The Phosphorylated Pathway of Serine Biosynthesis Is Essential Both for Male Gametophyte and Embryo Development and for Root Growth in Arabidopsis

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INTRODUCTION

The amino acid L-Ser is essential for the synthesis of proteins and other biomolecules required for cell proliferation, including nucleotides and Ser-derived lipids such as phosphatidyl Ser and sphingolipids (see Supplemental Figure 1 online). Besides its participation in metabolism, additional nonmetabolic functions for Ser have been described in mammals and plants. In mammals, the de novo synthesis of L-Ser is essential in the development and function of the central nervous system (Yoshida et al., 2004). Furthermore, L-Ser is the precursor of D-Ser, a well-documented neuromodulator (Mothet et al., 2000). Similarly, in plants, D-Ser has recently been attributed a signaling role in male gametophyte-pistil communication (Michard et al., 2011).

Ser biosynthesis in plants proceeds via different pathways (Figure 1). One is the glycolate pathway, which takes place in mitochondria and is associated with photorespiration (Tolbert, 1980, 1997; Douce et al., 2001; Bauwe et al., 2010; Maurino and Peterhansel, 2010). Additionally, alternative nonphotorespiratory mechanisms of Ser biosynthesis have been postulated (Kleczkowski and Givan, 1988). In quantitative terms, Ser production through the glycolate pathway is considered to be the most important, at least in photosynthetic cells (Tolbert, 1980; Douce et al., 2001). In this pathway, two Gly molecules are converted into one molecule of Ser in a reaction catalyzed by two enzymes, the Gly decarboxylase complex and the Gly hydroxymethyltransferase (Figure 1). Since the glycolate pathway is associated with photorespiration, it should be active mainly in green tissues during daylight hours. It therefore follows that alternative pathways of Ser biosynthesis may be required in the dark and/or in nonphotosynthetic organs. However, the biological significance of the coexistence of several Ser biosynthetic pathways in plants is still not understood.

A nonphotorespiratory pathway, the so-called glycerate pathway, synthesizes Ser by the dephosphorylation of 3-phosphoglycerate (3-PGA) (Kleczkowski and Givan, 1988) (Figure 1). This pathway includes a reversed sequence of reactions from a portion of the oxidative photosynthetic carbon cycle linking 3-PGA to Ser (3-PGA-glycerate-hydroxypyruvate-Ser), with these reactions catalyzed by enzymes such as 3-PGA phosphatase, glycerate dehydrogenase, Ala-hydroxypyruvate aminotransferase, and Gly hydroxypyruvate aminotransferase. The existence of enzymatic activities of this pathway in plants has been demonstrated...
(Kleczkowski and Givan, 1988). However, the extent to which this pathway could be functional in plants is as yet unknown, and genes coding for the specific enzymes of the pathway have not been cloned and/or characterized.

A second nonphotorespiratory pathway, the phosphorylated pathway of Ser biosynthesis (PPSB), synthesizes Ser via phosphoserine from 3-PGA as a precursor (Handford and Davies, 1958). Supporting evidence for the PPSB in plants derives from the isolation and characterization of the enzyme activities of this route (Slaughter and Davies, 1968; Larsson and Albertsson, 1979; Walton and Woolhouse, 1986). This pathway, which is conserved in mammals and plants, defines a branch point for 3-PGA from glycolysis and involves three enzymes catalyzing sequential reactions: 3-phosphoglycerate dehydrogenase, 3-phosphoserine aminotransferase, and 3-phosphoserine phosphatase (PSP) (Figure 1). In humans, the PPSB plays a crucial role in cell proliferation control. This pathway can divert a substantial fraction of the glycolytic flux (carbon metabolism) into Ser biosynthesis (nitrogen metabolism) and can contribute to cell proliferation and oncogenesis (Bachelor et al., 2011; Locasale et al., 2011; Pollari et al., 2011; Possemato et al., 2011). For instance, an enhanced PPSB results in an increased cell proliferation rate, which is associated with certain breast cancers (Locasale et al., 2011; Possemato et al., 2011). By contrast, downregulation of the PPSB causes a restriction in tumor cell proliferation (Possemato et al., 2011).

Unlike in mammals, the functional significance of the PPSB in plants is not yet known. Although some genes of the pathway have been cloned and the enzymes that they encode have been biochemically characterized in Arabidopsis thaliana (Ho et al., 1998, 1999a, 1999b; Ho and Saito, 2001), no genetic evidence for the physiological functions of these genes has been provided to date. Synthesis of amino acids takes place mainly in mature roots and source leaves, which export N through the phloem-xylem system to supply sinks, such as flowers and seeds (Lam et al., 1996). Specifically, Ser is easily transported through the phloem (Riens et al., 1991; Hunt et al., 2010). This would imply that the Ser synthesized in photosynthetic cells through the photorespiratory pathway could be supplied to non-photosynthetic organs. Similarly, Ser synthesized through non-photorespiratory mechanisms in roots could contribute to the amino acid supply to sinks such as seeds and flowers. However, the relevance of each Ser biosynthetic pathway in different organs and under different environmental conditions remains to be defined.

In previous studies, we characterized the plastidial glycolytic glyceraldehyde 3-phosphate dehydrogenase (GAPCp) family in Arabidopsis (Muñoz-Bertomeu et al., 2009, 2010a, 2010b, 2011a, 2011b). The gapcp1 gapcp2 double mutants show a drastic developmental phenotype, including arrested primary root growth, dwarfism, and male sterility. We concluded that GAPCps play an important role in plant development as their...
activity affects Ser supply to roots (Muñoz-Bertomeu et al., 2009; Muñoz-Bertomeu et al., 2010a). We specifically hypothesized that GAPCp activity is essential for supplying the precursor 3-PGA for the PPSB in the plastids of nonphotosynthetic organs. From these results, it could be inferred that the PPSB may play an important role in specific plant developmental events.

This study addresses the functional significance of the PPSB in plant metabolism and development by targeting the last enzyme of this pathway, PSP1. Here, we show that PSP1 is essential for male gametogenesis and embryogenesis and that it is indispensable for postembryonic root development. Although it is clear that metabolic changes and developmental reprogramming are tightly related, the connecting links are not known. This study highlights the relevant function of the PPSB in connecting primary metabolism with development.

RESULTS

Expression Pattern and Subcellular Localization of PSP

In The Arabidopsis Information Resource database (http://www.arabidopsis.org), we found a single gene (At1g18640) coding for PSP (PSP1). This gene was previously cloned, and the encoded protein possesses in vitro PSP activity (Ho et al., 1999a). However, an exhaustive study of its expression pattern, which could give clues about its functions, was lacking.

Analysis of the PSP1 promoter using the promomer tool (Winter et al., 2007) revealed that the promoter regions were significantly enriched in consensus sequences present in important genes regulating anther development, such as the floral homeotic genes AGAMOUS and AGAMOUS2 (see Supplemental Table 1 online). An in silico analysis of PSP1 expression based on microarray databases (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi and https://www.genevestigator.com/gv/) revealed a constitutive pattern of expression through the main Arabidopsis development stages (see Supplemental Figure 2 online). At the organ level, PSP1 displayed a high expression level in hypocotyls, early stages of root, flower (stages 9 to 12), seed and embryo (globular stage) development, and in shoot apices (vegetative, inflorescence, and transition apex). We assessed the expression patterns of PSP1 by RT-PCR. At both seedling and adult stages, PSP1 was expressed in all organs studied, and no significant differences in expression between organs were found (Figure 2A). When PSP1 expression was studied under different growth conditions, an 8-h exposure to darkness induced the gene expression in the aerial parts, but not in roots, whereas longer exposures (24 h) repressed PSP1 expression on both roots and aerial parts (Figure 2B). Ser, the product of PSP1 activity, did not repress PSP1 expression in the light, but repressed it after 8-h exposure to darkness in aerial parts.

Analysis of promoter-β-glucuronidase (GUS) fusions confirmed that PSP1 is expressed in all organs studied, but it displayed a highly specific cell tissue pattern (Figure 3A). At the seedling stage, PSP1 was expressed mainly in the vegetative apex, veins, the distal zone of cotyledons, and in stomata. In roots, PSP1 was expressed mainly in the transition zone between roots and shoots, along the root vasculature, in the proximal part of the meristems and in the root cap columella. At the adult stage, PSP1 transcripts were detected in all organs studied, but once again, a very specific cell tissue pattern was observed. In flowers, GUS activity was associated mainly with anthers, stigma, and pollen grains (Figure 3A). In leaves, PSP1 was expressed mainly in the distal zone and veins. To further confirm and extend the analysis of the PSP1 expression pattern, we stably expressed a PSP1-GFP (for green fluorescent protein) protein fusion construct under the control of the PSP1 promoter (ProPSP1:PSP1) in wild-type plants. GFP fluorescence corroborated the cell tissue–specific PSP1 expression pattern in the roots and reproductive organs (Figure 3B). In seedling roots, PSP1 was clearly expressed in the entire meristematic zone. In flowers, PSP1 was expressed in pollen, anthers, and carpels. In later developmental stages, PSP1 was expressed in siliques and seeds, especially in embryos.

Using a 35S-PSP1-GFP construct, Ho et al. (1999a) demonstrated that PSP1 was localized in plastids. Our results
Figure 3. Tissue and Subcellular Localization of PSP1.
employing the native PSP1 promoter for intracellular visualization of PSP1 corroborated that it is localized in both chloroplasts and nongreen plastids in leaves (Figure 3C). In roots, PSP1 also displayed a plastidial localization. Similar results were obtained when the PSP1-GFP construct was expressed under the control of the 3SS promoter (see Supplemental Figure 4 online).

The Homozygous psp1 Mutation Is Embryo Lethal

In order to shed light on the in vivo function of PSP1 in Arabidopsis, a reverse genetic approach was followed. Two independent T-DNA insertion mutant lines affecting PSP1 were identified in the mutant collections (Salk-062391 and GK877F12), but only the Salk-062391 line could be reconfirmed. This line was named psp1.1. The presence and genomic location of the T-DNA insertion was verified by PCR amplification of genomic DNA and by sequencing of the PCR products. The T-DNA insertion was localized in the fourth intron, 1153 nucleotides downstream of the start codon of the PSP1 gene (see Supplemental Figure 4 online). Genotyping plants from the original seed stock identified only wild-type (PSP1/PS1) and heterozygous (PSP1/psp1.1) individuals, with no visual phenotype seen. An analysis of the segregation of the mutant psp1.1 allele was conducted in a population of 264 seeds obtained from heterozygous PSP1/psp1.1 plants. No homozygous individuals (psp1.1/psp1.1) could be rescued based on PCR genotyping (Table 1). The segregation analysis of the population displayed a 1:2 ratio (PSP1/PS1:PS1/psp1.1; \( \chi^2 = 1.38; P > 0.05 \)), which is typical of a Mendelian segregation with a lethal phenotype for psp1.1/psp1.1 individuals. To corroborate these results, a second segregation analysis was conducted based on the kanamycin resistance conferred by the T-DNA insertion in the mutant allele. Of 1183 seedlings produced from heterozygous PSP1/psp1.1 plants, 66.1% were kanamycin resistant and 33.9% were kanamycin sensitive (Table 1). These results do not match the expected 75% resistant individuals for viable psp1.1/psp1.1 mutants. On the contrary, the observed results match a 1:2 segregation (PSP1/PS1:PS1/psp1.1; \( \chi^2 = 0.17; P > 0.05 \)), indicating a lethal phenotype associated with the mutant psp1.1 allele. PSP1/psp1.1 plants were visually indistinguishable from the wild type, indicating the recessive nature of the mutant allele.

Further evidence for the role of PSP1 was obtained by analyzing point mutations in psp1 alleles, which were identified by the Seattle Arabidopsis TILLING (for targeting-induced local lesions in genomes) Project (http://tilling.fhcrc.org/). A new psp1 allele was identified (psp1.2), which resulted in the substitution of Ser-178 for Phe (S178F). According to SIFT (for sorting intolerance from tolerant) software analysis, this mutation is predicted to affect protein functions (SIFT score < 0.05). Specifically, the Ser-178-Phe mutation, with the highest position-specific scoring matrix score of all the putative mutants identified, is situated in the PSP1 conserved active site, at an essential position for catalytic activity, suggesting that it could be lethal. In a population of 58 individuals coming from heterozygous PSP1/psp1.2 mother plants, no homozygous psp1.2/psp1.2 could be identified by genotyping. Once again, the segregation analysis matched a 1:2 ratio (PSP1/PS1:PS1/psp1.2; \( \chi^2 = 0.42; P > 0.05 \)) corroborating the essential role of PSP1 in early Arabidopsis developmental stages.

To investigate whether the lethality associated with psp1 mutations is due to the male gametophyte, female gametophyte, or embryo defects, reciprocal outcrosses of PSP1/psp1.1 plants as male/female parent (donor/recipient) and the wild type as female/male parent (recipient/donor) were performed, and the segregation of the mutant allele was studied based on the antibiotic resistance conferred by the T-DNA insertion (Table 1). The results indicate that the male and female mutant gametophytes were transmitted with an efficiency of 79 and 86.4%, respectively, which provides evidence that psp1.1 triggers an embryo-lethal phenotype.

To further confirm this, siliques from heterozygous PSP1/psp1.1 plants were dissected at different developmental stages. From 8 d after pollination (DAP), we observed a population of abnormal seeds, which was randomly distributed along the length of the siliques (Figure 4A). At 15 DAP, mutant seeds were white and started to deflate. At 22 DAP, mutant seeds turned dark brown and were completely deflated. Segregation analysis of a population of 5064 seeds obtained from heterozygous PSP1/psp1.1 plants revealed a 1:4 ratio (20.9% mutant:79.1% normal seeds; \( \chi^2 = 2.52; P > 0.05 \)). This ratio agrees with the expected 20.4%-79.6% ratio for psp1.1/psp1.1:PS1/PS1:PS1/psp1.1:PS1/psp1.1 calculated according to the transmission efficiency for the PSP1 and psp1.1 alleles (Table 1). To characterize the nature of the seed nonviability, we examined the embryos in developing siliques of heterozygous PSP1/psp1.1 plants (Figure 4B). At 2 DAP, all the embryos examined reached a similar developmental stage (octant stage according to Capron et al., 2009). However, at 5 DAP, some of the embryos showed delayed development (early globular versus triangular stage). According to microarray databases, PSP1 expression is maximal at the globular stage (see Supplemental Figure 2 online). The developmentally delayed embryos could be assigned to mutant seeds at 7 DAP (heart versus mid torpedo) and 10 DAP (heart versus early cotyledon stage). The terminal aborted embryos (15 DAP) were albino and could be classified as early curled cotyledons (L1) according to the SeedGenes database.

Figure 3. (continued).

(A) Left: Expression of GUS under the control of the PSP1 promoter in apical meristem (I), cotyledons (II), guard cells (III), root and shoot vasculature (IV, V, and VII), and root meristem (VI) of 6- to 10-d-old plants. Right: Expression of GUS in anthers (I and II), carpels (I and IV), pollen (III), siliques (V), and leaves (VI) of adult plants. Bars = 0.5 mm in I, II, IV, V, VII, VIII, IX, XI, XII, and XIII and 50 µm in III, VI, and X.

(B) PSP expression in roots and floral organs visualized by PSP1-GFP expression under the control of PSP1 promoter. Pg, pollen grain. Bars = 0.25 mm.

(C) Chloroplastic/plastidic localization of PSP1 by stable expression of PSP1-GFP fusion protein under the control of PSP1 promoter in mesophyll, stomata, and root cells. Bars = 50 µm.
In the adult stage, transgenic pspl1/pspl1 ProPSP/PSP1 plants were fertile and visually indistinguishable from the wild type. However, when ProPSP/proPSP1 plants were transformed with a ProPSP-GFP cDNA under the control of the 35S promoter (Pro35S:PSP1), the resulting homozygous pspl1/pspl1 transgenic lines in the segregating population were sterile, producing small silique with no seeds (Figures 5A and 5B). A PSP1 overdose effect was not the cause of the observed sterility phenotype since the wild type expressing Pro35S:PSP1 was fertile (Figures 5A and 5B).

As the 35S promoter exhibits very low expression, or none, in the Arabidopsis tapetum (Grienenberger et al., 2009; Muñoz-Bertomeu et al., 2010b), we performed an ontogenic serial analysis of anther and pollen development from pspl1/pspl1 Pro35S:PSP1 plants compared with wild-type and pspl1/pspl1 ProPSP/PSP1 plants. For that purpose, floral buds were classified from stages 7 to 12 according to the landmark events described by Smyth et al., (1990) and analyzed by transmission electron microscopy (Figure 6A). In the pspl1/pspl1 Pro35S:PSP1 anthers, tetrads of microspores were formed (stage 7 according to Sanders et al., 1999) and progressed in their development till the polarized microspore stage (stage 9). These initial steps of microspore development in pspl1/pspl1 Pro35S:PSP1 were similar to those observed in the wild type. However, after the polarized microspore stage, pspl1/pspl1 Pro35S:PSP1 microspores initiated a degeneration process characterized by detachment of microspore protoplasm from the cell wall. At the end of the maturation process (stage 12), most of pspl1/pspl1 Pro35S:PSP1 pollen grains showed partially shrunken or completely collapsed protoplasm. As in the wild type, pollen from pspl1/pspl1 ProPSP/PSP1 anthers developed and matured normally.

### Table 1. Segregation of the pspl1 Mutant Allele in the Progeny of Self-Crossed and Reciprocal Outcrossed Plants

<table>
<thead>
<tr>
<th>Self-Crosses</th>
<th>No. of Progeny</th>
<th>Genotypes of Progeny (PCR)</th>
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<tr>
<td>PSP1/pspl1.1</td>
<td>264</td>
<td>PSP1/PSP1 (97 (36.7)%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PSP1/pspl1.1 (167 (63.3)%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pspl1.1/pspl1.1 (0 (0.0)%)</td>
</tr>
<tr>
<td>PSP1/pspl1.1 Pro35S/PSP1</td>
<td>192</td>
<td>PSP1/PSP1 (54 (28.1)%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PSP1/pspl1.1 (102 (53.1)%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pspl1.1/pspl1.1 (36 (18.8)%)</td>
</tr>
<tr>
<td>PSP1/pspl1.1 ProPSP/PSP1</td>
<td>192</td>
<td>PSP1/PSP1 (50 (26.0)%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PSP1/pspl1.1 (99 (51.6)%)</td>
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<td></td>
<td></td>
<td>pspl1.1/pspl1.1 (43 (22.4)%)</td>
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<tr>
<td>Pspl1.1/pspl1.1 ProPSP/PSP1</td>
<td>72</td>
<td>PSP1/PSP1 (0 (0.0))</td>
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<td></td>
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<td>PSP1/pspl1.1 (0 (0.0))</td>
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<td>pspl1.1/pspl1.1 (72 (100)%)</td>
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<table>
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<tr>
<th>Self-Crosses</th>
<th>No. of Progeny</th>
<th>Antibiotic Resistance</th>
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<tr>
<td>PSP1/pspl1.1</td>
<td>1183</td>
<td>Kanr (%) 782 (66.1)%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kans (%) 401 (33.9)%</td>
</tr>
<tr>
<td>PSP1/pspl1.1 Pro35S/PSP1</td>
<td>1229</td>
<td>Kanr (%) 899 (73.1)%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kans (%) 330 (23.9)%</td>
</tr>
<tr>
<td>PSP1/pspl1.1 ProPSP/PSP1</td>
<td>365</td>
<td>Kanr (%) 268 (73.4)%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kans (%) 97 (26.6)%</td>
</tr>
<tr>
<td>Pspl1.1/pspl1.1 ProPSP/PSP1</td>
<td>479</td>
<td>Kanr (%) 479 (100)</td>
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<td>Kans (%) 0 (0.0)</td>
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<th>Reciprocal Outcrosses</th>
<th>No. of Progeny</th>
<th>Antibiotic Resistance</th>
<th>Transmission Efficiency (%)</th>
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<td>PSP1/pspl1 × Pspl1/pspl1.1</td>
<td>111</td>
<td>Kanr (%) 62 (55.9)%</td>
<td>79.0</td>
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<tr>
<td>PSP1/pspl1.1 × Pspl1/pspl1.1</td>
<td>151</td>
<td>Kanr (%) 81 (53.6)%</td>
<td>86.4</td>
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</table>

Kanr, kanamycin resistant; Kans, kanamycin sensitive.

- *Significantly different from the expected 1:2:1 ratio for normal Mendelian segregation (χ² = 89.84; P < 0.001). Not significantly different from the 1:2 ratio for embryo-lethal effect (χ² = 1.38; P > 0.05).
- *Not significantly different from the expected 1:2:1 ratio for normal Mendelian segregation (χ² = 4.13; P < 0.05).
- *Not significantly different from the expected 1:2:1 ratio for normal Mendelian segregation (χ² = 0.70; P > 0.05).
- *Not significantly different from the expected 1:2:1 ratio for normal Mendelian segregation (χ² = 2.25; P < 0.001).
- *Not significantly different from the expected 1:3 ratio for normal Mendelian segregation (χ² = 0.48; P > 0.05).
- *Transmission efficiency (%) = (mutant/wild type) × 100.
- *Not significantly different from the expected 1:1 ratio for equal transmission efficiency (χ² = 1.52; P > 0.05).
- *Not significantly different from the expected 1:1 ratio for equal transmission efficiency (χ² = 2.25; P < 0.05).

(http://www.seedgenes.org). PCR-based genotyping analysis revealed that the aborted embryos were psp1.1/pspl1.1 individuals.
Figure 4. psp1 Homozygous Mutants Die at the Embryo Stage.

(A) Silique from wild-type (WT) and heterozygous PSP1/psp1.1 plants at 15 and 22 DAP observed with a binocular microscope. In the PSP1/psp1.1 silique, a population of mutant seeds is observed. The right picture shows a close-up of the mutant seeds. Bars = 1 mm.

(B) The micrographs show the embryo development of the wild type and homozygous psp1.1/psp1.1 from the same silique at different stages observed with a differential interference contrast microscope (2, 5, 7, and 10 DAP) or with a binocular microscope (15 DAP). Bars = 50 μm.

(Figure 6C; see Supplemental Figure 5 online). As a consequence of the pollen degeneration, anther locules from psp1.1/psp1.1 Pro3SS:PSP1 plants were almost empty at the late stages of pollen development compared with those of the wild type or psp1.1/psp1.1 ProPSP:PSP1 (see Supplemental Figure 6 online). To confirm which step of gametophyte development was affected by psp1.1 mutation, we performed Hoechst staining of microspore nuclei from psp1.1/psp1.1 Pro3SS: PSP1 plants at different developmental stages (Figure 6B). For this experiment, the most normally shaped microspores at the last stages of development were selected since most of them were seriously malformed. Nucleus staining corroborated that tetrad and polarized microspore development in psp1.1/psp1.1 Pro3SS:PSP1 anthers was normal but that subsequent to the polarized microspore stage, development was arrested and no bicellular or tricellular pollen was found. These results indicate that microspores from psp1.1/psp1.1 Pro3SS:PSP1 are unable to undergo the double mitosis necessary to reach the mature pollen stage. Scanning electron microscopy revealed that mature pollen from the psp1.1/psp1.1 Pro3SS:PSP1 line was shrunken and collapsed and unable to germinate when cultured in vitro (Figure 6C).

Analysis of anther development indicated that the tapetal cell layer was formed in psp1.1/psp1.1 Pro3SS:PSP1 anthers, but tapetum cells displayed irregular development, with partially shrunken protoplasm that was detached from the cell wall at critical stages of pollen development (stages 9 and 10 according to Sanders et al., 1999) compared with wild-type cells (Figure 6D). This may indicate a loss of turgor in the tapetum cells. It is important to note that pollen in psp1.1/psp1.1 Pro3SS:PSP1 anthers initiated the degeneration process at those developmental stages (Figure 6A). A delay in the programmed cell death of the tapetum layer was also observed, since this layer was still present at developmental stage 12 while it was absent in wild-type or psp1.1/psp1.1 ProPSP:PSP1 anthers (Figure 6D; see Supplemental Figure 6 online).

The observed morphological alteration in the tapetum of psp1.1/psp1.1 Pro3SS:PSP1 anthers correlated with the expression of PSP1 in this cell layer, specifically at stage 9 (Figure 6E; see Supplemental Figure 7 online). PSP1 was also clearly expressed in pollen, mainly in stage 12 and beyond (Figure 6E; see Supplemental Figure 7 online).

We next conducted segregation analysis of self-fertilized PSP1/psp1.1 plants that were homozygous for constructs Pro3SS:PSP1 and ProPSP:PSP1. Based on PCR genotyping, and unlike the segregation of self-fertilized PSP1/psp1.1 plants, the segregation of PSP1/psp1.1 Pro3SS:PSP1 displayed a typical Mendelian 1:2:1 ratio (PSP1/PSPPSP1/psp1.1:psp1.1:psp1.1; \( \chi^2 = 4.13, P > 0.05 \)) (Table 1). The same was true for the segregation of self-fertilized PSP1/psp1.1 ProPSP:PSP1 (\( \chi^2 = 0.7, P > 0.05 \)); Table 1). Assuming a hypothesis of male sterility associated with the psp1.1 allele, we should expect psp1.1/psp1.1 Pro3SS:PSP1-GFP individuals to be sterile and psp1.1/psp1.1 ProPSP:PSP1 to be fertile. In both cases, the observed results agreed with the expected calculations according to the transmission efficiency for the PSP1 and psp1.1 alleles (Figure 7). For instance, in the segregation analysis of PSP1/psp1.1 Pro3SS:PSP1, we obtained 18.8% sterile homozygous individuals, which agreed with the expected 20.4%. In the segregation analysis of psp1.1/psp1.1 ProPSP:PSP1, we obtained 26% fertile homozygous individuals, which almost perfectly matched the expected 25%. Based on kanamycin resistance, the segregation analysis of both self-fertilized PSP1/psp1.1 Pro3SS:PSP1 and PSP1/psp1.1 ProPSP:PSP1 matched the 1:3 ratio (sensitive:resistant, \( \chi^2 = 2.25 \) and 0.48, respectively, \( P > 0.05 \)), unlike the 1:2 ratio observed in the PSP1/psp1.1 segregation analysis.
Role of PSP1 in Vegetative Development

To study the function of PSP1 in the vegetative development, we obtained six independent conditional psp1.1/psp1.1 mutant lines from the segregating population of transformed PSP1/psp1.1 plants with a PSP1-GFP construct under the control of the inducible heat shock promoter HS18.2 (ProHSP18.2:PSP1). Based on the induction pattern, two lines, which showed the lowest protein background expression level under noninduced conditions, were selected for further analysis (Figure 8A).

In plates and under noninduced conditions, psp1.1/psp1.1 ProHSP18.2:PSP1 (conditional psp1.1/psp1.1 mutants) showed no significant difference in fresh weight (FW) of the aerial parts of the plant but the root FW was drastically reduced compared with the wild type (see Supplemental Figure 8 online). This reduction in root FW was associated with a short-root phenotype (Figures 8B to 8D), which corresponds well with the specific expression pattern of PSP1 in root meristems (Figures 3A and 3B). The root growth inhibition of the conditional psp1.1/psp1.1 mutants was rescued by both heat shock induction of PSP1 and Ser supply to the growth medium (Figures 8B to 8D). Consequently, both treatments also recovered normal root FW values compared with controls (see Supplemental Figure 8 online).

Further evidence for the role of PSP1 in root development is provided by the phenotypic analysis of transgenic lines with reduced PSP1 expression. For this purpose, we used artificial microRNA (amiRNA) lines. These lines phenocopied the short-root phenotype (Figure 9) and the Ser rescue of root growth of conditional psp1.1/psp1.1 mutants (data not shown).

At the adult stage, the six conditional psp1.1/psp1.1 mutant lines analyzed showed a sterile phenotype before induction and were fertile after heat shock treatment (Figure 5), thus confirming the essentiality of PSP1 for embryo and/or anther development. However, unlike the short-root phenotype, it was not possible to complement the sterile phenotype of these conditional psp1.1/psp1.1 mutants by Ser supply to the growth medium (data not shown).

In order to further test the function of PSP1 in embryo development, ovules of noninduced conditional psp1.1/psp1.1 mutants were cross-pollinated with pollen from heterozygous PSP1/psp1.1 plants. As in heterozygous PSP1/psp1.1 plants, a population of aborted seeds with abnormal embryo development could be observed. In this case, the segregation analysis of 435 seeds revealed a 1:1 ratio (48% mutant:52% normal seeds; $\chi^2 = 0.66; P > 0.05$) as expected.
Figure 6. PSP1 Expression Is Essential for Proper Tapetum and Pollen Development.

(A) Developmental analysis by transmission electron microscopy of pollen (from stages 7 to 12 according to Sanders et al., 1999) in wild-type plants (WT) and homozygous psp1.1/psp1.1 plants transformed with PSP1-GFP cDNA under the control of the 35S promoter (Pro35S:PSP1). Bars = 20 μm.
Metabolic Characterization of Lines Overexpressing and Underexpressing PSP1

PSP activity was increased by 75% in the wild type expressing Pro35S:PSP1 (Oex) and reduced by 57% in amiRNA lines compared with controls (58.5 ± 2.2, 14.5 ± 1.2, and 33.3 ± 4.8 µmol Pi mg protein⁻¹ h⁻¹ in Oex, amiRNA, and the wild type, respectively). The root growth rate in the Oex did not significantly differ from that of the wild type (Figure 9). However, metabolomic analysis revealed a clearly altered metabolite content in both amiRNA and Oex plants compared with the wild type (Table 2; see Supplemental Table 2 online). In the aerial part of both amiRNAs and Oex plants, total amino acid increased compared with the wild type, but Oex plants showed the greatest increase (27% versus 61%, respectively). In roots, metabolite changes differed from those found in the aerial parts and even occasionally, showed an inverse trend. In this organ, amiRNA lines displayed a greater increase in total amino acid levels compared with the wild type (32% versus 15% increase, respectively). Given that no perfect inverse correlation of metabolite levels between Oex and amiRNA lines was found, we focused on either those metabolites that significantly changed more than 1.5 times in only one of the lines (overexpressed or underexpressed) compared with the wild type or those changes in which the Oex/amiRNA ratio was higher than 1.5 or lower than 0.5.

In aerial parts, significant increases in Pro and Orn were noted in Oex lines, while Trp increased in amiRNA lines compared with the wild type (Table 2). A clear significant increase was also found in organic acids participating in either glycolysis or in the tricarboxylic acid (TCA) cycle (citric, glyceric, fumaric, and malic) in the amiRNA lines (but not the Oex lines) in comparison to the wild type. Regarding sugars and sugar alcohols, galactitol, myoinositol, maltose, and raffinose were significantly increased in the Oex lines compared with the wild type. By contrast, 1-O-methyl glucopyranoside significantly increased in amiRNA lines compared with the wild type.

In roots, a change in the Trp levels was observed, being significantly increased in amiRNA but decreased in Oex lines compared with the wild type (Table 2). A drastic reduction in the glyceric acid level was found in the Oex plants, while the level of this metabolite was not significantly altered in the amiRNA plants. By contrast, other glycolytic intermediates, such as Fru-6-P and Glc-6-P, increased in the Oex lines. Finally, Ser levels increased in both aerial parts and roots of both amiRNAs and Oex plants.

Neither the total soluble protein and starch contents of roots and aerial parts of the Oex lines nor the total soluble protein content of the amiRNA lines in roots and aerial parts were significantly altered in comparison to the wild type (Table 2). However, the starch content of amiRNA lines was increased by 40% in the aerial parts in comparison to the wild type.

DISCUSSION

The existence of the PPSB in plants has been known since the 1950s. However, the quantitative contribution and physiological significance of this pathway had not been defined in plants, mainly due to the coexistence of two other pathways with the same metabolic function. By following a gain- and loss-of-function approach in Arabidopsis, we provide here both molecular and genetic evidence for the essential role of the PPSB in plants.

Our data provide strong evidence concerning PSP1 expression in different cell types and under differing environmental conditions, which affords clues as to its function. We have shown that PSP1 is constitutively expressed in all Arabidopsis organs but displays a highly specific cell-type pattern in reproductive organs and roots, with expression in the tapetum, root cap columella, meristematic zone, and root elongation zone. This expression pattern corresponds with the proposed function of PSP1 in embryo, root, and pollen development. It is interesting to note that we found a high PSP1 expression level in the aerial parts of seedlings in comparison to that in roots. These results could indicate a role of the PPSB in green organs, even during the light period. According to our expression data, we suggest that the PPSB may play a more ubiquitous role in different plant organs and under different environmental conditions than initially believed. However, the essentiality of the pathway may be highly cell-type specific.

Inhibiting or enhancing PSP1 expression drastically altered the primary metabolism of Arabidopsis. Although it is difficult to give a complete picture of the metabolic changes associated with PSP1 activity, some conclusions can be drawn from our data. Silencing PPSB in the aerial parts resulted in elevated glyceric

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Figure 6. (continued).

(B) Developmental analysis of pollen (from tetrad to tricellular stage) in wild-type and psp1.1/psp1.1 Pro35S:PSP1 plants visualized by Hoechst staining of microspore nucleus. Bars = 25 µm.
(C) Scanning electron micrographs of pollen grains (left) and light microscopy images of pollen germination assays (right) from the wild type, the wild type transformed with Pro35S:PSP1 (PSP1/PSP1 Pro35S:PSP1), and the endogenous PSP1 promoter (PSP1/PSP1 ProPSP:PSP1); heterozygous PSP1/psp1.1 and homozygous psp1.1/psp1.1 plants transformed with Pro35S:PSP1 and the endogenous PSP1 promoter (ProPSP:PSP1). Bars = 20 µm (left pictures) and 200 µm (right pictures).
(D) Analysis by transmission electron microscopy of anther development (from stages 7 to 12 according to Sanders et al., 1999) in wild-type plants and homozygous psp1.1/psp1.1 plants transformed with Pro35S:PSP1. Top images in each stage: cross sections of anthers. Bottom images in each stage: close-up of tapetum cells. Arrows point to tapetum cells. Bars = 20 µm.
(E) PSP1 expression in the tapetum and pollen at different anther development stages (stages 9 and 12 in anther and pollen, respectively) visualized by confocal microscopy of a PSP1-GFP fusion protein under the control of the endogenous PSP1 promoter expressed in the wild type. GFP fluorescence and the merge images are presented. Bars = 75 µm.
acid, whose phosphate derivatives are important biochemical inter-
mediates in glycolysis, specifically 3-PGA, where glycolysis and
the PPSB diverge. The increase in glyceric acid in the
PSP1-silenced lines was accompanied by an increment in all intermediates
of the TCA cycle that we measured. In the Oex lines these inter-
mediates did not increase and some were even reduced in com-
parison to the wild type. It thus seems reasonable to assume that
inhibition of the PPSB in the aerial parts is able to increase the
glycolytic flux to the TCA cycle, which would in turn have im-
portant consequences for plant growth and development.

In roots, silencing the PPSB did not produce the same effect
on glycolytic metabolites and the TCA cycle as in the aerial parts, although Oex plants clearly showed a lowered glyceric acid level, hinting at a depletion of 3-PGA in these lines as a result of enhancing the PPSB. The different behavior of the TCA cycle in roots and aerial parts could be related to the lack

of photosynthetic and photorespiratory activity in roots, which
provide respiratory substrates and Ser to the aerial parts. In
support of this hypothesis is the considerable body of evi-
dence for regulatory links between photorespiration and the
TCA cycle (Timm et al., 2012).

Altering the PPSB also clearly changed the biosynthesis of
several amino acids. The amino acid Trp proved to be the com-
mon link between aerial parts and root metabolism in both
PSP1-underexpressing and -overexpressing lines. The Oex/amiRNA Trp
t ratio was 0.2 in both aerial parts and roots. This amino acid de-

v- tives from phospho- enol pyruvate, a metabolite downstream of
3-PGA in the glycolytic pathway.

As expected, we observed that the Ser levels of both aerial
parts and roots of Oex plants increased. It is conceivable that all
of the other alterations observed in the metabolite profiling of
these lines could be a consequence of this increase in Ser. We
also saw that the Ser level increased in the amiRNA plants, which was somewhat unanticipated. The elevated Ser level in the aerial parts of amiRNA plants could be explained by an activation of the other pathways for Ser biosynthesis, especially the glycolate pathway, in order to compensate for the lack of PPSB activity in nonphotosynthetic organs. Ser provided through the glycolate pathway could be transported to amiRNA roots through the phloem, thus accounting for the high Ser levels noted in these organs. However, a cause/effect correlation between Ser deficiency and the root developmental arrest observed in amiRNA lines cannot be rationalized unless other parameters are taken into account. Ser measurements reflect the mean amino acid content in the whole organ. Thus, it is possible that Ser deficiency is restricted only to specific cell types, such as root meristematic and columella cells where PSP1 is specifically expressed, and that such local Ser deficiencies provoke root growth inhibition. Thus, the high Ser level in whole amiRNA roots could result from the inhibition of root growth.
growth, which in turn could mask Ser deficiency in specific cell types. One likely consequence of root growth inhibition in amiRNA lines is that most of the amino acids determined were also accumulated in this organ. In fact, the level of 19 out of 23 measured amino acids increased in amiRNA roots compared with the wild type. Similarly, the starch level in the aerial parts measured amino acids increased in amiRNA roots compared with the wild type. Similarly, the starch level in the aerial parts increased in amiRNA lines compared with wild type. One likely consequence of root growth inhibition in amiRNA lines is that most of the amino acids determined were synthesized autonomously by the embryo itself. At the globular-heart transition stage, many metabolic pathways are established in the embryo. Specifically, the first step of differentiation of proplastids, where PSP1 is localized, occurs at the globular-to-heart transition phase (Devic, 2008). The delay in psp1.1/psp1.1 mutant embryo development at the globular stage is probably related to the establishment of the PPSB in the embryo.

The role of Ser and/or Ser derivatives in plant reproduction has been recently demonstrated (Michard et al., 2011; Yamaoka et al., 2011). Michard et al. (2011) showed that o-Ser is involved in a plant signaling mechanism between male gametophyte and pistil, affecting pollen tube growth and morphogenesis. Yamaoka et al. (2011) showed that mutants of phosphatidylserine synthase (PSS1), an enzyme involved in phosphatidylserine biosynthesis, exhibit similar phenotypes as psp1.1/psp1.1 mutants, that is, diminished fertility due to inhibition of pollen maturation and to a high embryo abortion rate. However, we discovered differences between the mutants. For instance, reciprocal crossing revealed that the male mutant allele pss1 has a decreased transmission rate. We found no significant difference in the transmission efficiency of the psp1.1 mutant and male and female alleles compared with the wild-type allele, indicating no gametophytic defect unless gametes are produced by homozygous anthers (psp1.1/psp1.1 Pro35S:PSP1-GFP). The segregation analysis of heterozygous PSP1/psp1.1 and cross-pollinated ovules of noninduced conditional psp1.1/psp1.1 mutants with pollen from heterozygous PSP1/psp1.1 plants corroborated that male psp1.1 alleles are viable if produced by heterozygous anthers where PSP is expressed. According to our data, the only possibility that defects in the male psp1.1 gametophyte could be specifically associated with PSP activity in pollen is if premeiotic gene expression in diploid heterozygous microsporocytes enables all mutant gametophytes to survive the loss of PSP1 in homozygous gametenes, as has already been suggested for other essential genes (Muralla et al., 2011). In our case, this possibility is improbable, since we were unable to detect PSP1 expression in premeiotic sporogenous cells.

Several of the genes regulating tapetum development have been found to affect pollen viability and/or development (Colcombet et al., 2005; Yang et al., 2007). Accordingly, our results indicate a temporal correlation between PSP expression in the tapetum (stages 9 and 10 according to Sanders et al., 1999) and the developmental alterations in psp1.1/psp1.1 Pro35S:PSP1-GFP tapetum and microspores. These stages are known to be critical for pollen maturation (Ge et al., 2010). PSP1 was also expressed in pollen but in later stages (stages 11 to 14) where psp1.1/psp1.1 Pro35S:PSP1-GFP mutant microspores already displayed altered development.

Cells from the tapetum and microspores develop as "communicating" neighbors. Early in development, tapetum cells...
expression during the post-tetrad stages in the tapetum and that an essential step in microspore development is PSP1 cell layer during the critical stages of pollen maturation suggest tapetum along with the specific PPSB activity affects the proper development of both cell types, microspore maturation. We propose that the absence of PSP1 are involved in the secretion of molecules into the locule for anthers, we contend that it is possible that incorrect co-

are involved in the secretion of molecules into the locule for microspore maturation. We propose that the absence of PSP1 activity affects the proper development of both cell types, which in turn affects pollen maturation. The developmental modifications observed in the psp1.1/psp1.1 Pro35S:PSP1-GFP tapetum along with the specific PSP1 expression in this cell layer during the critical stages of pollen maturation suggest that an essential step in microspore development is PSP1 expression during the post-tetrad stages in the tapetum and not in the microspore itself. Proper timing of programmed cell death in the tapetum is required for normal microsporogenesis (Kawanabe et al., 2006). Since we found delayed degeneration of the tapetum cell layer in the psp1.1/psp1.1 Pro35S:PSP1-GFP anthers, we contend that it is possible that incorrect coordination of the timing between the degeneration of tapetum and microspore development could ultimately have affected pollen maturation.

During embryo and anther development, cells actively divide. The root apical meristem, where PSP1 is clearly expressed, also undergoes intense mitotic activity. Our results indicate that the PPSB plays an essential role in these nonphotosynthetic, actively dividing cells. In the last years, considerable information linking the PPSB with the regulation of cell proliferation and oncogenesis in mammals has appeared (Baeget al., 2011; Locasale et al., 2011; Pollari et al., 2011; Possemato et al., 2011). Similarly, the function of this pathway in plant embryo, anther, and root development could be manifested via the regulation of cell proliferation. In both mammals and plants, Ser is crucial for methyl group transfer by providing one carbon units associated with primary metabolism in plants and animals. (Eichler et al., 1981). Thus, Ser could be the link connecting the PPSB with the regulation of cell cycle progression. Given the evidence for the one-carbon metabolism taking place in Arabidopsis plastids (Zhang et al., 2010), the arrested root and embryo development in the psp1.1/psp1.1 mutant would suggest that the PPSB is required to supply Ser for the proper functioning of one-carbon metabolism in some non-photosynthetic cells. If this idea proves correct, it opens up the possibility of evolutionarily conserved signaling mechanisms associated with primary metabolism in plants and animals.

### Conclusions

PSP1 is expressed in photosynthetic and nonphotosynthetic organs but plays an essential function only in very specific cell types, probably because the other Ser pathways in these cell types cannot compensate for the lack of PPSB activity. We
provided molecular, morphologic, physiological, and genetic evidence for the essential role of the PPsb in male gametophyte and embryo development. We have also proven that this pathway is indispensable for proper root growth. It is well known that metabolism is intimately related to development, but the connecting links between these processes remain unknown. Although it is difficult to separate the metabolic and regulatory functions of a metabolic pathway, the PPsb might be one of the main actors connecting primary metabolism with development.

Finally, we provided tools that will allow the future study of this pathway under different environmental conditions and its interactions with other Ser biosynthetic pathways.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana seeds (ecotype Columbia 0, Col-er 105) were supplied by the European Arabidopsis Stock Center (Scholl et al., 2000). Unless stated otherwise, seeds were sown on 0.8% agar plates containing one-fifth-strength Murashige and Skoog (MS) medium with Gamborg vitamins as previously described (Muñoz-Bertomeu et al., 2009). Some plates were supplemented with 0.1 mM Ser as indicated in the legends to the figures. The optimal concentration of Ser was previously determined (Muñoz-Bertomeu et al., 2009). For the selection of Psp1 transgenic plants, half-strength MS plates supplemented with 0.5% Suc and appropriate selection markers were used. Some plantlets and seeds were also grown in greenhouse conditions as described elsewhere (Muñoz-Bertomeu et al., 2009). Where indicated, conditional psp1.1/psp1.1 mutants were treated for 45 min at 37°C daily before and after pollination.

Primers

All primers used in this work are listed in Supplemental Table 3 online.

Mutant Isolation and Characterization

The psp1.1 (SALK_062391) allele of the At1g18640 gene was identified in the SGNAL Collection database at the Salk Institute (Alonso et al., 2003). Mutants were identified by PCR genotyping using gene-specific primers and left border primers of the T-DNA insertion. PSP_RP and PSP_LP were the gene-specific primers for psp1.1 allele. The T-DNA insertion was confirmed by sequencing the fragment amplified by the T-DNA internal borders (LBB1.3) and gene-specific primers.

Tilling mutants were provided through the Seattle TILLING Project (http://tilling.hcrg.org/), which performed a high-throughput reverse genetic screening to identify ethyl methanesulfonate–induced mutations in the Col-er 105 background (Till et al., 2003). As a result, a psp1.1 allele (psp1.2) with a S178F amino acid substitution was identified. Tilling mutants were identified by PCR amplification of the target sequences with primers S178F2 and S178FRev and by restriction analysis of the PCR product with the enzyme EcoRV, which differentiates between the wild-type and the mutant alleles.

Cloning and Plant Transformation

For gene promoter-reporter fusions, a 1.4-kb fragment corresponding to the native Psp1 promoter was fused to the GUS gene in pCAMBIA1303, giving ProPsp-GUS. The cDNA corresponding to Psp1 (At1g18640) was placed under the control of three different promoters: the constitutive 35S promoter (Pro35S), the native Psp1 promoter (ProPsp), and the heat shock–inducible promoter of gene At5g59720 (ProHSP18.2) (Matsuhara et al., 2000) giving constructs Pro35S:Psp1, ProPsp:Psp1, and ProHSP18.2:Psp1, respectively. The Psp1 cDNA was cloned in the pCR8/GW/TOPO plasmid and subcloned in the plasmid pMDC83 (Curtis and Grossniklaus, 2003). This plasmid allowed us to clone Psp1 in frame with a GFP cDNA at the C-terminal position (PSG-GFP), giving the Pro35S:Psp1 vector. This vector was used to obtain constructs ProPsp:Psp1 and ProHSP18.2:Psp1. The 35S promoter of Pro35S:Psp1 was exchanged with the native Psp1 promoter of ProPsp-GUS, giving the ProPsp:Psp1 vector. The 35S promoter of Pro35S:Psp1 was also exchanged for the promoter region of gene Psp1.2, giving the ProHSP18.2:Psp1 vector. The HSP18.2 promoter was obtained from PT7101 plasmid (Matsuhara et al., 2000).

amiRNAs were generated to target Psp1 using the Web microRNA designer (http://wmd2.weigelworld.org/cgi-bin/mirnatoools.pl). The amiRNAs were cloned according to the protocol by Rebecca Schwab in Detlef Weigel’s laboratory (http://wmd2.weigelworld.org/themes/amiRNA/pics/Cloning_of_artificial_microRNAs.pdf). All PCR-derived constructs were verified by DNA sequencing. Additional cloning information is provided in Supplemental Table 4 online.

Arabidopsis plants were transformed with the different constructs using the floral dipping method (Clough and Bent, 1998) with Agrobacterium tumefaciens carrying psou. For the amiRNA and GUS lines, the wild type was used. As psp1.1/psp1.1 is embrylo lethal, whenever necessary, the progeny of heterozygous plants (Psp1/psp1.1) was transformed with the different constructs. Transformants were selected by antibiotic selection, while homozygous psp1.1/psp1.1, heterozygous Psp1/psp1.1, and the wild type were identified by PCR genotyping using gene-specific primers (PSP_RP and PSP_LP) and left border primers of the T-DNA insertions (LBB1.3). At least four independent single insertion homozygous T3 lines were obtained for all different constructs. After characterization by RT-PCR, two to five different lines were selected for further analysis depending on the experiment. We used both syngenic wild-type lines as well as wild-type Columbia-0 as controls for our studies. Syngenic plants were the wild-type progeny from the segregation of heterozygous transgenic Psp1/psp1.1 plants. For amiRNAs, we used as controls the wild type used for transformation with the amiRNAs.

RT-PCR

Total RNA was extracted from seedlings and adult plants using the NucleoSpin RNA II kit (Macherey-Nagel). RNA (0.5 to 1 μg) was reverse transcribed using polyT primers and the Power SYBR Green PCR Master Mix (Applied Biosystems) with the Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. Real-time quantitative PCR was performed using a 5700 sequence detector system (Applied Biosystems) with different internal standards were selected (Czechowski et al., 2005) depending on the efficiency of the primers. Relative mRNA abundance was calculated using the comparative cycle threshold method according to Pfaffl (2001). Primers used for PCRs are listed in Supplemental Table 3 online.

Pollen Germination and GUS Activity Assays

In vitro pollen germination assays were done using the optimized solid medium described by Boavida and McCormick (2007) as previously described (Muñoz-Bertomeu et al., 2010b). For GUS activity assays, plant organs were incubated in GUS buffer (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid [X-GlcA; Duchefa] overnight at
37°C. Plant material was cleared in 70% ethanol before microscopy observation. At least six independent transgenic lines showed identical GUS-staining patterns, which differed only in the expression level of GUS. Images were acquired with a Leica DM1000 microscope and a Leica DC350 digital camera.

Microscopy
For transmission electron microscopy of anther and pollen, inflorescences containing buds at different developmental stages from wild-type, psp1.1/psp1.1 Pro35S:PSP1-GFP, and psp1.1/psp1.1 ProPSP: PSP1-GFP plants were collected and classified into different developmental groups according to Smyth et al. (1990). Buds from the same group were removed from plants, fixed with 2.5% glutaraldehyde, and postfixed in 1% osmium. Tissue was dehydrated in an ethanol series and infiltrated with LR-White resin. Ultrathin sections (60 nm) were examined with a JEOL 1010 transmission electron microscope at 60 kV equipped with a MegaView III digital camera with Analysis software.

For differential interference contrast microscopy, siliques from heterozygous PSP1/psp1.1 plants were dissected longitudinally at 2, 5, 7, and 10 DAP. Ovules from individual siliques were morphologically classified, cleared in Hoyer’s solution, and observed with a Nikon ECLIPSE E800 compound microscope equipped with Nomarski differential interference contrast optics and a Nikon DXM1200F digital camera with Nikon ACT-1 software. Bright-field photographs of 15-d-old hand-dissected embryos submerged in water were obtained using a stereoscopic zoom microscope Nikon SMZ 1500 equipped with a DS-R1 digital sight camera.

For scanning electron microscopy, pollen was mounted on standard stubs and coated with gold-palladium prior to observation under a field emission microscope (Hitachi S-4100). GFP fluorescence was observed with a confocal microscope (Leica TCS-SP).

For nucleus staining with Hoechst, inflorescences containing buds at different developmental stages from wild-type and psp1.1/psp1.1 Pro35S:PSP1-GFP plants were collected and classified in different groups as described for transmission electron microscopy. Buds from the same group were dissected on a microscope slide, and pollen grains released from anthers were stained with 10 µg/mL Hoechst dye. Samples were observed with Nikon Eclipse E800 compound microscope equipped with an epifluorescence module and a Nikon DXM1200F digital camera with Nikon ACT-1 software.

Metabolite Determination and PSP Activity
Aerial parts and roots of 18- to 21-d-old wild-type, Oex, and amiRNA lines were used to determine metabolite content. Starch was determined with the Encytec starch kit (ATOM). The protein content was quantified using the Bio-Rad protein assay kit. The levels of other metabolites were determined in derivatized methanol extracts by gas chromatography–mass spectrometry using the protocol defined by Lisec et al. (2006).

PSP activity was measured in at least six biological replicates of wild-type, Oex, and amiRNA root extracts according to Ho et al. (1999a).

Immunoblotting
For immunoblots, crude protein extracts from whole wild type and conditional psp1.1/psp1.1 mutants were obtained by harvesting 0.5 g plant material in liquid nitrogen and grinding in 1 mL ice-cold homogenization buffer (140 mM NaCl, 8 mM Na2HPO4, 7H2O, 2 mM KH2PO4, and 10 mM KCl, pH 7.4) with 1% protease inhibitor cocktail (Sigma-Aldrich; P-8399), followed by two successive centrifugations (15,000g for 15 min at 4°C). Protein samples were quantified with Bradford reagent (Bio-Rad) using BSA as a standard, and then 100 µg total proteins was separated in 8% SDS-PAGE. Proteins were electrotransferred onto immunoblot nitrocellulose membranes (Bio-Rad) using the Mini Tran-Blot Cell (Bio-Rad) for 1 h at 100 V with transfer buffer (17 mM Tris, 192 mM Gly, and 20% [v/v] methanol). Immunoblots were probed with anti-GFP antibodies (Molecular Probes; ref. A-11122) at a final dilution of 1:20,000. Ponceau S-stained membranes are shown as loading controls. Cross-reacting bands were detected using the ECL select Western-blotting detection reagent kit (Amersham Biosciences).

Statistics
Experimental values represent mean values and ±; n represents the number of independent samples. P values were calculated with a Student’s t test (two-tailed) using Microsoft Excel. The level of significance was fixed at 5% (0.05), representing the probability of error if the hypothesis of a significant difference between mean values was accepted. In the segregation analysis, data were tested to evaluate if the observed values agreed with Mendelian proportions using the χ² test. P values higher than 0.05 indicate that the observed values are not significantly different from the expected ratio. P values lower than 0.001 indicate that the observed values differ significantly from the expected ratio.

Accession Number
Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession number At1g18640 (PSP).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Simplified Scheme Showing the Main Metabolites Synthesized from Serine.

Supplemental Figure 2. In Silico Analysis of PSP1 Expression Pattern.

Supplemental Figure 3. Subcellular Localization of PSP1 by Stable Expression of PSP1-GFP Fusion Construct under the Control of the 3SS Promoter in Arabidopsis.

Supplemental Figure 4. Genomic Organization of psp1.1 T-DNA Mutant Line.

Supplemental Figure 5. Developmental Analysis of Pollen in Homozygous psp1.1/psp1.1 Plants Transformed with PSP1-GFP cDNA under the Control of the Endogenous PSP1 Promoter by Transmission Electron Microscopy.

Supplemental Figure 6. Light Microscopy Images of Sectioned Anthers from Wild-Type and Homozygous psp1.1/psp1.1 Plants Transformed with PSP1 under the Control of Either the 3SS Promoter (Pro3SS:PSP1) or the Endogenous PSP1 Promoter (ProPSP:PSP1) at Stage 12.

Supplemental Figure 7. PSP1 Expression in the Anther and Pollen at Different Anther Development Stages.

Supplemental Figure 8. Fresh Weight of Wild-Type and Conditional psp1.1/psp1.1 Mutant Lines under Different Growth Conditions.

Supplemental Table 1. Significantly Enriched Consensus Sequences in the Promoter Regions of PSP1.

Supplemental Table 2. Metabolite Levels in the Aerial Parts and Roots of Wild-Type, PSP1-Silenced, and PSP1-Overexpressing Plants.

Supplemental Table 3. List of Primers Used in This Work.

Supplemental Table 4. Constructs.
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AUTHOR CONTRIBUTIONS


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The Phosphorylated Pathway of Serine Biosynthesis Is Essential Both for Male Gametophyte and Embryo Development and for Root Growth in Arabidopsis

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