

single intron, encodes a patatin-like protein that is highly expressed in the sturdy mutant and therefore is likely to be the STURDY gene. Transgenic studies confirmed that the sturdy phenotype resulted from the overexpression of the STURDY gene.
gans (Paiva et al., 1983; Vancanneyt et al., 1989) and other species as well (Vancanneyt et al., 1989; Ganal et al., 1991). Although regarded as a storage protein, patatin has enzymatic activity. It exhibits esterase activity on a broad range of lipid substrates (Rosahl et al., 1987; Andrews et al., 1988; Högen and Willmitzer, 1990). These findings suggest that patatin or patatin-like proteins may possess other biological functions. We currently are investigating the enzymatic activities of the predicted STURDY gene product. Studying STURDY should enhance our understanding of the function(s) of patatin. This stiff inflorescence stem is the first reported phenotype caused by overexpression of a patatin-like protein. With the progression of the Arabidopsis genome sequencing project, many more patatin-like proteins are being discovered in Arabidopsis. Although they may not always share a high degree of homology at the nucleotide level, their protein sequences clearly indicate that they belong to the class of patatin-like proteins. It will be interesting to see whether overexpression of other patatin-like proteins will result in a similar phenotype.

The cross sections show that the inflorescence stem structure organization of the sturdy mutant retains a similar pattern to wild type, whereas the stem mass is significantly increased. It has been reported that the stem cell mass could be altered by varying the endogenous levels of auxins and cytokinins in transgenic plants (Medford et al., 1989; Romano et al., 1991). In addition, a phospholipase A2 involved in signal transduction of a disease-resistant reaction appeared to be a patatin (Senda et al., 1996). Therefore, it could be hypothesized that STURDY plays a role in the signal transduction pathway of hormone responses. It is interesting that mutations in a receptor protein kinase, ERECTA, cause a similar phenotype to sturdy (Torii et al., 1996). It is suggested that ERECTA participates in the coordination of cell growth pattern. We currently are investigating the

Figure 5. The floral phenotype of the transgenic plants overexpressing STURDY. A, The floral phenotype of 10 independent transgenic lines. B, RNA gel-blot analysis. RNA (6 μg) isolated from the leaves of the 10 transgenic lines was hybridized to the STURDY cDNA probe. On the bottom is an ethidium bromide stain of an agarose gel loaded with the RNA (1 μg per lane) used in the gel blot.
relationship between \textit{ERECTA} and \textit{STURDY}. Modifications in the biochemical composition of the \textit{sturdy} mutant alternatively may be responsible for the strengthened inflorescence stem phenotype. Slightly brittle leaves and an increase in the number of lignin-containing cells in stems of the \textit{sturdy} mutant indicate biochemical alternations in cellular components by overexpressing of \textit{STURDY}. Further detailed analysis on the physiology and biochemistry of the \textit{sturdy} mutant is required to determine the cause of the mutant phenotype.

Although most genetic screens are based on mutants originating from loss-of-function, the activation T-DNA-tagging screen provides an alternative approach to identify mutants that are caused by gain-of-function. A related approach, with a complete CaMV-35S promoter oriented outward from a transposable \textit{Ds} element, has also been used to isolate gain-of-function mutations (Wilson et al., 1996; Schaffer et al., 1998; Fridborg et al., 1999). These approaches enhance the possibility of discovering new genes in pathways that have been exhaustively studied by conventional genetic screens, or genes that have either no or lethal mutant phenotypes when inactivated. A particular useful feature of these methods of gene discovery is the potential for direct application of isolated genes. For example, in a genetic screen for an agronomically desirable phenotype, conventional loss-of-function approaches are likely to isolate genes that require inactivation for their application. In contrast, activation T-DNA and transposon tagging identify genes that can be applied by overexpression. Because gene knockout technologies such as antisense, chimeric gene targeting (Hohn and Puchta, 1999) and homologous recombination (Puchta and Hohn, 1996) are still commercially prob-

---

Figure 6. Homology between \textit{STURDY} amino acid sequence and those of \textit{patain} and \textit{patatin-like proteins} from \textit{Arabidopsis} and other plant species. A consensus sequence between homologous proteins was identified and is shown at the top of the alignment. The amino acids in black boxes are those identical to the consensus sequence, and the amino acids in gray boxes are those similar to the consensus sequence. The positions of each sequence used in the alignment are listed on the right. The accession numbers of these sequences are \textit{STURDY}, AL138648; \textit{Arabidopsis} \textit{patain-like protein 1} (AtPLP1), AC004697; \textit{Arabidopsis} \textit{patain-like protein 2} (AtPLP2), AL049655; \textit{Arabidopsis} \textit{patain-like protein 3} (AtPLP3), Z99707; tobacco, U68484; rubber, AJ223038; and potato, A24142.
lematic, overexpression remains the most useful technique for genetic engineering.

The patatin gene family provided a model system for studying developmental and metabolic gene expression in the late 1980s and early 1990s. Since then, research interests in patatin have declined due to the lack of physiological relevance. By using an activation T-DNA-tagging approach, we found that a patatin-like protein, STURDY, is implicated in stem structure. Although many questions are yet to be answered, our findings shed new light on the biological function of patatin and, hopefully, provoke renewed interest in research related to patatin. Meanwhile, we continue to study the strengthened stem phenotype caused by overexpression of STURDY and apply the technology to crop species to enhance lodging resistance.

MATERIALS AND METHODS

Plasmid Construction

The binary vector, pMON29963, used to generate the activation T-DNA-tagging population was constructed similar to methods described by Hayashi et al. (1992). It contains four stacked enhancers each corresponding to the −90 to −342 region of the original CaMV-35S promoter (Fang et al., 1989). This enhancer repeat was inserted into the T-DNA region 15 bp from the right border. Near the left border was the neomycin phosphotransferase II gene (NPTII) driven by the nopaline synthase promoter conferring kanamycin resistance. All other genetic elements used in the binary vector were identical to those described by Ye et al. (1999).

PCR primers, 5′-CTTCTAGAGATGGATCTCAGGG-3′ and 5′-CGGGATCTTAAACGGCCGTCAGGAGG-3′, were designed according to the sequences at the beginning and end of the STURDY reading frame (Fig. 4) with the addition of restriction sites XbaI and BamHI for convenient cloning. These primers were used in a RT-PCR reaction to amplify a 1.1-kb cDNA fragment from RNA extracted from the leaves of sturdy mutant and RNA (20 μg) isolated from leaves (L2), stems (S), flowers (F), and roots (R) of wild type were hybridized to the STURDY cDNA probe. On the bottom is an ethidium bromide stain of an agarose gel loaded with the RNA (1 μg per lane) used in the gel blot.

Plant Material, Growth Conditions, and Transformation

Seeds of Arabidopsis ecotype Columbia (Col-0) were obtained from Lehle Seeds (Tucson, AZ). Plants were grown in potting soil (Scotts, Marysville, OH) in a growth chamber at 24°C with a 16-hr photoperiod (120 μmol m⁻² sec⁻¹). The binary Ti plasmids pMON29963 and pMON42154 were introduced into Arabidopsis by Agrobacterium tumefaciens-mediated transformation. The resulting plasmid, pMON42154, contains the STURDY cDNA under transcriptional control of an enhanced 35S promoter. The −90 to −342 region of the original CaMV-35S promoter (Fang et al., 1989) was quadrupled to produce the enhanced 35S promoter.
Isolation of Genomic DNA and DNA Gel-Blot Analysis

Genomic DNA was isolated from leaves of Arabidopsis plants as previously described (Coleman and Kao, 1992). Genomic DNA (3 μg) was digested with restriction enzymes, separated on a 0.7% (w/v) agarose gel, and transferred to nylon membranes (Roche Molecular Biochemicals). Prehybridization, hybridization, washing, and detection of the membrane were conducted using the non-radioactive DIG system (Roche Molecular Biochemicals) following the manufacturer’s protocols.

Isolation of Total RNA and RNA-Blot Analysis

Total RNA was isolated from leaves, inflorescence stems, roots, and flowers of wild-type and sturdy mutants by TRIzol Reagent from Life Technologies (Gaithersburg, MD) following the manufacturer’s protocols. Total RNA samples (2, 6, or 30 μg) were electrophoresed on 1% (w/v) agarose/formaldehyde gels and transferred to nylon membranes (Roche Molecular Biochemicals). The blots were analyzed by the same DIG system described above following the manufacturer’s protocols for RNA gel-blot analysis.

Genomic Library Construction and Screening

Approximately 30 μg of genomic DNA was isolated from sturdy using the same method described in the DNA gel-blot analysis. The genomic library was constructed by partial digestion with BglII and then using the Lambda FIX II/XhoI Partial Fill-In Vector Kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions. The same non-radioactive DIG system was also used for screening the library. Using the CaMV-35S enhancer as a probe, three independent clones were isolated from about 15,000

Figure 8. Cross sections of inflorescence stems of sturdy and wild-type plants. A and B, sturdy and wild type, respectively. C and D, Close-up sturdy and wild type. Arrows point to the branching vascular bundles. Bars = 100 μm. co, Cortex; f, interfascicular fibers; ph, phloem; pi, pith; x, xylem.
Sequence Analysis

The genomic and cDNA clones were sequenced using in-house facilities (ABI Prism 377, Perkin Elmer, Foster, CA). The STURDY gene, including its promoter region containing CAAT and TATA boxes, coding region, and poly-A signal, was identified by GENSCAN1.0, a software written by Chris Burge (Stanford University, CA) modeling Arabidopsis. The STURDY coding region and its translated protein sequence were used to search the GenBank database by applying BLASTN and BLASTP 2.0.8 (Altschul et al., 1997). Homology alignment of various patatin and patatin-like protein sequences was done by the MegAlign software (DNASTAR Inc.) using the clustal method with PAM250 residue weight table. Conserved amino acid residues were defined by no larger than three distance units.

Microscopy

Tissue samples were collected from first and second internodes of wild-type and sturdy plants (14–15 cm) in the growth chamber. Tissue (approximately 5-mm slices) was vacuum infiltrated in a fixative solution containing 4% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde in 100 mM phosphate buffer (pH 7.0). Tissue samples were then placed in 30% (v/v) ethanol. Further processing was done on a Tissue-Tek VIP automated processor (Sakura Finetek, Torrance, CA) through a series of graded ethanol wash steps: 50%, 75%, and 85% (v/v) ethanol at 37°C for an hour each; 95% and 100% (v/v) ethanol at 40°C and 45°C for 2 h each; and finally with paraffin at 58°C for 10 h with four solution changes. Sections 6 μm thin were cut from embedded tissue using a microtome (Reichert Jung 2030 model, Leica Co, Deerfield, IL) and mounted on Probeon Plus slides (Fisher Scientific, Pittsburgh), which were then dried overnight at 37°C.

For toluidine blue O stain, slides were deparaffinized using a series of xylene and ethanol washes (5 min each) and then rehydrated. Slides were stained for 5 min in 1% (w/v) toluidine blue O (Fisher Scientific) in 1% (w/v) Borax (Sigma) and then rinsed in water for 2 min and dehydrated quickly through a series of ethanol washes (95%, 95%, 100%, and 100% [v/v] for 1 min each). These were subsequently placed into two changes of xylene and mounted with Permount (Fisher Scientific). Slides were observed under a microscope (BH-2; Olympus, Melville, NY).

Acknowledgments

We thank Debbie Stone, Wendi Zumalt, and Stacy Minor for plant transformation; Mark Woerner and Richard Ornber for their expertise and suggestions in microscopy; and Claire Cajacob, Charles Romano, and Tedd Elich for their valuable comments on the manuscript. Received June 15, 2000; returned for revision August 16, 2000; accepted September 26, 2000.

Literature Cited


