FRAGILE FIBER3, an Arabidopsis Gene Encoding a Type II Inositol Polyphosphate 5-Phosphatase, Is Required for Secondary Wall Synthesis and Actin Organization in Fiber Cells

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Type II inositol polyphosphate 5-phosphatases (5PTases) in yeast and animals have been known to regulate the level of phosphoinositides and thereby influence various cellular activities, such as vesicle trafficking and actin organization. In plants, little is known about the phosphatases involved in hydrolysis of phosphoinositides, and roles of type II 5PTases in plant cellular functions have not yet been characterized. In this study, we demonstrate that the FRAGILE FIBER3 (FRA3) gene of Arabidopsis thaliana, which encodes a type II 5PTase, plays an essential role in the secondary wall synthesis in fiber cells and xylem vessels. The fra3 mutations caused a dramatic reduction in secondary wall thickness and a concomitant decrease in stem strength. These phenotypes were associated with an alteration in actin organization in fiber cells. Consistent with the defective fiber and vessel phenotypes, the FRA3 gene was found to be highly expressed in fiber cells and vascular tissues in stems. The FRA3 protein is composed of two domains, an N-terminal localized WD-repeat domain and a C-terminal localized 5PTase catalytic domain. In vitro activity assay demonstrated that recombinant FRA3 exhibited phosphatase activity toward PtdIns(4,5)P2, PtdIns(3,4,5)P3, and Ins(1,4,5)P3, with the highest substrate affinity toward PtdIns(4,5)P2. The fra3 missense mutation, which caused an amino acid substitution in the conserved motif II of the 5PTase catalytic domain, completely abolished the FRA3 phosphatase activity. Moreover, the endogenous levels of PtdIns(4,5)P2 and Ins(1,4,5)P3 were found to be elevated in fra3 stems. Together, our findings suggest that the FRA3 type II 5PTase is involved in phosphoinositide metabolism and influences secondary wall synthesis and actin organization.

INTRODUCTION

Phosphoinositides are phospholipids present ubiquitously in all eukaryotic cell membranes. They are generated by sequential phosphorylation of phosphatidylinositol by specific kinases. Studies in yeast and animals have documented that phosphoinositides are important signaling molecules that regulate a variety of cellular functions, including cell differentiation and proliferation, cell death, vesicle trafficking, and actin cytoskeleton organization (Takenawa and Itoh, 2001). One of the best-studied phosphoinositides is phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2). PtdIns(4,5)P2 not only serves as a precursor for the production of signaling molecules diacylglycerol and inositol 1,4,5-triphosphate [Ins(1,4,5)P3], but also by itself regulates vesicle trafficking and actin organization by binding to and modulating the activity of actin-associated proteins, including profilin, α-actinin, vinculin, and gelsolin (Takenawa and Itoh, 2001).

Termination of phosphoinositide signaling is mediated by inositol polyphosphate phosphatases (PTases) that hydrolyze the 3-, 4-, or 5-position phosphate from the inositol ring of phosphoinositides. In plants, all forms of phosphoinositides except PtdIns(3,4,5)P3 have been detected (Mueller-Roeber and Pical, 2002), and PtdIns(4,5)P2 has been shown to be important for the polar growth of pollen tubes (Kost et al., 1999). However, little is known about the phosphatases that remove phosphate from phosphoinositides in plants (Mueller-Roeber and Pical, 2002).

PTases are traditionally classified into four groups based on the position of phosphate they hydrolyze, 1-, 3-, 4-, or 5-phosphatases. The best characterized group is inositol polyphosphate 5-phosphatases (5PTases), which is further divided into four types according to their distinct substrate specificities for inositol phosphates and/or phosphoinositides (Majerus et al., 1999). Type I 5PTases hydrolyze only water-soluble substrates Ins(1,4,5)P3 and Ins(1,3,4,5)P4. Type II 5PTases hydrolyze not only water-soluble Ins(1,4,5)P3 and Ins(1,3,4,5)P4 but also phosphoinositides PtdIns(4,5)P2 and PtdIns(3,4,5)P3.

In plants, several genes encoding inositol polyphosphate 1- or 5-phosphatases and a group of putative SAC domain phosphoinositide phosphatases have been identified (Berdy et al., 2001; Sanchez and Chua, 2001; Xiong et al., 2001; Despres et al., 2003; Zhong and Ye, 2003). It has been shown that mutation of a bifunctional enzyme with 3′(2′),5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities in the...
Figure 1. Alterations of Stem Strength and Fiber Secondary Wall Thickness by the fra3 Mutation.
Arabidopsis thaliana fiery1 mutant causes altered sensitivities to abscisic acid (ABA) induction (Quintero et al., 1996; Xiong et al., 2001). Eleven putative type I 5PTase genes have been identified in the Arabidopsis genome (Berdy et al., 2001), and two of them, At5PTase1 and AtIP5P2, exhibit typical type I 5PTase substrate specificity [i.e., they hydrolyze only water-soluble inositol phosphates Ins(1,4,5)P3 and Ins(1,3,4,5)P4] (Berdy et al., 2001; Sanchez and Chua, 2001). Recently, it was shown that another 5PTase, AtPTase11, displays phosphatase activity toward phospholipids (Ercetin and Gillaspy, 2004). Overexpression of At5PTase1 or AtIP5P2 in transgenic Arabidopsis plants has been demonstrated to reduce the Ins(1,4,5)P3 level and alter the ABA-induced plant responses (Sanchez and Chua, 2001; Burnette et al., 2003). Mutation of the COTYLEDON VASCULAR PATTERN2 (CVP2) gene, which encodes a putative type I 5PTase, has been shown to alter leaf venation pattern, Ins(1,4,5)P3 level, and ABA sensitivity (Carlund and Nelson, 2004). In addition, overexpression of the human type I 5PTase in tobacco (Nicotiana tabacum) causes a significant reduction in the Ins(1,4,5)P3 level but no apparent effects on cell growth and morphology (Perera et al., 2002).

Although several type I 5PTases have been studied in plants, the type II 5PTases have not been characterized except for a report of the presence of four putative type II 5PTase genes in the Arabidopsis genome (Berdy et al., 2001). Mutations of several human and yeast type II 5PTases have been shown to cause dramatic effects on development or stress response. Mutations of human OCR1L have been linked to oculocerebrorenal syndrome of Lowe, a rare X-linked developmental disorder. In Lowe syndrome, it was found that the 5PTase activity of OCR1L is abolished, and concomitantly there is an increase in the level of PttdIns(4,5)P2, which is thought to be the cause of the syndrome (Zhang et al., 1998). Mutation of the type II 5PTase domain in the yeast Inp51p has been shown to cause an elevation of PttdIns(4,5)P2 and confer cold tolerance (Stolz et al., 1998). However, the biochemical activities and cellular functions of type II 5PTases in plants remain unknown.

In this study, we demonstrate that the FRAGILE FIBER3 (FRA3) gene, which is required for the secondary cell wall formation and mechanical strength of fiber cells, encodes a type II 5PTase. We show that the fra3 mutation dramatically reduces secondary wall thickening, and this alteration is associated with abnormal actin organization in fiber cells. The FRA3 protein consists of an N-terminal localized WD-repeat domain and a C-terminal localized 5PTase catalytic domain. We demonstrate that FRA3 exhibits type II 5PTase activity in vitro and shows the highest substrate affinity toward PttdIns(4,5)P2. Moreover, we have found that the endogenous levels of PttdIns(4,5)2 and Ins(1,4,5)P3 are elevated in fra3 stems. Together, our findings provide genetic evidence suggesting that the FRA3 type II 5PTase plays an essential role in cell wall synthesis and actin organization.

RESULTS

The fra3 Mutations Cause Defects in Secondary Wall Thickening in Fiber Cells and Xylem Vessels

Arabidopsis inflorescence stems develop three to four layers of fiber cells between vascular bundles, and the mechanical strength of mature stems is largely attributable to these interfascicular fibers (Zhong et al., 1997, 2001). To investigate the molecular mechanisms underlying fiber cell formation, we isolated two allelic fra3 mutants defective in stem strength. Quantitative analysis showed that the breaking strength of fra3 and fra3-2 stems was reduced by approximately threefold compared with that of the wild type (Figure 1A), indicating that the fra3 mutations cause a dramatic reduction in the mechanical strength of stems.

Because interfascicular fibers contribute largely to the mechanical strength of stems, we investigated the fiber morphology in the fra3 mutants. Both fra3 and fra3-2 mutants exhibited the same cellular phenotypes; therefore, only the data from the fra3 mutant are presented in this study. Cross-section examinations revealed that although the interfascicular fiber cells in the wild type had relatively uniform and thick secondary walls (Figure 1D), those in the fra3 mutant showed an apparent reduction in wall thickness (Figure 1E). It was noted that the wall thickness appeared not to be uniform among fra3 fiber cells, and some of the fiber cells with extremely thin walls looked larger in diameter than wild-type ones (Figure 1E). Transmission electron microscopy further confirmed that the fra3 mutation caused a dramatic reduction in the wall thickness among fiber cells (Figures 2A to 2D, Table 1). It was also observed that the wall thickness of xylem vessels was slightly reduced in the fra3 mutant compared with the wild type (Figures 1B, 1C, 2E, and 2F, Table 1). Quantitative analysis of cell wall composition demonstrated that the fra3 mutation resulted in a 37% reduction in glucose but no major effects on other sugar content (Table 2). This suggests that the fra3 mutation mainly affects cellulose content, which is consistent with the dramatic decrease in secondary wall thickness in fiber cells. Longitudinal sections showed no apparent differences in the length of fibers and pith cells between the wild type and the fra3 mutant (Figures 1F and 1G). No anatomical changes were observed in other cell types in the fra3 mutant. These results

Figure 1. (continued).

Inflorescence stems of 8-week-old plants were used for breaking force measurement, and their bottom internodes were examined for fiber cell morphology. co, cortex; en, endodermis; ep, epidermis; if, interfascicular fiber; ph, phloem; pi, pith; x, xylem. Bars = 60 μm in (B) to (E) and 190 μm in (F) and (G).

(A) Breaking force measurement showing that the force to break stems apart was about three times lower in fra3 and fra3-2 than in the wild type.

(B) and (C) Cross sections of stems showing vascular bundles in the wild type (B) and fra3 (C).

(D) Cross section of an interfascicular region of the wild type showing fiber cells with thick walls.

(E) Cross section of an interfascicular region of fra3 showing fiber cells with thin walls. Note that some fiber cells (arrows) had extremely thinner walls.

(F) and (G) Longitudinal sections of interfascicular regions showing long fiber cells in both the wild type (F) and fra3 (G).
indicate that the stem strength defect caused by the fra3 mutation is a result of reduced fiber cell wall thickness.

Except for the defective secondary wall thickening and stem strength phenotypes, the fra3 mutant did not exhibit any abnormality in plant growth and development. Both dark- and light-grown fra3 seedlings were indistinguishable from the wild type. Mature fra3 plants had similar height as the wild type and underwent reproductive growth as normally as the wild type (data not shown).

The fra3 Mutation Alters Actin Organization in Fiber Cells

Because cell wall synthesis is influenced by cytoskeletons, we next investigated whether the fra3 mutation caused any defects
in cytoskeleton organization (Figure 3). Immunolabeling experiments showed that the organization of cortical microtubules in developing fiber cells did not exhibit any noticeable differences between the wild type and fra3 (Figures 3J and 3K), indicating that the fra3 mutation did not affect cortical microtubule organization. Examination of the organization of filamentous actin (F-actin) showed that although the F-actin in developing wild-type fiber cells formed a fine network (Figures 3A and 3B), the F-actin in developing fra3 fiber cells was apparently disrupted (Figures 3C and 3D). The F-actin cables in fra3 fiber cells appeared to lose their fine network pattern and became much thicker compared with the wild type (Figures 3E to 3H). Some of the thick F-actin cables appeared to be caused by bundling, which is a reminiscence of F-actin bundling caused by the actin drug latrunculin B (Mathur et al., 2003). Quantitative measurement of the width of actin cables based on their fluorescent signals showed that compared with the wild type, the width of F-actin cables in the tip and middle regions of fra3 fiber cells increased by 72 and 43%, respectively (Figure 3I). Examination of the cytoskeleton in elongating pith cells of the fra3 mutant showed no apparent alterations in the organization of F-actin (Figures 3L and 3M) and cortical microtubules (data not shown) compared with the wild type. These results demonstrated that the fra3 mutation caused an alteration in F-actin organization in fiber cells, which is consistent with the defective fiber phenotype observed in the fra3 mutant.

Map-Based Cloning of the FRA3 Gene

To investigate the molecular nature that was responsible for the defective fiber phenotype, we pursued the map-based cloning of the FRA3 gene. Using codominant amplified polymorphic sequences (CAPS) markers, we mapped the fra3 locus near the g11447 marker on chromosome 1. Fine mapping with additional CAPS markers revealed that the fra3 locus was located within a 27-kb region in the BAC clone F5I14 (Figure 4A). Based on the gene annotations of F5I14 from the Arabidopsis genome database, this 27-kb region contains six genes. Sequencing of these genes from the fra3 and fra3-2 mutants revealed point mutations in one of these genes, F5I14.11 (At1g65580) (Figure 4A). The fra3 mutant carries a C-to-T mutation, and the fra3-2 mutant contains a G-to-A mutation. The fra3 mutation causes loss of a BstNI site in the gene, which was confirmed by digestion of PCR-amplified DNA fragment with BstNI (data not shown). Transformation of the wild-type F5I14.11 gene into the fra3 and fra3-2 mutants completely restored the fiber wall thickness, F-actin organization, and stem strength to the wild-type level (Figures 4C to 4F). Together, these results unequivocally demonstrated that the point mutations in the F5I14.11 gene are responsible for the fra3 mutant phenotypes; therefore, the F5I14.11 gene represents the FRA3 gene.

Sequence Analysis of the FRA3 Gene and Its Encoded Protein

To perform molecular characterization of the FRA3 gene, we isolated the full-length FRA3 cDNA from an Arabidopsis stem cDNA library. Comparison of the sequences of FRA3 gene and its cDNA revealed that the FRA3 gene consists of 11 exons and 10 introns with 6341 bp in length from the start codon to the stop codon (Figure 4A). The longest open reading frame in the FRA3 cDNA is 3306 bp long, and it encodes a protein of 1101 amino acids with a predicted molecular mass of 121.6 kD and a predicted pI of 5.9. The fra3 mutation occurred in the ninth exon and fra3-2 in the third exon of the FRA3 gene (Figure 4A). Comparison of the wild-type and fra3 cDNA and their deduced amino acid sequences showed that the fra3 missense mutation changed an Ala codon GCC to a Val codon GTC (Figure 4B). The fra3-2 mutation changed a Trp codon TGG to a stop codon TGA (Figure 4B), which results in a truncated protein with deletion of 681 amino acid residues.

A search of the GenBank database showed that the deduced FRA3 protein contains a domain that shares high sequence similarity to a group of type II 5PTases from yeast and animals. All known 5PTases contain a conserved catalytic domain that is ~350 amino acid residues in length. Within this domain, two highly conserved motifs have been shown to be important for the 5-phosphatase activity (Majerus et al., 1999). The putative 5-phosphatase catalytic domain of FRA3 protein shares 28% identity and 48% similarity with the catalytic domain of human type II 5PTase (Figure 5A). In particular, the two conserved 5PTase catalytic motifs (GDNXY/FR and PA/SWC/TDR) are completely conserved in the FRA3 5PTase domain (Figure 5B). It was noted that the fra3 missense mutation occurred at the Ala residue in motif II (Figure 5B), suggesting that the Ala residue is important for FRA3 function. The fra3-2 non-sense mutation caused deletion of the whole 5PTase domain (Figure 6C), indicating that fra3-2 is devoid of the 5PTase function.

### Table 1. Wall Thickness of Fibers and Vessels in the Stems of Wild Type and fra3 Mutant (μm)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fiber Cells</th>
<th>Vessels</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
<td>3.54 ± 0.25</td>
<td>2.23 ± 0.18</td>
</tr>
<tr>
<td>fra3</td>
<td>2.11 ± 0.45</td>
<td>1.64 ± 0.21</td>
</tr>
</tbody>
</table>

The wall thickness was determined from transmission electron microscope micrographs of fibers and vessels. Data are means ± SE from 25 cells.

### Table 2. Cell Wall Sugar Composition of the Stems of the Wild Type and fra3 Mutant (mg/g Dry Cell Wall)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Arabinose</th>
<th>Rhamnose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>277 ± 0.7</td>
<td>144 ± 4.2</td>
<td>17.4 ± 0</td>
<td>15.7 ± 0.4</td>
<td>13.5 ± 0.5</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td>fra3</td>
<td>174 ± 16</td>
<td>136 ± 4.9</td>
<td>17.8 ± 0.7</td>
<td>13.2 ± 0.0</td>
<td>12.1 ± 0.6</td>
<td>3.9 ± 0.2</td>
</tr>
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Cell wall materials were prepared from mature stems of 10-week-old plants. Data are means ± SE of two independent assays.
Figure 3. Alteration of F-Actin Organization in Developing Fiber Cells of the fra3 Mutant.

Longitudinal sections of stems were immunolabeled for F-actin and microtubules with monoclonal antibodies against actin or α-tubulin and fluorescein isothiocyanate–conjugated secondary antibodies. Fluorescence-labeled F-actin and cortical microtubules were visualized under a confocal microscope. Bars = 18 μm in (A) to (D), 6 μm in (E) to (H), 11 μm in (J) and (K), and 12 μm in (L) and (M).

(A) and (B) The tip (A) and middle (B) regions of a wild-type fiber cell showing fine F-actin network.

(C) and (D) The tip (C) and middle (D) regions of a fra3 fiber cell showing thick F-actin cables.

(E) and (F) High magnification of areas from (A) and (B), respectively, showing fine F-actin cables.

(G) and (H) High magnification of areas from (C) and (D), respectively, showing thick F-actin cables.

(I) Quantitative measurement of the width of fluorescence-labeled F-actin cables in the tip and middle regions of fiber cells.

(J) and (K) Fiber cells showing cortical microtubules aligned in parallel in both the wild type (J) and fra3 (K).

(L) and (M) Pith cells showing the fine F-actin network in both the wild type (L) and fra3 (M).
In addition to the putative 5PTase domain located at the C-terminal region, FRA3 contains an N-terminal region with ~500 amino acids in length. A search of the GenBank conserved domain database revealed the presence of a WD-repeat domain in the FRA3 N-terminal region (Figure 6A). The putative FRA3 WD-repeat domain shows 23% identity and 35% similarity to the conserved WD-repeat domain. The WD repeat is a structurally conserved motif that comprises a 44- to 60-amino acid sequence with a GH dipeptide 11 to 24 residues from its N terminus and a WD dipeptide at the C terminus. It has been shown that neither the GH dipeptide nor the WD dipeptide is completely conserved among WD repeats. Instead, the WD repeat is a conserved structural motif that forms a four-stranded antiparallel β sheet (Smith et al., 1999). WD-repeat proteins are defined as having at least four or more WD repeats that are required to form a β propeller structure (Smith et al., 1999). Further analysis of the FRA3 amino acid sequence using a program that identifies protein repeats revealed that FRA3 contains six WD repeats (Figure 6B). Each WD repeat in FRA3 was predicted to form four β strands by the secondary structure prediction program. Because FRA3 contains more than four WD repeats that are required for formation of a β propeller structure, FRA3 is by definition also a WD-repeat protein. Together, these results show that FRA3 is composed of two conserved domains, a WD-repeat domain and a 5PTase domain (Figure 6C).

Expression Pattern of the FRA3 Gene

To investigate the expression pattern of the FRA3 gene, we first used the semiquantitative RT-PCR method to detect its expression in various organs. The FRA3 gene was found to be expressed at similar levels in seedlings, stems, roots, and flowers, but a much lower expression level was detected in mature leaves (Figure 7A). This indicates that the FRA3 gene is expressed...
ubiquitously in various Arabidopsis organs. The fra3 and fra3-2 point mutations did not affect the accumulation of FRA3 mRNA (Figure 7B).

We next used the β-glucuronidase (GUS) reporter gene to further study the FRA3 gene expression pattern. A 2-kb 5' upstream sequence and the entire exon and intron regions of the FRA3 gene were ligated in frame with the GUS reporter gene in the binary vector pBI101. The construct was introduced into both wild-type and fra3 plants. Because the construct completely rescued the fra3 mutant phenotypes (data not shown), it indicates that the region of the FRA3 gene used in the GUS reporter construct contains all elements responsible for the expression of the endogenous FRA3 gene. Examination of GUS activity in the transgenic plants showed GUS expression in young roots (Figure 8A), cotyledons (Figure 8B), leaves (Figure 8C), and flowers (Figure 8D). In rapidly elongating inflorescence stems, the GUS signals were present in nearly all tissues (Figure 8E). However, the signals gradually became more concentrated in vascular bundles and interfascicular fiber cells when the internodes were near cessation of elongation (Figure 8F). In nonelongating internodes in which fiber cells undergo massive secondary wall deposition (Ye et al., 2002), the GUS staining was predominantly seen in interfascicular fibers and vascular bundles (Figures 8G and 8H). The predominant expression of the FRA3 gene in developing fibers and vascular cells is consistent with the defective fiber and vessel phenotypes seen in the fra3 mutant.

FRA3 Possesses Phosphatase Activity toward PtdIns(4,5)P2, PtdIns(3,4,5)P3, and Ins(1,4,5)P3

The finding that FRA3 contains a putative type II 5PTase domain prompted us to investigate its phosphatase activity. We expressed His-tagged full-length FRA3 protein in yeast and purified the recombinant FRA3 protein on a nickel-nitrilotriacetic acid agarose (Ni-NTA) column (Figure 9A). The purified protein was assayed for its phosphatase activity using various phospholipids and water-soluble inositol phosphates as substrates. It was found that FRA3 hydrolyzed phosphate from both PtdIns(4,5)P2 and PtdIns(3,4,5)P3 but not from other phospholipids, including PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P2, and PtdIns(3,5)P2 (Figure 9B). FRA3 was also capable of hydrolyzing phosphate from Ins(1,4,5)P3 but not Ins(1,3,4,5)P4. The fra3 mutant protein, which contains a missense mutation in the catalytic motif II, did not show any detectable phosphatase activity.
activity toward any substrates, indicating that the mutation completely abolishes its enzymatic activity. These results demonstrate that FRA3 is indeed a 5PTase. Because it hydrolyzes both phospholipids and water-soluble inositol phosphate, FRA3 belongs to the type II 5PTase group.

FRA3 Exhibits Differential Magnesium Sensitivity toward Different Substrates

Because the enzymatic activity of 5PTases in yeast and animals requires divalent cations, such as Mg^{2+} (Majerus et al., 1999), we investigated whether FRA3 exhibited similar biochemical properties as its yeast and animal counterparts. It was found that in the presence of the divalent cation chelator EDTA, FRA3 did not

Figure 6. Sequence Analysis of the WD Repeats of FRA3.

(A) Sequence alignment of the N-terminal region of FRA3 with the conserved WD-repeat domain (WD40 domain, cd00200) from the GenBank conserved domain database. Gaps (marked with dashes) were introduced to maximize the sequence alignment. Identical and similar amino acid residues are shaded with black and gray, respectively. (B) Alignment of the six WD repeats in FRA3. The WD repeats were predicted using a program that identifies protein repeats. The shaded sequences shown in each repeat represent three of the four putative β strands (a, b, and c) as predicted with the secondary structure prediction program. (C) Diagram of the FRA3 protein showing the organization of the WD repeats and the 5-phosphatase domain. The numbered bars represent the six WD repeats. The hatched region represents the 5-phosphatase domain in which the two conserved motifs are marked with black bars. The fra3 and fra3-2 mutation sites are marked.

Figure 7. Gene Expression Analysis of FRA3 by Semiquantitative RT-PCR.

(A) Expression of the FRA3 gene in various Arabidopsis organs. The expression level of a ubiquitin gene was used as an internal control. The seedlings used were 2 weeks old. Mature leaves were from 6-week-old plants. Flowers and mature roots were from 8-week-old plants. Stems I and II were from 4- and 8-week-old plants, respectively. (B) Expression of the FRA3 gene in seedlings of the wild type, fra3 mutants, and a representative fra3 complementation line (fra3 compl.). (C) Overexpression of wild-type FRA3 cDNA (35S::FRA3) or mutant fra3 cDNA (35S::fra3) in wild-type seedlings under the control of the 35S promoter of Cauliflower mosaic virus. Shown are four representative lines of 35S::FRA3 and three representative lines of 35S::fra3.
The FRA3 gene, including a 2-kb 5’ upstream sequence and the entire exon and intron region, was ligated in frame with the GUS reporter gene and transformed into wild-type Arabidopsis. Various organs from the transgenic plants were stained for GUS activity. fp, fiber precursor; if, interfascicular fiber; pi, pith; x, xylem. Bars = 165 μm in (E) to (G) and 330 μm in (H).

(A) and (B) Primary root (A) and cotyledon (B) from 3-d-old seedlings showing the GUS staining in all tissues.

(C) Leaf from 10-d-old seedlings showing the GUS staining in mesophyll cells and veins.

(D) Flower showing the GUS staining in various floral parts.

(E) Section from young elongating internodes showing GUS staining in all tissues.

(F) Section from internodes at the stage near the cessation of elongation showing intensive GUS staining in vascular bundles and interfascicular regions.

(G) Section from nonelongating internodes showing the GUS staining predominantly in vascular bundles and interfascicular fibers.

(H) High magnification of (G) showing fiber cells with thick secondary walls and strong GUS staining.
indicating that FRA3 requires Mg\(^2+\) for the enzymatic activity (Figure 10A). It was interesting to find that the FRA3 phosphatase activity toward Ins(1,4,5)P\(_3\) is more sensitive to pH (Figure 10B). The optimal temperature for the FRA3 activity toward PtdIns(4,5)P\(_2\) and PtdIns(3,4,5)P\(_3\) was between 22 and 37°C, whereas that toward Ins(1,4,5)P\(_3\) was around 22°C (Figure 10C). These results indicate that the FRA3 phosphatase activity toward Ins(1,4,5)P\(_3\) is more sensitive to pH and temperature compared with the phospholipid substrates.

**FRA3 Exhibits the Highest Substrate Affinity toward PtdIns(4,5)P\(_2\)**

To test the substrate affinity of FRA3, we examined the kinetic properties of FRA3. The FRA3 phosphatase activity was assayed using various concentrations of PtdIns(4,5)P\(_2\), PtdIns(3,4,5)P\(_3\), and Ins(1,4,5)P\(_3\). The \(K_m\) and \(V_{max}\) values were calculated by the Lineweaver-Burk analysis. It was found that the \(K_m\) values of FRA3 phosphatase activity toward PtdIns(4,5)P\(_2\), PtdIns(3,4,5)P\(_3\), and Ins(1,4,5)P\(_3\) were 63, 299, and 1040 \(\mu M\), respectively (Figures 11A to 11C). The \(K_m\) values of FRA3 were comparable with those of human type II 5PTase [180 \(\mu M\) for PtdIns(4,5)P\(_2\) and 1150 \(\mu M\) for Ins(1,4,5)P\(_3\)] (Ijuin et al., 2000). The kinetic analysis demonstrates that FRA3 has a much higher substrate affinity toward PtdIns(4,5)P\(_2\) than PtdIns(3,4,5)P\(_3\) and Ins(1,4,5)P\(_3\), suggesting that FRA3 might preferentially hydrolyze PtdIns(4,5)P\(_2\) in vivo.

**The fra3 Mutation Causes an Elevation in the Endogenous Levels of PtdIns(4,5)P\(_2\) and Ins(1,4,5)P\(_3\)**

To investigate whether abolishment of the phosphatase activity by the fra3 mutation caused any accumulation of its substrates in vivo, we measured the amount of endogenous Ins(1,4,5)P\(_3\) and PtdIns(4,5)P\(_2\) in wild-type and fra3 plants. In fra3 seedlings, although the level of Ins(1,4,5)P\(_3\) was not altered (Figure 12A), the level of PtdIns(4,5)P\(_2\) slightly increased compared with the wild type (Figure 12B). In fra3 stems, the levels of both Ins(1,4,5)P\(_3\) (Figure 12C) and PtdIns(4,5)P\(_2\) (Figures 12D) were elevated compared with the wild type. These results demonstrate that the fra3 mutations cause an accumulation of PtdIns(4,5)P\(_2\) and Ins(1,4,5)P\(_3\) in the inflorescence stems, which is consistent with the defective fiber and vessel phenotypes in the fra3 mutant.

It has been shown previously that the sensitivity of seed germination to ABA treatment could be altered by a change in Ins(1,4,5)P\(_3\) level (Sanchez and Chua, 2001; Burnette et al., 2003; Carland and Nelson, 2004). Comparison of the seed germination of fra3 with that of the wild-type revealed no changes in the sensitivity to ABA inhibition (data not shown), which was in agreement with the unaltered level of Ins(1,4,5)P\(_3\) in seedlings.

**Overexpression of the FRA3 5PTase Does Not Alter Plant Growth and Development**

To study whether overexpression of the FRA3 5PTase affects plant growth and development, we introduced the wild-type...
cDNA driven by the 35S promoter of Cauliflower mosaic virus into wild-type plants. A total of 72 transgenic plants were obtained, and none of them showed any abnormality in plant growth and development. RT-PCR analysis confirmed that 25 of the transgenic lines accumulated a much higher level of FRA3 mRNA than the wild type (four representatives are shown in Figure 7C). It is worth noting that the mutant fra3 cDNA was overexpressed as a control (Figure 7C) in the overexpression study, and no abnormality in plant growth and development was observed. It has been reported that overexpression of several type I 5PTases did not cause any altered phenotypes in normal plant growth and development (Sanchez and Chua, 2001; Perera et al., 2002; Burnette et al., 2003; Carland and Nelson, 2004).

DISCUSSION

Type II 5PTases have been known in yeast and animals to regulate the level of phosphoinositides and thereby influence various cellular activities such as actin cytoskeleton and vesicle trafficking (Takenawa and Itoh, 2001). In plants, several genes encoding putative 5PTases have been identified, and overexpression of two type I 5PTase genes has been shown to affect ABA sensitivity (Sanchez and Chua, 2001; Burnette et al., 2003). However, the putative type II 5PTase genes have not been characterized, and their roles in plant cellular functions are not known. Our finding that FRA3 encodes a type II 5PTase that is essential for actin organization and secondary wall synthesis in fiber cells provides a functional study of a type II 5PTase in plants.

FRA3 Is a Type II 5PTase

Two lines of evidence demonstrate that the FRA3 gene encodes a type II 5PTase. First, FRA3 possesses a domain that shows the highest sequence similarity to the catalytic domains of type II 5PTases from yeast and animals. The two conserved motifs that are known to be essential for the 5-phosphatase activity are completely conserved in FRA3. The importance of these two catalytic motifs was further evidenced in the fra3 mutant, in which a missense mutation of the Ala residue in motif II completely abolishes the FRA3 phosphatase activity. The Ala/Ser residue in motif II is conserved in all known 5PTases (Majerus et al., 1999). Our finding that the fra3 mutation completely abolishes its phosphatase activity provides biochemical evidence for the functional importance of the Ala/Ser residue in motif II of 5PTases. Secondly, recombinant FRA3 protein is able to hydrolyze phosphate from both phospholipids [PtdIns(4,5)P2 and PtdIns(3,4,5)P3] and water-soluble inositol polyphosphate [Ins(1,4,5)P3], which is a feature of type II 5PTases from yeast and animals (Majerus et al., 1999). The available evidence

Various concentrations of Mg^{2+} were included in the assay buffer. The lowest Mg^{2+} used for the activity assay was 0.1 mM as shown in the figure. (B) Effects of pH on the FRA3 phosphatase activity toward its substrates. (C) Effects of temperature on the FRA3 phosphatase activity toward its substrates.
suggests that the FRA3 type II 5PTase shares both sequence and catalytic activity conservations with those from yeast and animals.

It is of interest to find that in addition to fibers and vascular tissues, the FRA3 gene is also expressed in other tissues. However, the defects caused by the fra3 mutations are only seen in fiber cells and xylem vessels. This fact suggests that other FRA3-like 5PTases might compensate for the loss of FRA3 in other cell types. The Arabidopsis genome contains three additional genes that encode proteins with high sequence similarity to FRA3. One of these FRA3-like proteins, At5PTase14, exhibits the same phosphatase activity as FRA3 [i.e., it shows activity toward PtdIns(4,5)P2, PtdIns(3,4,5)P3, and Ins(1,4,5)P3] (Zhong and Ye, 2004). In addition, the lack of alteration of Ins(1,4,5)P3 level in the fra3 seedlings might be because of compensation by type I 5PTases that are known to regulate the Ins(1,4,5)P3 level (Sanchez and Chua, 2001; Burnett et al., 2003; Carland and Nelson, 2004).

It is intriguing to note that FRA3 is capable of hydrolyzing PtdIns(3,4,5)P3, although PtdIns(3,4,5)P3 has not yet been detected in plants. A putative Tyr phosphatase from Arabidopsis has also been shown to be able to hydrolyze PtdIns(3,4,5)P3 (Gupta et al., 2002). Because of the lack of evidence for the existence of PtdIns(3,4,5)P3 in plants, the functional importance of FRA3 phosphatase toward PtdIns(3,4,5)P3 is currently unknown.

**FRA3 Contains a WD-Repeat Domain**

In addition to the phosphatase catalytic domain, some type II 5PTases in yeast and animals possess additional modules, such as SAC domain, Rho GTPase activating protein domain, and Pro-rich domain (Whisstock et al., 2002). The SAC domain in synaptojanins has been shown to hydrolyze 3- or 4-position phosphate from PtdIns(3)P, PtdIns(4)P, or PtdIns(3,5)P2, and together with the activity of the type II 5PTase domain, it has been thought to be able to completely terminate the phosphoinositide signaling molecules (Hughes et al., 2000). The Rho GTPase activating protein domain in OCRL1 has been demonstrated to interact with Rac GTPase, which was proposed to play a role in localizing OCRL1 to the trans-Golgi network (Faucherre et al., 2003).

It is extremely interesting to find that FRA3 contains a WD-repeat domain, which is not found in any known 5PTases from yeast and animals. WD-repeat proteins have been found in all eukaryotes, and some of them have been shown to be involved in many important biological processes, including signal

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**Figure 11.** Substrate Affinity of the FRA3 Phosphatase.

The kinetic activity of FRA3 phosphatase was assayed in the presence of various concentrations of substrates. The results were analyzed by Lineweaver-Burk plots to determine the $K_m$ and $V_{max}$ values.

(A) The kinetic property of FRA3 toward PtdIns(4,5)P2. PtdIns(4,5)P2 (6 to 400 μM) was incubated with 0.25 μg of recombinant FRA3 protein.

(B) The kinetic property of FRA3 toward PtdIns(3,4,5)P3. PtdIns(3,4,5)P3 (6 to 400 μM) was incubated with 0.5 μg of recombinant FRA3 protein.

(C) The kinetic property of FRA3 toward Ins(1,4,5)P3. Ins(1,4,5)P3 (50 to 800 μM) was incubated with 0.5 μg of recombinant FRA3 protein.
transduction, RNA synthesis/processing, chromatin assembly, vesicle trafficking, cytoskeletal assembly, cell cycle control, and apoptosis (Li and Roberts, 2001). It has been proposed that the WD repeats in these proteins create a stable platform for protein–protein interactions (Smith et al., 1999). Indeed, some of the WD-repeat proteins have been shown to be involved in the assembly of multiprotein complexes, such as the β subunit of the G protein, the TAFII transcription factor, and the E3 ubiquitin ligase complexes (Li and Roberts, 2001). In plants, many WD-repeat proteins have been identified (van Nocker and Ludwig, 2003), and the WD-repeat domain in COP1 has been shown to interact with other effectors (Holm et al., 2001). The possible role of the WD-repeat domain in the FRA3 protein remains to be investigated.

Magnesium Concentration Differentially Regulates the FRA3 Phosphatase Activity toward Phosphoinositides and Ins(1,4,5)P₃

Biochemical studies of 5PTases in yeast and animals have shown that 5PTases require Mg²⁺ for their catalytic activity. It was proposed that the 5PTases share a common mechanism for catalysis with a family of Mg²⁺-dependent nucleases, including DNase I and the apurinic/apyrimidinic base excision repair endonucleases (Whisstock et al., 2002). Because FRA3 shows high sequence similarity to 5PTases from yeast and animals, it was not surprising to find that FRA3 also requires Mg²⁺ for its activity. However, it was intriguing to find that the optimal Mg²⁺ concentration required for the FRA3 phosphatase activity toward Ins(1,4,5)P₃ and phosphoinositides differs dramatically. This finding suggests that in plant cells, the free Mg²⁺ concentration, which may fluctuate depending on the physiological status of the cell, might influence the FRA3 phosphatase activity toward phosphoinositides and Ins(1,4,5)P₃.

FRA3 Is Required for Normal Actin Organization in Fiber Cells

The fact that the fra3 mutation alters the normal actin organization in fiber cells indicates that regulation of the phosphoinositide metabolism by the FRA3 phosphatase is essential for actin organization. In agreement with this finding, it has been shown that mutations of OCRL1, a human type II 5PTase, cause a decrease in long actin stress fibers and an increase in punctate F-actin staining (Suchy and Nussbaum, 2002). Both human synaptojanins and yeast synaptojanin homologs, which contain type II 5PTase domains, are known to be important for actin organization (Mitchell et al., 2002).

It has been shown in yeast and animals that PtdIns(4,5)P₂ regulates actin polymerization by modulating actin regulatory proteins, such as cofilin, profilin, and capping proteins CapZ and gelsolin (Takenawa and Itoh, 2001). In plants, PtdIns(4,5)P₂ has also been shown to be able to bind profilin (Kovar et al., 2001). Because FRA3 is able to hydrolyze PtdIns(4,5)P₂ in vitro and its mutation causes an accumulation of PtdIns(4,5)P₂ in vivo, it is
reasonable to propose that the abnormal actin organization seen in the fra3 mutant is a result of the alteration in PtdIns(4,5)P$_2$ level.

**FRA3 Influences Secondary Wall Synthesis in Fiber Cells**

The fra3 mutant was isolated based on its reduced fiber strength phenotype. Anatomical examination revealed that the fra3 mutation dramatically reduced the secondary wall thickness in fiber cells, which likely accounts for the weak fiber strength phenotype. A prominent phenotype in fra3 is that the reduction in wall thickness is not uniform among fiber cells. Occasionally, some fiber cells are nearly devoid of secondary walls. This phenotype is reminiscent of that caused by mutation of the ROOT HAIR DEFECTIVE3 gene in the fra4 mutant. The fra4 mutant exhibited aggregation of actin cables in fiber cells, which was proposed to partially account for the defective secondary wall thickening phenotype (Hu et al., 2003). Because mutation of the FRA3 gene also resulted in altered actin organization, it is possible that the defective secondary wall thickening seen in the fra3 mutant is caused partially by the altered actin organization. This is in agreement with the known functions of F-actin in facilitating the transport of vesicles carrying cell wall polysaccharides and vesicles containing cellulose-synthesizing enzymes. An alteration in actin organization might impede the transport of these vesicles, thereby causing a reduction in the overall cell wall synthesis. The possibility that the signaling molecules PtdIns(4,5)P$_2$ and Ins(1,4,5)P$_3$, which are substrates for FRA3, might directly affect secondary wall synthesis could not be excluded.

In summary, we have demonstrated that FRA3 belongs to the type II 5P-tase group and exhibits the highest substrate affinity toward PtdIns(4,5)P$_2$. Mutation of the FRA3 gene was found to cause a dramatic reduction in secondary wall thickness, an alteration in actin organization, and an increase in PtdIns(4,5)P$_2$ and Ins(1,4,5)P$_3$ levels. We propose that FRA3 type II 5P-tase regulates the metabolism of phosphoinositides and that this regulation is important for normal actin organization and secondary wall synthesis.

**METHODS**

**Mutant Isolation and Map-Based Cloning**

Ethyl methanesulfonate–mutagenized M2 Arabidopsis thaliana (ecotype Columbia) plants were grown in a greenhouse, and their inflorescence stems were screened for mutants with reduced stem strength. The stem segments, in which developing fiber cells just began to deposit lignin as stained with phloroglucinol HCl, were used for immunolabeling of actin filaments and cortical microtubules in fiber cells. Elongating stems were used for immunolabeling of actin filaments and cortical microtubules in pith cells. Samples were fixed in PEMT buffer containing 1.5% (v/v) formaldehyde and 0.5% (v/v) glutaraldehyde. Aldehyde fixation has been shown to preserve actin filaments well in plant cells (Vitha et al., 2000), and the F-actin patterns revealed by histolocalization and green fluorescent protein–tagged talin are comparable (Frank et al., 2003). After fixation, the stem segments were sectioned longitudinally with a vibrotome into 100-μm-thick sections that were then processed for immunolabeling. Sections were incubated with monoclonal antibodies against chicken actin (ICN, Aurora, OH) or chicken α-tubulin (Sigma–Aldrich) and subsequently with fluorescein isothiocyanate-conjugated secondary antibodies. The fluorescence-labeled F-actin and microtubules were observed under a Leica TCS SP2 spectral confocal microscope (Leica Microsystems, Heidelberg, Germany). Images were saved and processed with Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA). The width of the fluorescence-labeled actin cables was measured using the ruler tool of Adobe Photoshop. A total of 125 actin cables were measured for calculation of the averaged width and standard errors.

**Expression Analysis**

Total RNA was isolated from leaves, stems, roots, flowers, and seedlings using a Qiagen RNA isolation kit (Qiagen, Valencia, CA). One microgram of the purified RNA was treated with DNase I to remove any potential genomic DNA contamination and then used for first strand cDNA synthesis. One-twentieth of the first strand cDNA was used for PCR amplification of FRA3 with gene-specific primers (5′-AGACATGACCT-TAAACCTACGTTG-3′ and 5′-AGAGCGCTTTGCGTGGTCTG-3′). The primers used for RT-PCR span six introns so that any potential
amplification from genomic DNA could be easily identified based on its larger size. No genomic DNA was amplified in the RT-PCR reactions. The PCR was performed for variable cycles to determine the logarithmic phase of amplifications for the samples. The RT-PCR reactions were repeated three times, and identical results were obtained. The expression of a ubiquitin gene was used as an internal control for determining the RT-PCR amplification efficiency among different samples.

For the GUS reporter gene study, the FRA3 gene containing a 2-kb 5′ upstream sequence and the entire exon and intron regions was PCR amplified by high-fidelity DNA polymerase with primers 5′-TGGAGGAGAATCTTTAGAAGTCTC-3′ and 5′-TGGACTGTGCAAGTTCTTCTTAGTG-3′, confirmed by sequencing, and ligated in frame with the GUS reporter gene in the pBl101 vector. The construct was introduced into wild-type and fra3 mutant plants by Agrobacterium-mediated transformation. Transgenic plants were selected on MS medium containing 50 μg/mL of kanamycin. For analysis of the GUS activity, seedlings or stem segments were first immersed in 90% ice-cold acetone for 20 min and then incubated in the GUS staining solution (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.5 mM ferri cyanide, 0.5 mM ferrocyanide, and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid) at 37°C. The tissues were cleared in 70% ethanol and then used directly for observation or hand sectioned before observation.

Generation of FRA3-Overexpressing Plants

The full-length wild-type or mutant FRA3 cDNA was PCR amplified using high-fidelity DNA polymerase from cDNAs derived from wild-type or fra3 mutant stems, respectively. The amplified cDNA was confirmed by sequencing and ligated downstream of the 35S promoter of Cauliflower mosaic virus in pBl121. The constructs were transformed into wild-type Arabidopsis plants using the Agrobacterium-mediated transformation procedure. Transgenic plants were selected on kanamycin, and the T2 generation was used for expression analysis and examination of any potential phenotypes caused by FRA3 overexpression.

Sequence Analysis

The WD-repeat domain and 5PTase catalytic domain in FRA3 were identified by searching the public database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). The domain sequences were aligned using the ClustalW 1.8 program (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). The WD repeats were identified using a protein repeats searching program (http://www.embl-heidelberg.de/~andrade/papers/rep/search.html) and further analyzed using the secondary structure prediction program (http://www.predictprotein.org/) to determine the four β strands that potentially form the antiparallel β sheet.

Expression of Recombinant Proteins in Yeast

Full-length cDNAs of wild-type FRA3 and mutant fra3 were PCR amplified using specific primers (5′-ATGGAGATGCTCAAAAAGATCAAAAC-3′ and 5′-CTATGGACTGTGCAAGTTCTTCTTAG-3′) and confirmed by sequencing. The cDNAs were ligated in frame into the yeast expression vector pYES2/NT, which is tagged with six His and the Xpress epitope (DYDDDDDK) at the N terminus (Invitrogen, Carlsbad, CA). The constructs were transformed into the yeast strain INVSc1 (Invitrogen). The expression of recombinant proteins was induced in the presence of 2% galactose for 24 h. After induction, yeast cells were broken using glass beads, and the crude protein extracts were passed through an Ni-NTA column for purification of recombinant proteins.

The expression of recombinant proteins was confirmed by immunoblot analysis. Two hundred nanograms of purified recombinant protein was separated on a 4 to 20% SDS gel and transferred onto a nitrocellulose membrane. The recombinant proteins were detected by incubation with the monoclonal antibody against the N-terminal tagged Xpress epitope (Invitrogen) and horseradish peroxidase–conjugated secondary antibodies. The control protein used was β-galactosidase expressed under the same conditions. Both recombinant wild-type FRA3 and mutant fra3 proteins were confirmed to be expressed in yeast cells.

Phosphatase Activity Assay

Purified recombinant proteins were used for inositol polyphosphate phosphatase activity assay. Phospholipids were dissolved in the reaction buffer (50 mM Tris-HCl, pH 7.0, 0.25% β-octylglucoside, and 1 mM phenylmethylsulfonyl fluoride) with the aid of sonication. Phosphatase activity was measured by mixing 0.5 μg of recombinant proteins and 50 μM of substrates in the reaction buffer and incubated at 22°C. The enzyme activity was linear for 30 min; thus, all assays described in this study were run for 15 min. The FRA3 activity toward Ins(1,4,5)P3 was assayed in the presence of 3 mM Mg2+ in the assay buffer, and the FRA3 activity toward PtdIns(4,5)P2 and PtdIns(3,4,5)P3 was assayed in the presence of 0.1 mM Mg2+. The free phosphate released from the substrates was detected by the malachite green method (Kodama et al., 1986). The control protein used for the assays was β-galactosidase in replace of FRA3 in the reaction mixtures. All assays were run in duplicates and repeated at least twice, and identical results were obtained.

To test the effects of Mg2+ concentration on the FRA3 activity, different concentrations of MgCl2 were added in the reaction buffer. For analysis of the effects of pH and temperature, the FRA3 phosphatase activity was assayed under the same conditions as described above except for various pH or temperatures. For the kinetic analysis, the FRA3 activity toward a range of concentrations of substrates was assayed under the same conditions as described above except that pH 6.5 was used for Ins(1,4,5)P3.

Measurement of the Endogenous Levels of Ins(1,4,5)P3 and PtdIns(4,5)P2

Ten-day-old seedlings grown on agar containing MS medium and inflorescence stems of 8-week-old plants grown in a greenhouse were collected for measurement of Ins(1,4,5)P3 and PtdIns(4,5)P2. Tissues were ground into a fine powder in liquid nitrogen with a mortar and pestle, and the fresh weight of the ground tissues was determined in preweighed tubes. For extraction of Ins(1,4,5)P3, 1 g of fresh ground tissues were mixed with 0.8 mL of 20% ice-cold perchloric acid and incubated on ice for 20 min. The mixture was centrifuged at 4,000g for 20 min, and the supernatant was saved and neutralized to pH 7.5 with 1.5 M KOH containing 60 mM Heps and 5% universal pH indicator (Fisher Scientific, Pittsburgh, PA). The neutralized solution was centrifuged at 2,000g for 15 min, and the supernatant was saved for Ins(1,4,5)P3 assay. The amount of Ins(1,4,5)P3 was measured using the Ins(1,4,5)P3-[3H] Biotrak Assay System (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s protocol. Serial dilutions of the samples and all the controls were included in the assay according to the manufacturer’s recommendation. The amount of Ins(1,4,5)P3 in the samples was interpolated from a standard curve generated with known amount of Ins(1,4,5)P3. The assays were performed in duplicates and the experiments were repeated twice.

For extraction of PtdIns(4,5)P2, 1 g of fresh ground tissues was mixed with 2.1 mL of methanol/chloroform:12 M HCl (80:20:1, v/v/v) for 15 min. After further extraction by addition of 0.7 mL of chloroform and 1.25 mL of 0.1 M HCl, the chloroform phase was separated from the aqueous phase by centrifugation at 1,000g for 15 min. The lipid-containing chloroform phase was saved and dried in a stream of nitrogen. PtdIns(4,5)P2 in the dried sample was converted to Ins(1,4,5)P3 by addition of 250 μL KOH.
and heating at 100°C for 15 min. After cooling on ice, the solution was neutralized to pH 7.5 with 20% of perchloric acid containing 5% universal pH indicator. The neutralized solution was extracted with 1-butanol:light petroleum ether (5:1, v/v), and the lower aqueous phase containing Ins(1,4,5)P$_3$ was saved for Ins(1,4,5)P$_3$ assay. The samples were spiked with $[^{3}H]$ PtdIns(4,5)P$_2$ to determine the efficiency of conversion of PtdIns(4,5)P$_2$ to Ins(1,4,5)P$_3$. The amount of PtdIns(4,5)P$_2$ was calculated based on the conversion efficiency.

**Acknowledgments**

We thank Beth Richardson and John Shields for their help with microscopy and the editor and reviewers for their constructive comments. This work was supported by grants from the USDA and the Department of Energy.

Received August 31, 2004; accepted September 14, 2004.

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FRAGILE FIBER3, an Arabidopsis Gene Encoding a Type II Inositol Polyphosphate 5-Phosphatase, Is Required for Secondary Wall Synthesis and Actin Organization in Fiber Cells
Ruiqin Zhong, David H. Burk, W. Herbert Morrison III and Zheng-Hua Ye
Plant Cell 2004;16;3242-3259; originally published online November 11, 2004;
DOI 10.1105/tpc.104.027466

This information is current as of July 9, 2017

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