Cloning and Expression of a Wheat (Triticum aestivum L.) Phosphatidylserine Synthase cDNA

OVEREXPRESSION IN PLANTS ALTERS THE COMPOSITION OF PHOSPHOLIPIDS*

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We describe the cloning of a wheat cDNA (TaPSS1) that encodes a phosphatidylserine synthase (PSS) and provides the first strong evidence for the existence of this enzyme in a higher eukaryotic cell. The cDNA was isolated on its ability to confer increased resistance to aluminum toxicity when expressed in yeast. The sequence of the predicted protein encoded by TaPSS1 shows homology to PSS from both yeast and bacteria but is distinct from the animal PSS enzymes that catalyze base-exchange reactions. In wheat, Southern blot analysis identified the presence of a small family of genes that cross-hybridized to TaPSS1, and Northern blots showed that aluminum induced TaPSS1 expression in root apices. Expression of TaPSS1 complemented the yeast cha1 mutant that lacks PSS activity and altered the phospholipid composition of wild type yeast, with the most marked effect being increased abundance of phosphatidylserine (PS). Arabidopsis thaliana leaves overexpressing TaPSS1 showed a marked enhancement in PSS activity, which was associated with increased biosynthesis of PS at the expense of both phosphatidylinositol and phosphatidylglycerol. Unlike mammalian cells where PS accumulation is tightly regulated even when the capacity for PS biosynthesis is increased, plant cells accumulated large amounts of PS when TaPSS1 was overexpressed. High levels of TaPSS1 expression in Arabidopsis and tobacco (Nicotiana tabacum) led to the appearance of necrotic lesions on leaves, which may have resulted from the excessive accumulation of PS. The cloning of TaPSS1 now provides evidence that the yeast pathway for PS synthesis exists in some plant tissues and provides a tool for understanding the pathways of phospholipid biosynthesis and their regulation in plants.

Although phosphatidylserine (PS) is a minor phospholipid component of plant membranes (1), it is likely to have roles in addition to contributing to the structure of lipid bilayers. In mammalian and yeast cells, PS is normally distributed asymmetrically across the plasma membrane with the inner surface containing most of the PS (2, 3). Externalization of PS on the plasma membrane is an early indicator of apoptosis in mammalian cells (4, 5). Phosphatidylserine is required for activation of protein kinase C (6), is implicated as a key element in the coagulation of blood (3), and also allows phagocytes to recognize apoptotic cells (7). By comparison, there is little information available describing the roles of PS in plants although a recent report describes the migration of PS to the outer surface of the plasma membrane being associated with apoptosis in plant cells (8).

In Saccharomyces cerevisiae, PS is synthesized by the action of phosphatidylserine synthase (PSS; CDP-diacylglycerol:L-serine 1-phosphatidyltransferase, EC 2.7.8.8) that catalyzes the condensation of L-serine with CDP-diacylglycerol (9). The synthesis of PS in yeast is the first committed step in the major pathway for the de novo synthesis of phosphatidylethanolamine (PE) and phosphatidylcholine (PC; Fig. 1A), the two most abundant phospholipids of yeast membranes. In mammalian cells, by contrast, PS is synthesized by exchange of the polar head group of an existing phospholipid with L-serine (10) in a so-called base-exchange reaction (PSS-BE; Fig. 1B) whereas PC and PE are synthesized by pathways that do not involve PS (11). Although the enzymes involved in the synthesis of PS by both types of reactions share the same trivial name, phosphatidylserine synthase, the biochemistry of the reactions is different, and there is minimal amino acid sequence similarity between the two types of enzymes.

In plant cells the major pathway for the synthesis of PS is uncertain and may differ according to the type of tissue and species in question (12). A PSS-base exchange enzyme activity has been demonstrated in castor bean endosperm (13), whereas another report described a PSS activity in extracts of spinach leaves (14), but there are no subsequent reports to verify the presence of PSS in other plant species or tissues. Feeding of radiolabeled precursors to plant cells has produced equivocal results in identifying the major pathways for phospholipid biosynthesis. Some results have suggested that PC is synthesized primarily by pathways that do not require PS, but it has not been possible to completely rule out that PS contributes to PC biosynthesis (15, 16). Despite the paucity of direct experimental evidence in plants, a dogma has developed that PS synthesis in higher eukaryotic cells, including plant cells, proceeds exclusively by the base-exchange pathway (11, 17).

As a result of screening a wheat cDNA library for clones that conferred enhanced aluminum resistance to yeast, we isolated a cDNA encoding a PSS that catalyzes the formation of PS by

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U91983. § to whom correspondence should be addressed: Plant Industry, Commonwealth Scientific and Industrial Research Organisation, GPO Box 1600, Canberra Australian Capital Territory 2601, Australia. Tel.: 61 2 6246 5047; Fax: 61 2 6246 5000; E-mail: e.delhaize@pi.csiro.au.

The abbreviations used are: PS, phosphatidylserine; PSS, phosphatidylserine synthase; PE, phosphatidylethanolamine; PC, phosphatidylycerol; PI, phosphatidylinositol; PG, phosphatidylglycerol; PCR, polymerase chain reaction.
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Phosphatidylserine synthase catalyzes the condensation of CDP-diacylglycerol with L-serine. The resulting PS serves as a substrate for the condensation of CDP-diacylglycerol with L-serine. The resulting PS serves as a substrate for the condensation of CDP-diacylglycerol with L-serine.

A

Phosphatidic acid

CDP-diacylglycerol

PI

PG

B

PC/PE

PSS-BEI

PS

PSS-BEI

PE

FIG. 1. The major biosynthetic pathways for PS in yeast (A) and mammalian cells (B). The yeast enzymes that compete for the common substrate CDP-diacylglycerol are also shown. The enzymes catalyzing the various yeast reactions are as follows: arrow 1, CDP-diacylglycerol synthase; arrow 2, phosphatidylglycerol synthase (PGS); arrow 3, phosphatidylethanolamine synthase; arrow 4, phosphatidylglycerophosphate synthase; arrow 5, phosphatidylglycerophosphate synthase. In mammalian cells two phosphatidylserine synthases, with different substrate specificities, catalyze base-exchange reactions (PSS-BEI) from pre-existing phospholipids to synthesize PS.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Media—A wheat cDNA library was prepared from an aluminum-resistant wheat line and expressed in the yeast (S. cerevisiae) strain InVSc2 (Invitrogen, Carlsbad, CA) strain (1). Other yeast strains (FY833: MATα his3-D1 ura3-52) and mammalian cells (2) were quantitated using a Pusendorf-Imager (Fujifilm BAS 2000 PhosphorImager) with MACBAS (version 2.5E) software to normalize expression levels relative to rRNA expression as determined with the yeast rRNA probe pTA250.2 (26). Genes homologous to the predicted proteins encoded by the cDNAs were identified using the BLASTX algorithm (27).

Wheat DNA was purified by the method of Doyle and Doyle (28), and Southern hybridization analyses were performed as described by Sambrook et al. (29).

Transgenic Plants—The TaPSS1 cDNA was ligated to plant expression vectors under the control of the cauliflower mosaic virus promoter. The Arabidopsis transformation was performed using the pART7/pART27 vector system (30) in Agrobacterium, and transgenic plants were generated by vacuum infiltration (31). For tobacco (Nicotiana tabacum), transgenic plants were generated by transformation using the binary vector pLLEX201-3 (32). Tobacco was then transformed with this vector using Agrobacterium and co-cultivation of leaf explants (33). Control plants were transformed with the vectors alone. For tobacco, seed (T1) plants were selected from the primary transgenics (T0) and grown for analysis of phospholipids and PSS activity. For Arabidopsis, kanamycin-resistant T1 plants were selected and segregating T2 lines were analyzed. In both cases, TaPSS1 overexpressing lines could be easily identified in the segregating lines on the basis of their phenotype.

Phospholipid and PSS Assays—Yeast cultures (5 ml of LPM-galactose medium) at a starting A600 of 1.0 were labeled with KH32PO4 (0.74 MBq; Amersham Pharmacia Biotech) for 1 or 24 h. Phospholipids were extracted from yeast cells using procedures described by Homann et al. (35) and were separated by thin layer chromatography using either a single dimension with chloroform:methanol:acetic acid:water (5:4:1:1 by volume) as the solvent system or with a two-dimensional procedure where chloroform:methanol:water (1:1:0.5 by volume) was used for the first dimension and chloroform:methanol:water (1:1:0.5 by volume) was used for the second dimension. The identity of the various phospholipids was determined by comparison to standard phospholipids (Sigma), and the thin layer chromatography plates were analyzed with a PhosphorImager system (Molecular Dynamics) to quantify the 32P incorporated into the various phospholipids. Yeast extracts were prepared and assayed for PSS activity according to Homann et al. (35).
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Statistical analysis of the phospholipid composition in yeast and Arabidopsis was complicated by the high variability between replicates for the total 32P incorporated. Therefore the statistical analyses were performed on the proportion of 32P appearing in each phospholipid fraction. The data were transformed with the arcsine function to normalize the distribution and to minimize the variance between the means. For each phospholipid a t test was used to compare the means of the different genotypes.

RESULTS

Isolation of a Wheat cDNA That Encodes PSS—A wheat cDNA encoding PSS was isolated from a yeast expression library that was screened for cDNAs whose expression enhanced aluminum resistance. After screening over 2 million yeast transformants, eight cDNAs encoding six different genes were found to confer aluminum resistance to the yeast strain INVSc2. Sequencing of these cDNAs identified one (TaPSS1) whose predicted translation product showed sequence similarity to PSS from other organisms. Fig. 2 shows that the predicted protein encoded by the TaPSS1 open reading frame is similar to PSS from S. cerevisiae (54% identity; GenBank™ accession number D00171), B. subtilis (GenBank™ accession number P39923) and H. pylori (GenBank™ accession number AE000614) were aligned with the deduced amino acid sequence of TaPSS1. Identical amino acids are shown with dark shading, and similar amino acids are shown with light shading.

FIG. 2. Alignment of the predicted amino acid sequence encoded by the TaPSS1 open reading frame with the predicted polypeptides encoded by PSS genes from yeast and bacteria. Sequences of PSS from yeast (S. cerevisiae; GenBank™ accession number D00171) B. subtilis (GenBank™ accession number P39923) and H. pylori (GenBank™ accession number AE000614) were aligned with the deduced amino acid sequence of TaPSS1. Identical amino acids are shown with dark shading, and similar amino acids are shown with light shading.

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Fig. 3. Northern and Southern blot analysis of wheat using TaPSS1. A, Northern hybridization; each lane contains 10 μg of total RNA from the tissues indicated. Probes were TaPSS1 (PSS) or the 26S rRNA gene (rRNA). Roots were treated with 10 μm aluminum for the times indicated (in hours, see Snowden and Gardner (25)). The aluminum induction experiments were performed twice, and quantitation of the transcript levels showed a 3.5-fold (experiment shown) and 5-fold increase after 8 h exposure in two independent experiments. B, Southern hybridization; each lane contains 20 μg of wheat genomic DNA cleaved with DraI (lane 1), SstI (lane 2), XbaI (lane 3), and AvaII (lane 4). The blot was probed with TaPSS1, and the final wash stringency was 0.2× SSC at 65 °C. kb, kilobase pairs; bp, base pairs.

TaPSS1 Complements the Yeast cho1 Mutant—The similarity of the protein encoded by the TaPSS1 open reading frame to PSS from yeast and bacteria suggested that it encodes a plant PSS. Yeast cho1 mutants are defective in PSS activity and require exogenously supplied choline or ethanolamine for growth (37, 38). A cho1 mutant was constructed by PCR-mediated disruption in the yeast strain FY833. Fig. 4 shows that expression of the wheat TaPSS1 gene complemented the choline requirement of the cho1 mutant, demonstrating that TaPSS1 encodes a functional plant PSS enzyme.

Expression of TaPSS1 in Yeast Alters the Composition of Phospholipids and Confers PSS Activity to a Yeast cho1 Mutant—Fig. 5 shows that expression of the wheat TaPSS1 gene changed the phospholipid profile of yeast strain InVSc2. Short term 32P-labeling experiments (Fig. 5A) showed a large increase in abundance of label into PS primarily at the expense of phosphatidylinositol (PI). Longer term 32P-labeling experiments (Fig. 5B) also showed increased PS biosynthesis at the expense of PI, but the effects were less dramatic than in the short term experiments. This difference was expected, since short term experiments label phospholipid intermediates in the various pathways, and the 32P in each phospholipid pool gives an indication of the rate of biosynthesis. By contrast, longer term 32P experiments more accurately reflect the steady state phospholipid composition in yeast cells (38). In long term experiments expression of TaPSS1 increased the amount of PS and PE and decreased the amount of PI, whereas the amount of PC was unchanged (Fig. 5B). Similar results were obtained for yeast strain FY833 (data not shown), which did not show enhanced aluminum resistance with expression of TaPSS1.

Overexpression of TaPSS1 in the yeast cho1 disruptant resulted in measurable PSS activity, to levels greater than those obtained by overexpression of the yeast CHO1 gene (Table I). PSS activity was undetectable in the disruption mutant transformed with the plasmid vector alone.

Overexpression of TaPSS1 in Plants Results in Increased Accumulation of PS and Is Associated with Necrotic Lesions on Leaves—When TaPSS1 cDNA was overexpressed in Arabidopsis and tobacco under the control of the cauliflower mosaic virus promoter, a number of transgenic plants showed changes in morphology and necrotic lesions. Arabidopsis plants showing the most severe phenotypes were stunted (Fig. 6D), and the necrotic lesions on tobacco typically appeared as spots with leaves being distorted and asymmetric (Fig. 6, B and C). Lesions and altered morphology were found in plants with the highest level of TaPSS1 transcript (data not shown).

One of the Arabidopsis lines that showed a severe phenotype, D244-24, was analyzed for its phospholipid composition by 32P labeling. After 24 h labeling with 32P, the proportion of radioactivity found in the various classes of major leaf phospholipids showed a dramatic difference in D244-24 compared with wild type Arabidopsis plants (Fig. 7A). The overexpression of TaPSS1 resulted in an increase of 32P incorporation into PS and a corresponding decrease of 32P incorporation into PI, as found for yeast. In addition, there was a decrease in 32P found in phosphatidylglycerol (PG) but an increase in labeled PC. Phosphatidylglycerol, which is not a major phospholipid of yeast cells, is a major phospholipid in leaves and is found in the lamellae of chloroplasts (1). Similar results were found for PS in roots (32P incorporated into PS as a percent of total phospholipids: control, 0.56 ± 0.17; D244-24, 7.95 ± 2.63, means ±
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FIG. 5. Composition of phospholipids labeled with 32P in IN-VSc2 yeast cells expressing TaPSS1 compared with vector alone. Cells were labeled with 32P for 1 h (A) or 24 h (B) before they were analyzed for phospholipid composition. The amount of 32P incorporated into the major phospholipids is expressed as a percent of the total 32P incorporated into phospholipids. The means from four independent transformants for each plasmid are shown ± S.E. and statistically significant differences in proportion of phospholipids between plasmids are shown by * (p < 0.05) or **(p < 0.01). Analysis by two-dimensional thin layer chromatography showed that phosphatidyl-dimethylethanolamine (PDE) comprised about 5% of the 32P incorporated into phosphatidyl-dimethylethanolamine (PDE) + PE.

We reasoned that, as in yeast cells, longer term labeling would more accurately reflect the steady state composition of plant phospholipids. Longer term 32P labeling (7 days) still showed a much greater 32P incorporation into PDE for the TaPSS1 transgenic lines compared with control plants (Fig. 7B). As in the 24-h experiment, label incorporated into PI and PG was reduced, although the effect was less pronounced than in the short term experiment.

Phosphatidylserine synthase activity was readily detected in crude homogenates prepared from leaves of Arabidopsis and tobacco overexpressing TaPSS1 but was either undetectable or very low for control plants that were transformed with the vector alone (Table I). The levels of TaPSS1 expression activity in different transgenic lines were related to the severity of the phenotypes described above (data not shown).

DISCUSSION

Four lines of evidence support the conclusion that the wheat TaPSS1 gene encodes a functional PSS enzyme as follows: (i) there is homology between the protein encoded by TaPSS1 and known PSS proteins from yeast and bacteria; (ii) TaPSS1 complements the yeast cho1 mutant; (iii) overexpression of TaPSS1 in plants and in a disrupted cho1 mutant of yeast yields measurable PSS activity in both types of organisms; and (iv) overexpression of TaPSS1 in yeast and plants changes the phospholipid composition in both these organisms with an increase in PS content being the most marked effect. Our cloning of a functional plant PSS gene provides the first strong evidence for the existence of PSS in higher eukaryotic cells. The role of PSS in wheat could be primarily for PS biosynthesis; or alternatively, in some plant species or tissues, it may play a key role in the biosynthesis of PC, as occurs in yeast.

Complementation of the yeast cho1 mutant provided strong evidence for TaPSS1 gene function. The fact that there is a single gene encoding PSS in the yeast genome (39) precludes the possibility that TaPSS1 is enhancing endogenous PSS in yeast by transactivation of another gene. A marked difference between yeast PSS and wheat PSS is the absence of an acidic N-terminal sequence in the wheat protein (Fig. 2). However, overexpression of a truncated form of the yeast CHO1 gene that
lacks sequence encoding the acidic N-terminal region of the protein was also able to complement the cho1 mutant. The truncated yeast gene produced less than one-tenth the amount of active enzyme produced by the wild type gene, even though comparable amounts of protein were produced by expression of the genes (40). This lower activity was attributed to the truncated form not being correctly inserted into membranes. In our case, the wheat TaPSS1 gene yielded even greater enzyme activity than the wild type CHO1 gene when overexpressed in yeast (Table I). The significance of the lack of an acidic domain in the wheat enzyme is not known, but this does not appear to limit its activity in yeast.

The plasma membrane is a primary barrier to the entry of aluminum into cells. A change in its lipid composition could conceivably alter the resistance of the cell by excluding aluminum, by altering the activity of specific membrane proteins involved in conferring aluminum resistance, or by altering the response of the yeast cell to external stimuli. Aluminum ions bind to phospholipids, and the binding affinity under acidic conditions is greatest for anionic phospholipids such as PG and PI (41). Several features of our results suggest further links between phospholipids and aluminum resistance. The wheat PSS transcript was induced in response to aluminum stress, and overexpression of PSS activity increased aluminum resistance in a third strain. However, the change in phospholipid composition in itself does not appear to be sufficient to confer aluminum resistant to all yeast genotypes, since expression of TaPSS1 in strain FY833 did not increase aluminum resistance despite similar changes in phospholipid composition as found for strain InVSc2. Preliminary results also indicate that overexpression of TaPSS1 in tobacco does not confer enhanced aluminum resistance.

In yeast, the major pathway for de novo PC biosynthesis is through the conversion of CDP-diacylglycerol and L-serine to PS (catalyzed by PSS) which is then converted sequentially to PE and PC by the action of PS decarboxylase and methyltransferases (9). The increased PE synthesis seen in yeast cells overexpressing PSS is consistent with this pathway since PS decarboxylase should convert some of the excess PS to PE. The observed increase in PS content at the expense of PI when TaPSS1 is expressed (Fig. 5) is also consistent with PSS and PI synthase competing for CDP-diacylglycerol as a common substrate (Fig. 1A).

In plants the major pathway for PC biosynthesis is not well defined, although much of the biochemical and physiological evidence suggest that the CDP-choline pathway predominates in some tissues (12, 15). The cloning of Brassica napus and Arabidopsis cDNAs that encode CTP:phosphocholine cytidylyltransferase, the enzyme that catalyzes the rate-limiting step in the conversion of choline to PC, provides support for the existence of this pathway (42, 43). Our cloning of a wheat cDNA that encodes a functional PSS protein now provides evidence for the existence of the yeast pathway for PS biosynthesis in wheat. Overexpression of TaPSS1 in Arabidopsis increased PS biosynthesis at the expense of both PI and PG, consistent with PSS, PI synthase, and PG-phosphate synthase competing for CDP-diacylglycerol as a common substrate (Fig. 1A). The rate of PC synthesis was also increased in the TaPSS1-overexpressing plants (Fig. 7, A and B), but PE synthesis did not increase. This result contrasts with that found in yeast, where PE biosynthesis increased but PC was little affected in both short and long term experiments. If the pathway to PC synthesis were the same in the transgenic plants as in yeast, then one would expect PE synthesis to be enhanced relative to PC because PE is the direct precursor of PC in this pathway. One possible explanation is that a base-exchange reaction of the type found in mammalian cells (Fig. 1B; PSS-BEI) converts PS and free choline directly to PC without proceeding through PE. The increase in PC may be a consequence of the plant cells attempting to reduce the amount of PS accumulated in the TaPSS1 overexpressers or, alternatively, may represent an active pathway for PC biosynthesis in wild type plants that is further enhanced in the transgenic plants.

As found in other organisms, PS is likely to be an essential phospholipid for growth of plant cells. In contrast to yeast and mammalian cells where PS can comprise up to 10% of the total phospholipids, in plants PS is a minor component comprising less than 2% of the total phospholipids found in leaves of many species (1). Results from our long term 32P-labeling experiments (Figs. 5 and 7) are consistent with these values for abundance of PS in yeast and plant cells. In mammalian cells overexpressing a PSS-base exchange enzyme, PS biosynthesis was greatly increased in vivo, but the steady state phospholipid composition was unchanged (44). This lack of effect on phospholipid composition was attributed to compensatory changes in activities of the pathways that synthesize and degrade PS to maintain overall phospholipid homeostasis. Recently, Kuge et al. (45) showed that PSS-base exchange activity is regulated by PS concentrations in the cell and that this can in itself
account for a large part of the PS homeostasis. By contrast, our results indicate that plant cells are unable to maintain phospholipid homeostasis when PSS activity is in excess since large changes in overall phospholipid composition were seen. We speculate that the necrotic lesions arise on plants expressing TaPSS1 at high levels because of a large increase in abundance of PS in membranes. This may have resulted in PS appearing on the outer surface of the plasma membrane by saturating the mechanisms that normally maintain phospholipid asymmetry across the membrane. The appearance of PS on the outer surface of cells is an early step in the apoptotic pathway, and in mammalian cells increased biosynthesis of PS precedes the appearance of PS on the outer surface of the plasma membrane (46). Furthermore, external application of PS to Chinese hamster ovary cells can in itself trigger apoptosis, whereas other phospholipids are not able to elicit this response (47).

In conclusion, we have isolated a wheat gene that encodes a functional PSS enzyme. Overexpression of TaPSS1 in yeast and plants alters the phospholipid composition of these organisms. Altering the expression of this gene in plants provides a new tool for defining the pathways of phospholipid biosynthesis and for understanding how these pathways are regulated. Altering the abundance of PS will also provide clues to the biological functions in plants of this minor, but potentially important, phospholipid.

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