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# Sulfurtransferases 1 and 2 Play Essential Roles in Embryo and Seed Development in *Arabidopsis thaliana**[S]

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**Sulfurtransferases (STRs) catalyze the transfer of a sulfur atom from a donor to a suitable acceptor molecule. The *Arabidopsis thaliana* genome encodes 20 putative STR proteins. The biological functions of most are unclear. We found that STR1 and STR2 play important roles in embryo/seed development. Mutation of *STR1* alone resulted in a shrunken seed phenotype, although growth and development of vegetative and reproductive organs were not affected. The shrunken seed phenotype was associated with the delayed/arrested embryo development, in most cases, at the heart stage. The embryo defect of *str1* mutant is not fully penetrant. Approximately 12.5% of embryos developed further and formed normal looking seeds. In severely shrunken seeds, no embryo could be identified after seed collection. Partially shrunken seeds that contained viable embryos could still germinate. However, cotyledons of the seedlings from such seeds were abnormal. An *STR1-GUS* fusion reporter revealed that the *STR1* gene was universally expressed, with high levels of expression in specific tissues/organs including embryos. The incomplete penetrance of *str1* embryo/seed phenotype is a result of functional *STR2*. Single *str2* mutant had no phenotype. However, no *str1*$^{-/-}$/*str2*$^{-/-}$ double mutant embryos were able to develop past the heart stage. Furthermore, *STR2* is haplo-insufficient in *str1* mutant background, and *str1*$^{-/-}$/*str2*$^{+/-}$ embryos were 100% lethal. These data provide new insights into the biological functions of the ubiquitous sulfurtransferase in *Arabidopsis* embryogenesis and seed development.**

Sulfurtransferases (STRs,[3] or STs), also known as rhodaneses, are ubiquitous enzymes that catalyze the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. In animals, sulfurtransferases function in cyanide detoxification (1–3), hydrogen sulfide detoxification (4), sulfur metabolism (5, 6), and synthesis or repair of iron-sulfur proteins (7, 8). However, information about their biological functions in plants is limited. In *Arabidopsis*, there are 20 putative sulfurtransferase proteins containing either one or two rhodanese domains. Based on amino acid sequence homology, these proteins are classified into six groups (Groups I–VI) (9). *STR* gene expression has been examined during plant defense response and senescence and under different growth conditions including various sulfate concentrations, phosphate deficiency, and different diurnal light/dark cycles (9–12). *AtSTR15* gene expression is enhanced by different hormone treatments (13, 14), and AtSTR13/SIR1 is a key regulator of many auxin-inducible genes (15). These findings suggest that sulfurtransferases may play divergent roles in plant growth, development, stress response, and metabolism.

STR1 and STR2 share high sequence identity. They are the only two members in the Group I sulfurtransferase, which is characterized by having two rhodanese domains. Recombinant STR1 and STR2 have similar $K_m$ values, and both prefer 3-mercaptopyruvate to thiosulfate as a substrate; as a result, they are also called mercaptopyruvate sulfurtransferase 1 (MST1) and MST2 (16–18). However, they have different localizations in cells. STR1 is localized in mitochondria, whereas STR2 is localized in the cytosol (19, 20). Their potential functions in plant senescence and cyanide detoxification have been investigated (11). It was reported that ethylene as well as its precursor, 1-aminocyclopropane-1-carboxylic acid, could induce the expression of *STR1*. *STR1* expression is also elevated in plants grown under low sulfate conditions and low phosphate conditions and in the presence of thiosulfate. However, total sulfurtransferase activity is significantly changed only under low phosphate conditions (9). In the *Arabidopsis* life cycle, *STR1* and *STR2* gene expression and total sulfurtransferase activity increase continuously as the plant ages, suggesting that STR1 and STR2 may be involved in senescence (9, 17, 18).

In this report, we demonstrate that *Arabidopsis STR1* and *STR2* play an important role in embryo and seed development. Null *str1* mutant alleles show a shrunken seed phenotype, a result of defective embryo development. Expression of a wild-type *STR1* or *STR1-GUS* fusion can fully complement the mutant phenotype. An *STR1-GUS* reporter revealed that the *STR1* gene is ubiquitously expressed, with high levels of expression in certain tissues/organs including embryos, which is consistent with a role in embryo development. In contrast, no change in the progress of senescence was observed in *str1*

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[S] The on-line version of this article (available at http://www.jbc.org) contains a supplemental method, Table I, and Figs. S1–S5.

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[3] The abbreviations used are: STR, sulfurtransferase; GUS, β-glucuronidase; MST, mercaptopyruvate sulfurtransferase; TST, thiosulfate sulfurtransferase; DAP, day(s) after pollination; DR, direct repeat; Rubisco, ribulose-bisphosphate carboxylase/oxygenase; T-DNA, transfer DNA.

mutant despite a reduction of ~80% of the total cellular MST activity. The embryo/seed developmental defect in *str1* single mutant is not fully penetrant, and about 12.5% of homozygous *str1* embryos are able to develop to full term and form normal looking seeds because of functional STR2. Single *str2* mutant has no embryo/seed defect. However, mutation of *STR2* in *str1* background results in complete embryo lethality. Neither $str1^{-/-}/str2^{-/-}$ nor $str1^{-/-}/str2^{+/-}$ embryo can develop past the heart stage. The *Arabidopsis* genome contains 20 *STR* genes, and the biological functions of most are unclear. This research provides new insight into the biological functions of STR1 and STR2 in plant embryo and seed development.

## EXPERIMENTAL PROCEDURES

*Plant Material and Growth Conditions*—Mutant and wild-type plants in *Arabidopsis thaliana* ecotype Columbia (Col-0) were used in all experiments. Plants were grown under long day conditions (16-h light/8-h dark cycle) with about 100 microeinsteins $m^{-2}$ $s^{-1}$ light at 22 °C. For observation of seedling phenotypes, sterilized seeds were plated on a half-strength Murashige and Skoog medium with 0.6% agar. Plates were incubated in a growth chamber at 22 °C under continuous light (70 microeinsteins $m^{-2}$ $s^{-1}$).

T-DNA insertion alleles of *STR1* (At1g79230), *str1-1* (SALK-015593), *str1-2* (SAIL_69_D10), *STR2* (At1g16460), and *str2* (SALK_067994) were obtained from the *Arabidopsis* Biological Resource Center (21, 22). Homozygous null mutants were screened by genomic PCR using gene-specific primers, 5′-gcaaatagttttggcgtctttc-3′ and 5′-taataaaggcagacaccgaaca-3′ for *str1*, and 5′-tgaagaagcagtttgaacagga-3′ and 5′-aaatctgtttatacgcggagga-3′ for *str2*. Data presented in this report were collected mostly from *str1-1* alleles, and those from *str1-2* alleles were supplied in the supplemental figures.

*Total RNA Extraction and RT-PCR*—Total RNA was extracted using TRIzol reagent (Invitrogen). After reverse transcription, *STR1* cDNA was amplified using gene-specific primers, 5′-atggcctcgacccttttct-3′ and 5′-taataaaggcagacaccgaaca-3′.

*Protein Extraction and Sulfurtransferase Activity Assay*—Protein was extracted from seedlings and various organs of mature plants as described (23). The concentration of protein extracts was determined by using a Bio-Rad protein assay kit. Sulfurtransferase activity was determined as reported (18). The MST enzymatic activity was assayed with 50 μg of total proteins in a reaction buffer consisting of 0.1 M Tris-HCl, pH 9.0, 10 mM KCN, 5 mM β-mercaptoethanol. The reaction was initiated by the addition of 3-mercaptopyruvate to a final concentration of 5 mM. The total volume of each assay reaction was 1 ml. After being incubated at 37 °C for 40 min, the reaction was stopped by the addition of 200 μl of acidic iron reagent (50 g $liter^{-1}$ $FeCl_3$, 200 ml $liter^{-1}$ 65% $HNO_3$). The absorption was read at 460 nm. The thiocyanate formation represents the activity. Thiosulfate sulfurtransferase (TST) enzymatic activity was assayed as described above and was initiated by the addition of 5 mM $Na_2S_2O_3$.

*Constructs and Plant Transformation*—To make the *STR1* complementation construct, we PCR-amplified the full-length *STR1* genomic DNA, including the 1109-bp region upstream of the ATG start codon and 260-bp region downstream of the stop codon, using *STR1-F1* (5′cccaagcttagaggtgttgcgcagagtcat3′) and *STR1-B1* (5′gctctagaaatcgttgagaaatttcctgg-3′) primers. The fragment was cloned into SmaI-digested pCAMBIA2300 to generate the final construct of $Pro_{STR1}$:*STR1*. For the GUS fusion construct, a SmaI site was introduced before the TGA stop codon by PCR using *STR1-S1* (5′gggtgaaagtctccaccgtaaaggctagcgac3′) and *STR1-G1* (5′gggtgaagaagattcaacactctctatgggc3′) primers. The cDNA of GUS was amplified by using primers (5′atgttacgtcctgtagaaaccccaacccg3′ and 5′tcattgtttgcctccctgctgcg3′) from the pBI121 vector. The fragment was then inserted into the SmaI-digested $Pro_{STR1}$:*STR1* to generate the final $Pro_{STR1}$:*STR1-GUS* construct. Both constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and then transformed into wild-type or *str1-1* mutant plants by the floral dipping method (24).

*DR5:GUS* reporter construct, which contains an auxin-responsive promoter with direct repeat (DR) elements, is commonly used to monitor endogenous auxin levels/activities (25–27). In this report, a *DR5:GUS* construct (25) in pBI101 backbone was transformed into Col-0 and *str1-1* plants. Embryos from *DR5:GUS* transgenic Col-0 and *str1-1* plants were dissected out of siliques and stained for GUS activity.

*Histological Analysis*—For GUS expression assays, various tissues from transgenic plants were incubated in GUS staining solution (10 mM EDTA, 0.1% Triton X-100, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, and 1 mg $ml^{-1}$ X-glucuronide in a 50 mM sodium phosphate buffer, pH 7.0) at 37 °C. After staining, the solution was replaced with 75% ethanol to remove chlorophyll. GUS expression was examined by using an Olympus microscope.

For morphological studies of embryos, the siliques were fixed in an ethanol:acetic acid (3:1, v/v) solution and then cleared in Hoyer's solution(28). The seeds were dissected out and observed on a microscope (Leica CTR 5000) equipped with Nomarski optics.

For vein patterning, cotyledons of 7-day-old seedlings were fixed in an ethanol:acetic acid (3:1, v/v) solution for 3 h and then treated with 75% ethanol to remove chlorophyll. The samples were cleared using Hoyer's solution, and vein patterns were observed with a microscope.

## RESULTS

*STR1 Is Required for Normal Seed Development in Arabidopsis*—The *STR1* (At1g79230) gene is located on chromosome 1 and has 12 exons and 11 introns. To determine the function of *STR1 in planta*, we identified two T-DNA insertion mutants, both in Columbia-0 (Col-0) background. SALK_015593 (*str1-1*) has an insertion in the seventh intron, and SAIL_69_D10 (*str1-2*) has an insertion in the seventh exon (Fig. 1*A*). Reverse transcription (RT)-PCR analysis of the total RNA from mutant seedlings was performed using *STR1* gene-specific primers. As shown in Fig. 1*B*, the *STR1* transcript was not detected in *str1-1* or *str1-2* mutant plants, demonstrating that both mutants are transcript-null.

We then compared the sulfurtransferase activities in total protein extracts prepared from 2-week-old seedlings, rosette leaves, flowers, and siliques of the wild-type and *str1-1* mutant

plants. Two sulfur donors, 3-mercaptopyruvate and thiosulfate, were used to determine the MST and TST activities, respectively. We found that total *Arabidopsis* protein extracts had very low TST activity (Fig. 2*A*), and in contrast, MST activity was much higher (Fig. 2*B*). In the *str1-1* mutant seedlings, TST and MST activities were both lower than those in the wild-type seedlings, especially the MST activity (Fig. 2, *A* and *B*). In extracts prepared from various organs of *str1-1* plants, MST activity was only about 20% of that in the wild type (Fig. 2, *B* and *C*). In addition, we found that MST activity was higher in flowers and siliques than in leaves and 2-week-old seedlings. The major reduction of MST activity in the *str1* mutant provided genetic evidence that the *STR1* gene encodes the major MST activity in *Arabidopsis* and that other members in the *STR* gene family are minor contributors. The high reduction of MST activity in the *str1* mutant is also consistent with the previous finding that recombinant STR1 enzyme prefers 3-mercaptopyruvate as its substrate rather than thiosulfate (18).

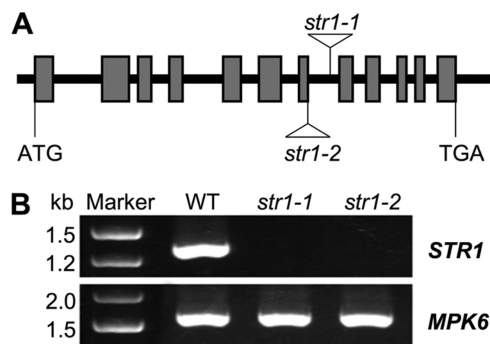


FIGURE 1. **Identification of two null alleles of *str1* mutant from T-DNA mutant collections.** *A*, structure of the *STR1* gene showing the positions of the T-DNA insertions in *str1-1* and *str1-2* mutants. *B*, levels of *STR1* transcripts in wild-type (WT), *str1-1*, and *str1-2* were determined by RT-PCR. The *MPK6* transcript level was used as a control.

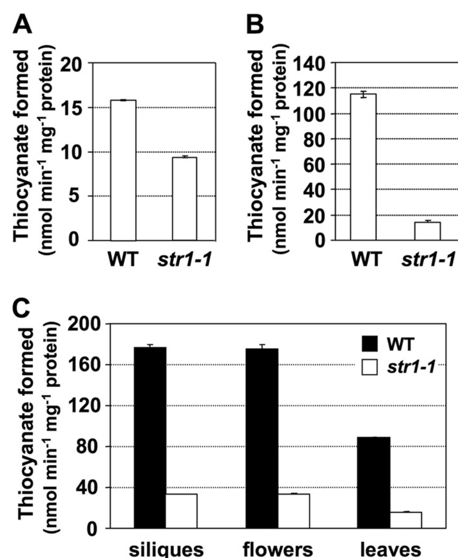


FIGURE 2. **Mutation of *STR1* gene greatly reduces the sulfurtransferase activity in *Arabidopsis*.** *A*, TST activity in WT and *str1-1* seedlings. *B*, MST activity in WT and *str1-1* seedlings. *C*, comparison of MST activity in various organs of WT and *str1-1* plants. Values are the means ± S.E. of three replicates.

The mutant *str1* plants displayed no visible phenotypic alterations with respect to plant size, morphology, and progress of senescence under our growth conditions. The rosette leaf size, mature plant height, flowers, and siliques in *str1* were all similar to wild type (Fig. 3, *A*–*E*), suggesting that the *STR1* gene is not essential to the vegetative growth/development and the formation of reproductive organs. However, the majority of seeds harvested from homozygous *str1* mutant plants were shrunken in size with a wrinkled appearance (Fig. 3, *F* and *G*), suggesting that the *STR1* gene is essential for normal seed development. To further investigate the seed phenotype in *str1* mutants, we calculated the percentage of shrunken seeds in siliques (supplemental Table 1). In siliques from wild-type plants, about 0.6% of seeds were shrunken. However, 87.5% of seeds in siliques from *str1-1* homozygous mutant plants were shrunken. The rest appeared to be normal, suggesting that the seed phenotype of *str1* mutant is not fully penetrant. About 20% of seeds were shrunken in siliques from *str1-1* heterozygous plants, indicating that only homozygous *str1* embryos may result in defective seeds. Theoretically, 25% of the progenies from a heterozygous plant should be homozygous. Because not all homozygous *str1* seeds were shrunken, we expected less than 25% of shrunken seeds from a heterozygous plant, which is consistent with our experimental observation.

FIGURE 3. **Mutation of *STR1* gene does not affect the growth/development of vegetative and reproductive organs but does cause the shrunken seed phenotype.** *A* and *B*, rosettes of the wild-type (*A*) and *str1-1* mutant (*B*). *C*, the mature wild-type (*left*) and *str1-1* (*right*) plants. The *inset* shows siliques from wild-type (*left*) and *str1-1* mutant (*right*) plants. *D* and *E*, top views of wild-type (*D*) and *str1-1* mutant (*E*) inflorescence. *F* and *G*, mature seeds from wild-type (*F*) and *str1-1* mutant (*G*). *Bars* = 1 cm.

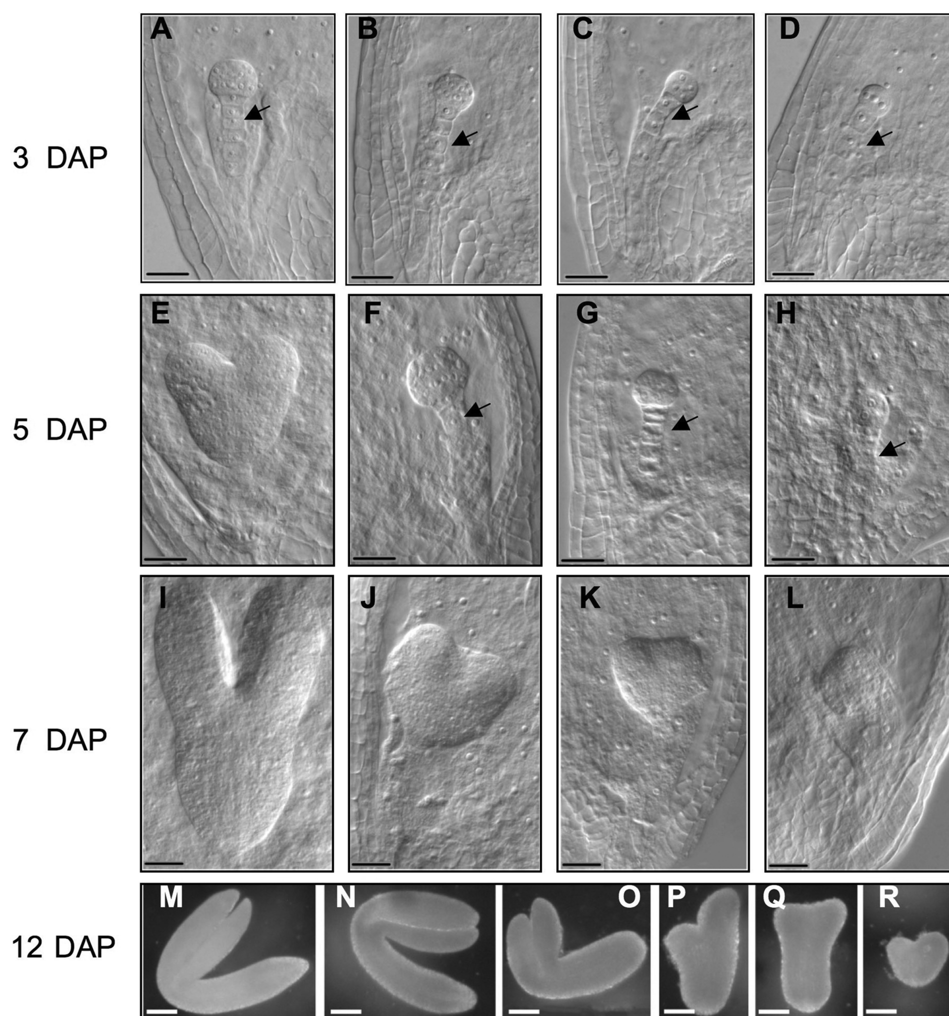


FIGURE 4. **Delayed embryo development in *str1* mutant.** Characterization of embryo development in wild-type and *str1-1* mutant at 3, 5, 7, and 12 DAP is shown. The siliques at various DAP were fixed and cleared in Hoyer's solution. The seeds were dissected out and observed under light microscopy. In the wild type, embryos developed through globular (*A*), heart (*E*), torpedo (*I*), and bent cotyledon (*M*) stages at 3, 5, 7, and 12 DAP, respectively. When compared with wild-type, embryo development was delayed in *str1-1* mutant. Embryos developed to globular (*B*) or octant (*C* and *D*) stages at 3 DAP, to globular stage (*F–H*) at 5 DAP, and to heart (*J* and *K*) and globular (*I*) stages at 7 DAP. At 12 DAP, embryos were in cotyledon (*N* and *O*), torpedo (*P* and *Q*), and heart (*R*) stages. *A–L*, Nomarski images of developing embryos. *Bars* = 25 $\mu$m. The *arrows* indicate the suspensor. *M–R*, pictures of dissected embryos from survival seeds. *Bars* = 100 $\mu$m.

To genetically characterize the shrunken seed phenotype of the *str1* mutant, we performed reciprocal crosses. When *str1-1* plants were used as the pollen donors and wild-type plants were used as the female parents, 0.87% of the F1 seeds were shrunken, which was consistent with the wild-type level ($p > 0.05$) (supplemental Table 1). This result suggests that the defective seed development was not a paternal effect due to defective pollen. When *str1-1* plants were pollinated with pollen grains from wild-type plants, the percentage of shrunken seeds was at the wild-type level (1.21%) as well ($p > 0.05$), ruling out the possibility of a maternal effect. Data from reciprocal crosses also demonstrated that *str1* is recessive and that the heterozygous $str1^{+/-}$ zygotes can develop normally and only the homozygous $str1^{-/-}$ zygotes have the potential to develop to shrunken seeds.

*Homozygous str1 Embryos Show Delayed Development, and Most Are Arrested at the Heart Stage*—Seed development can be divided into two major phases: embryo morphogenesis and seed maturation (29). Embryo morphogenesis is initiated by the double fertilization of the embryo sac that gives rise to the zygote (2n) and endosperm (3n). The time of pollination (0 days after pollination, DAP) is frequently used as the reference point to define the stages of seed development. *Arabidopsis* embryos have five different developmental stages, namely octant, globular, heart, torpedo, and bent cotyledon (30), and the suspensor degenerates at the late heart stage or early torpedo stage (31, 32).

In wild-type plants, the embryos developed through the globular, heart, torpedo, and bent cotyledon stages (Fig. 4*A*, *E*, *I*, and *M*) at 3, 5, 7, and 12 DAP, respectively. However, in *str1-1* mutant plants, only a small percentage of embryos reached the globular stage at 3 DAP (Fig. 4*B*); the others remained at the octant stage (Fig. 4, *C* and *D*). At 5 DAP, only 4.1% of the embryos (6/145) reached the heart stage; the others remained at the globular stage (Fig. 4, *F–H*). At 7 DAP, ~57.2% of the embryos (91/159) made it to the heart stage (Fig. 4, *J* and *K*), whereas the others remained at the globular stage (Fig. 4*L*). Dissection of *str1* seeds at 12 DAP revealed

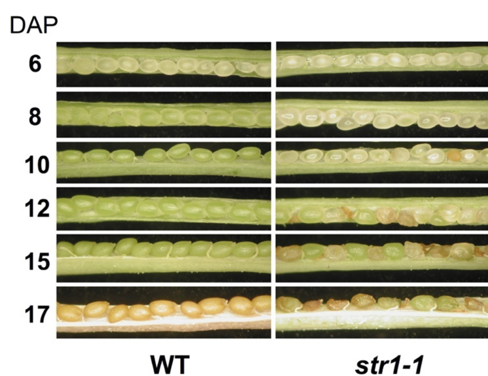


FIGURE 5. **Delayed development of *str1* mutant seeds.** The siliques at various DAP were dissected and observed under light microscopy. In *str1-1* mutant, brown shrunken seeds were randomly distributed throughout the silique beginning at 10 DAP.

embryos at different developmental stages, including heart, torpedo, and bent cotyledon stages (Fig. 4, *N–R*), and the majority were at the heart stage. At this time, the arrested *str1* embryos were bigger with more cells in comparison with the normal wild-type embryos at morphologically similar stage. For instance, the heart-shaped *str1* embryos at 12 DAP were ~200 $\mu$m in width (Fig. 4*R*), which is much bigger than the normal wild-type heart-stage embryos with ~50 $\mu$m in width (Fig. 4*E*). This observation suggests that the cell division continued to a certain extent in the affected *str1* mutant embryos. Eventually, ~12.5% *str1* embryos reached maturity and formed normal looking seeds (supplemental Table 1), and the rest formed seeds with different degrees of shrinkage. The most severely shrunken seeds (about 75% of the total) contained no embryo. The rest (12.5%) were partially shrunken seeds that were able to germinate (discussed later). The *str1-2* mutant had the same embryo phenotype as *str1-1* (supplemental Fig. S1). The suspensors of the delayed embryos appeared to be normal in both *str1* mutant alleles (Fig. 4 and supplemental Fig. S1).

At the beginning of the seed maturation stage (7 DAP), embryos are at the torpedo stage. They have already established the body structure of plants, exhibiting a polarity along an apical-basal axis (33, 34). Normal embryos at this stage also show bilateral symmetry along the apical-basal axis. Some *str1* embryos showed asymmetry in body structure, with two cotyledons of unequal sizes (Fig. 4, *P* and *Q*), which is likely the cause of an observed cotyledon defect (discussed later).

During the early seed maturation stage (7–10 DAP), *Arabidopsis* embryos grow rapidly to fill the seeds, whereas the endosperm is absorbed and reduced to a one-cell layer surrounding the embryo (35, 36). Although embryo development in the *str1* mutant was delayed, the endosperm development appeared to be normal (Fig. 4, *B–D* and *F–H*). Associated with the delayed embryogenesis, seed development was also affected in *str1* mutants (Fig. 5). When the embryos reached the torpedo stage at 6 DAP, the seeds became light green in wild-type plants as the embryos started to synthesize chlorophyll. However, the *str1-1* seeds remained white in appearance. At 8 DAP, the seeds in wild-type plants became green and expanded, but the seeds in *str1-1* mutant plants remained white and small. At 12 DAP, the embryos reached the bent cotyledon stage in wild-type plants. In *str1-1* mutant plants, there were brown shrunken seeds randomly distributed throughout the silique. Finally, the seeds in the wild-type plants showed brown color and were almost mature at 17 DAP. In contrast, only a small percentage of seeds were normal in *str1-1* mutants. The majority of seeds had a shrunken appearance at 17 DAP, and no embryos were present in the severely shrunken seeds. These results indicate that the defective embryogenesis is associated with and results in the shrunken seed phenotype in the *str1* mutant.

*Seed Germination and Cotyledon Morphology Is Affected in str1 Mutants*—The germination rate of seeds from *str1* plants was low in comparison with the wild type. After 9 days, nearly 100% of wild-type seeds germinated, whereas only about 25% of *str1* seeds did so (Fig. 6*A*). They included normal looking seeds and some partially shrunken seeds. About half of the *str1* seedlings showed defective cotyledons. Instead of two symmetric cotyledons in the wild type, these defective *str1* seedlings had only one abnormal cotyledon with the appearance of two cotyledons fused together in different degrees (Fig. 6, *D* and *E*, and supplemental Fig. S2). Examination of the vein pattern revealed that the single cotyledon was indeed formed from two merged cotyledons (Fig. 6, *H* and *I*, and supplemental Fig. S2), which is likely a result of defective embryogenesis. As shown in Fig. 4, *P* and *Q*, not all embryos were symmetrically developed. However, post-germination organ development and growth of these defective seedlings were normal. Although seedlings with a single cotyledon developed an unequally sized first pair of true leaves, they eventually grew to plants that were indistinguishable from the wild-type plants after being transferred to soil (Fig. 3). These results indicate that STR1 is not essential for post-germination growth and development and that the abnormal cotyledon development is due to defective embryogenesis.

*Genomic STR1 DNA Rescues the Shrunken Seed Phenotype of the str1 Mutant*—The identical mutant phenotype in two independent *str1* alleles strongly supported the role of STR1 in *Arabidopsis* embryo and seed development. A complementation experiment was performed to further verify the function of STR1. A construct with *STR1* cDNA driven by the 35S promoter (*35S:STR1*) was unable to rescue the *str1* mutant phenotype, suggesting the importance of a native promoter and/or elements in the introns. As a result, we generated a native *STR1* promoter construct ($Pro_{STR1}$:*STR1*) by using the full-length *STR1* genomic DNA that included the 1109-bp region upstream of the ATG start codon and the 260-bp region downstream of the stop codon. When transformed into the *str1-1* mutant plants, the construct was able to fully rescue the seed defects of homozygous *str1* mutant plants (Fig. 7). Two independent T3 homozygous transgenic lines (51-2 and 36-3) were selected for detailed analyses. At 12 DAP, the seeds in the transgenic lines were the same as those from the wild type (Fig. 7*A*), and mature seeds from both transgenic lines were normal (Fig. 7*B*). Seed germination rates and cotyledon patterns in the transgenic seedlings were also the same as those in the wild type. MST activity was measured to confirm the complementation at the biochemical level (Fig. 7*C*).

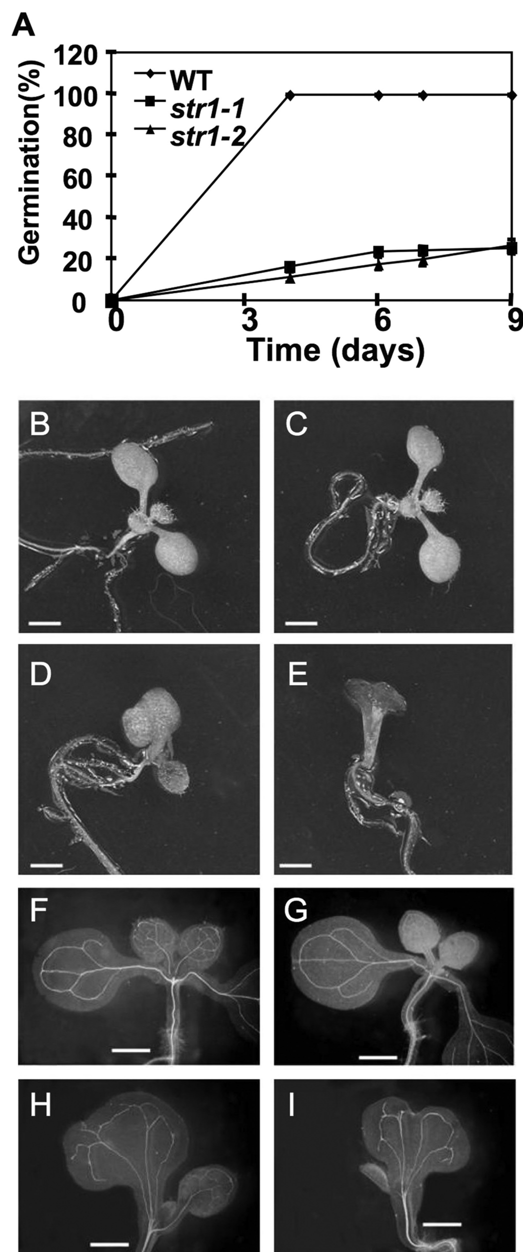


FIGURE 6. **Seed germination and the defective seedlings from the partially shrunken seeds.** *A*, comparison of the seed germination between wild-type and *str1* mutants. Sterilized seeds were sowed on solid Murashige and Skoog plates, kept in the dark at 4 °C for 3 days, and then moved to 22 °C under continuous light. Values are the means ± S.E. of three replicates. *B*, image of a 7-day-old wild-type seedling. *C–E*, 7-day-old *str1-1* mutant seedlings can have two normal cotyledons (*C*) or a single cotyledon (*D* and *E*). *F–I*, cotyledon vein patterns of seedlings shown in *B–E*, respectively. *F*, wild-type; *G–I*, *str1-1* mutant. *Bar* = 1 mm.

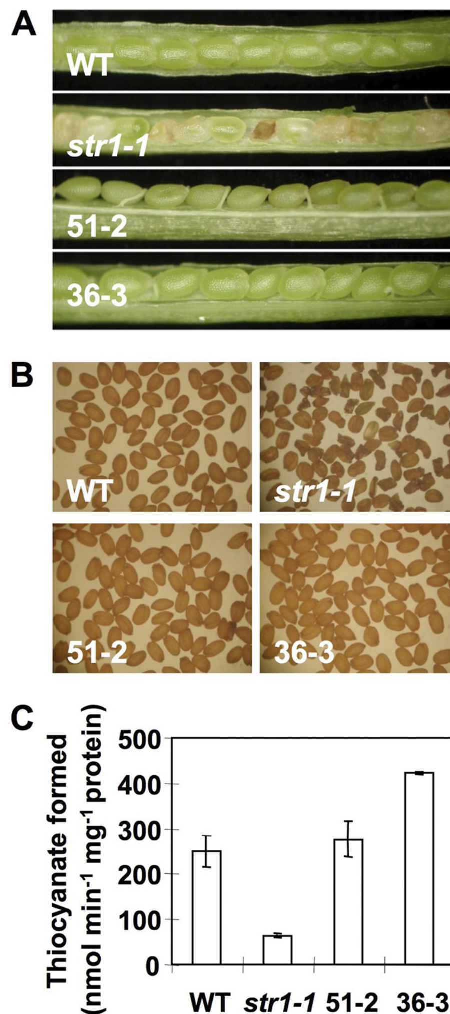


FIGURE 7. **Expression of a wild-type *STR1* gene can rescue the shrunken seeds phenotype.** *A*, siliques at 12 DAP were dissected and observed under a dissecting microscope. From *top* to *bottom*, they are: WT, *str1-1*, and two independent $Pro_{STR1}$:*Str1* transgenic homozygous lines (51-2 and 36-3) in *str1-1* mutant background. *B*, the mature seeds from WT, *str1-1*, and two independent transgenic lines (51-2 and 36-3). *C*, analysis of the MST activity in siliques from WT, *str1-1*, and transgenic lines (51-2 and 36-3). Values are the means ± S.E. of three replicates.

The MST activity in siliques from the rescued transgenic lines was restored to the wild-type level.

*Pattern of STR1 Gene Expression*—The constitutive 35S promoter-driven *STR1* cDNA construct failed to rescue the mutant phenotype of *str1* plants, suggesting the importance of native promoter. In addition, MST activity varies in different organs/developmental stages (Fig. 2). As a result, we attempted to determine the expression pattern of *STR1* using a GUS reporter. At first, a $Pro_{STR1}$:*GUS* construct was generated and transformed into wild-type plants. However, we were unable to detect GUS activity in the transgenic plants, indicating that elements in the full-length genomic DNA including the introns and/or 3′-UTR might be involved in the regulation of *STR1* gene expression. It is also possible that the STR1 protein is regulated at the post-translational level.

A $Pro_{STR1}$:*STR1-GUS* fusion construct was then generated by fusing the GUS cDNA to the last exon of the *STR1* genomic construct ($Pro_{STR1}$:*STR1*) and transformed into wild-type and *str1-1* plants. This $Pro_{STR1}$:*STR1-GUS* fusion construct fully rescued the seed defects of homozygous *str1-1* plants (supplemental Fig. S3), demonstrating that the STR1-GUS fusion protein is fully functional. GUS activity was detected in whole seedlings (Fig. 8*A*) and was particularly intense in cotyledon vein and root tips. In rosette leaves, the staining was stronger in veins and at the base of trichomes (Fig. 8*B*). Staining was also observed in inflorescence stems and flowers (Fig. 8, *C* and *D*). Although sepals and petals were entirely stained, the staining was stronger in the vascular tis-

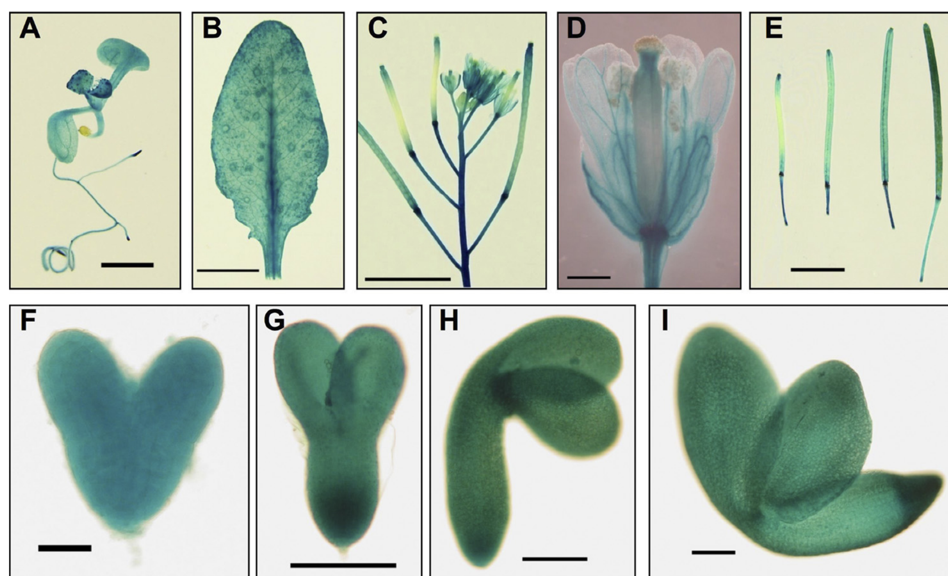


FIGURE 8. **Expression patterns of *STR1* gene at different stages and in different organs.** *A–E*, expression pattern of *Pro*$_{STR1}$*:STR1-GUS* in various plant organs. The results of GUS staining were observed on whole mounts of seedlings (*A*), leaf (*B*), inflorescence (*C*), flower (*D*), and siliques (*E*). *F–I*, expression pattern of *Pro*$_{STR1}$*:STR1-GUS* in embryo. Microscope observations of GUS activity were carried out on whole mounts of excised embryos aged 5 (*F*), 7 (*G*), 9 (*H*), and 12 (*I*) DAP. *Bars* = 2 mm in *A*, 0.5 cm in *B*, *C*, and *E*, 500 $\mu$m in *D*, 25 $\mu$m in *F*, and 100 $\mu$m in *G–I*.

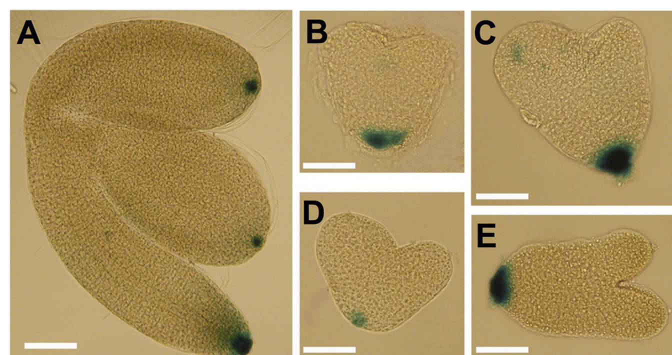


FIGURE 9. **Defective embryos in *str1* mutant have normal auxin reporter activity.** Embryos were dissected out of 9-DAP siliques of *DR5:GUS* (*A*) and *DR5:GUS/str1-1* (*B–E*) plants. After GUS staining, the embryos were imaged using a microscope equipped with a digital camera. *Bar* = 100 $\mu$m.

sue of sepals, petals, and stamen filaments. No staining was detected in pollen grains. Staining was detected in siliques and was particularly intense in the abscission zones and the apical ends of the siliques (Fig. 8*E*). GUS activity was observed in embryos at heart, torpedo, and bent cotyledon stages (Fig. 8, *F–I*). Particularly strong staining was observed in the apex of the cotyledon and root tips (Fig. 8, *G–I*). In summary, GUS reporter staining revealed that STR1 is universally expressed, with high levels of expression at specific locations, including embryonic shoot/root meristems, young leaves, root tips, flower abscission zones, fruit tips, and vasculatures.

*Defective str1 Embryos Maintain a Normal Auxin Reporter Activity*—The plant hormone auxin is known to play a vital role in embryo development (37–40). To determine whether the defective embryogenesis in *str1* mutant is associated with disturbed auxin activity, we compared *DR5:GUS* expression in the *str1* and wild-type embryos. *DR5* reporter constructs, which contain an auxin-responsive promoter with direct repeat (DR) elements, are commonly used to monitor endogenous auxin levels/activities (25–27). At 9 DAP, wild-type embryos developed to early bent cotyledon stage, whereas most *str1* embryos were still at the heart stage (Fig. 9). In wild-type embryos, DR5:GUS activity was observed in the root meristem and the tips of the developing cotyledons (Fig. 9*A*), which is consistent with previous reports (27, 41–43). In *str1* embryos arrested at the heart stage, it appears that auxin gradient/activity was still properly specified. As shown in Fig. 9, *B–E*, DR5:GUS activity was observed only at the basal end of the embryos, which is similar to the pattern in wild-type embryos at the heart stage (27, 41–43). The largely unperturbed *DR5:GUS* reporter gene activity suggests that the embryo defect in *str1* mutant is not a result of improper auxin distribution, and the function of STR1 in embryogenesis may be independent or downstream of auxin.

*Haplo-lethality of str2*$^{+/-}$ *in str1 Mutant Background*—Among the 20 putative *STR* genes in *Arabidopsis*, *STR2* (At1g16460) shares the highest sequence identity with *STR1*. Single *str2* mutant showed no decrease in MST activity, and no embryo/seed development defect was observed (Fig. 10, *A* and *B*). To determine whether the incomplete penetrance of embryo/seed defects in *str1* mutant is a partial loss-of-functional phenotype because of the presence of functional *STR2*, we attempted to generate *str1/str2* double mutant. We were unable to recover *str1*$^{-/-}$*/str2*$^{-/-}$ double mutant among the 133 F2 progenies that we genotyped. No *str1*$^{-/-}$*/str2*$^{+/-}$ offspring was identified either. However, many progenies with *str1*$^{+/-}$*/str2*$^{-/-}$ genotype were identified. Both *STR1* and *STR2* are located on chromosome 1. Physically, they are 24,180 kb apart, which is the equivalent of 97 centimorgans (1 centimorgan is about 250 kb on average (44)). The large number of *str1*$^{+/-}$*/str2*$^{-/-}$ plants identified in the F2 generation is consistent with the physical distance and the genetic linkage of the two genes. As a result, the lack of *str1*$^{-/-}$*/str2*$^{+/-}$ and *str1*$^{-/-}$*/str2*$^{-/-}$ progenies suggests that they might be embryo-lethal.

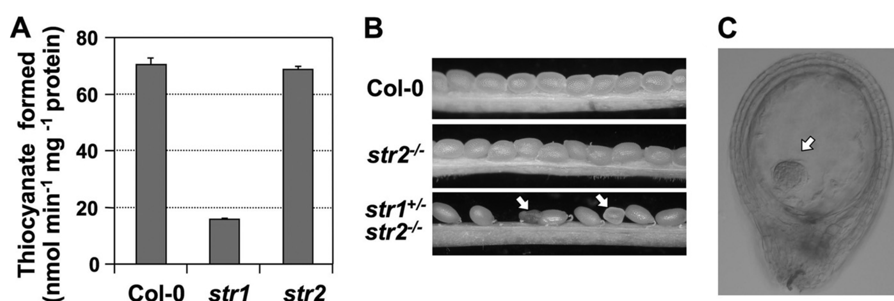


FIGURE 10. **Single *str2* mutant has no embryo/seed developmental defect, but it is haplo-insufficient in supporting the partial embryo/seed development seen in *str1* single mutant.** *A*, single *str2* mutant seedlings have a wild-type level of MST activity. MST activity in 14-day-old WT (Col-0), *str1*, and *str2* seedlings was measured. Values are the means ± S.E. of three replicates. *B*, siliques (12 DAP) from wild-type (Col-0), *str2*, and $str1^{+/-}/str2^{-/-}$ plants were dissected and observed under a dissecting microscope. The *arrows* indicate the abnormal seeds. *C*, a representative 12-DAP embryo from a $str1^{+/-}/str2^{-/-}$ plant that was arrested at globular stage. A microscope with Nomarski optics was used to take the picture. The *arrow* indicates the arrested embryo. *Bar* = 150 $\mu$m.

We then looked at the progenies of $str1^{+/-}/str2^{-/-}$ plants, which have the wild-type morphology. No $str1^{-/-}/str2^{-/-}$ plant was identified. The ratio of $str1^{+/-}/str2^{-/-}$ and $STR1/str2^{-/-}$ progenies was 86:45, close to 2:1 ($p > 0.05$), which again suggests that only the double homozygous zygotes are lethal. We also performed reciprocal crosses to determine the viability of $str1^{-}/str2^{-}$ gametes. When pollen grains from wild-type plants were used to pollinate $str1^{+/-}/str2^{-/-}$, we obtained 52 $str1^{+/-}/str2^{+/-}$ and 76 $STR1/str2^{+/-}$ F1 progenies. When pollen grains from $str1^{+/-}/str2^{-/-}$ plants were used to pollinate wild-type plants, we obtained 49 $str1^{+/-}/str2^{+/-}$ and 57 $STR1/str2^{+/-}$ F1 progenies. These results suggest that both male and female $str1^{-}/str2^{-}$ gametes are viable and that failure of getting $str1^{-/-}/str2^{-/-}$ plants from $str1^{+/-}/str2^{-/-}$ parents is a result of embryo lethality. To further enhance the chance of getting $str1^{-/-}/str2^{+/-}$ plants, we also crossed $str1^{+/-}/str2^{-/-}$ with $str1^{-/-}$ plants. However, only progenies with $str1^{+/-}/str2^{+/-}$ genotype (population of 263) were obtained, which confirms that $str1^{-/-}/str2^{+/-}$ zygotes are lethal.

There were two types of seeds in matured siliques of $str1^{+/-}/str2^{-/-}$ plants, normal looking and dark brown empty seeds. No shrunken seed was observed. All normal seeds germinated well, and their genotypes were either $str1^{+/-}/str2^{-/-}$ or $STR1/str2^{-/-}$. It is likely that the empty seeds were from $str1^{-/-}/str2^{-/-}$ zygotes, which aborted at an earlier stage. To determine the stage(s) of embryo lethality of $str1^{-/-}/str2^{-/-}$ zygotes, we dissected siliques from $str1^{+/-}/str2^{-/-}$ plants at different stages. After fixing and clearing, embryos were examined under a microscope with Nomarski optics. We found that most embryos were arrested at the globular stage and that none developed beyond the heart stage (Fig. 10*C*), suggesting that the embryos with $str1^{-/-}/str2^{-/-}$ genotype arrested at an earlier stage than $str1^{-/-}$ embryos. We found aborted embryos at the same stages in siliques from $str1^{+/-}/str2^{-/-}$ and $str1^{-/-}$ cross. In this cross, no plant with $str1^{-/-}/str2^{+/-}$ genotype was identified among the 263 F1 progenies, suggesting that $str1^{-/-}/str2^{+/-}$ embryos were all aborted as well. The embryo lethality of $str1^{-/-}/str2^{+/-}$, but not $str1^{+/-}/str2^{-/-}$ zygotes, suggests that haploid $str2^{+/-}$ is insufficient to maintain the development of embryos/seeds seen in the single $str1^{-/-}$ mutant. It also suggests that STR1 plays a more important role than STR2 in the process.

## DISCUSSION

Several laboratories have cloned *Arabidopsis STR1* and *STR2* genes and characterized their expression under various growth conditions (9, 16, 18, 20). Recently, microarray analysis revealed that *STR1* and *STR2* are expressed in *Arabidopsis* embryos (45). However, the exact biological function(s) of *STR1* and *STR2* remain unclear. In this study, we provide genetic evidence that *STR1* and *STR2* play important roles in *Arabidopsis* embryo and seed development. Mutation of the *STR1* gene results in a reduction of ~80% of cellular sulfurtransferase activity, which is associated with defective embryo/seed development. The majority of *str1* embryos arrested at the heart stage and eventually aborted. Only about 12.5% of the *str1* embryos developed to maturity and formed normal looking seeds. Single *str2* mutant has no embryo/seed phenotype. However, *STR2* is haploid-insufficient to maintain the development of embryos/seeds seen in the single $str1^{-/-}$ mutant. No $str1^{-/-}/str2^{+/-}$ progeny could be identified in populations from $str1^{+/-}/str2^{+/-}$ parental plants or a cross of $str1^{+/-}/str2^{-/-}$ and $str1^{-/-}$ plants. No $str1^{-/-}/str2^{-/-}$ progeny could be identified in populations from $str1^{+/-}/str2^{+/-}$ or $str1^{+/-}/str2^{-/-}$ parental plants either. As a result, STR1 is more important than STR2 in the process because $str1^{+/-}/str2^{-/-}$ embryos are fully viable. Sulfurtransferases are ubiquitous enzymes. Orthologs of *Arabidopsis* STR1 and STR2 exist in other plant species (supplemental Fig. 4). It is tempting to speculate that they play similar roles as *Arabidopsis* STR1/STR2, although direct evidence is still lacking.

A large number of embryo defective mutants have been identified through the screening of T-DNA insertional mutant collections (46). These mutants differ in their extent of abnormal embryo development, size and color of aborted seeds and embryos, efficiency of transmission through male and female gametes, capacity to produce mutant seedlings, and existence of other associated phenotypes (47). Genes involved in the regulation of sugar and lipid metabolisms are important to seed development. The *wrinkled1* (*wri1*) mutant seeds showed a decreased incorporation of sucrose and glucose into triacylglycerol, a result of reduced activities of key glycolytic enzymes including hexokinase- and pyrophosphate-dependent phosphofructokinase (48). As a result, *wri1*-null mutant seeds are not completely filled and give a wrinkled

appearance. WRI1 encodes an AP2/EREB domain transcription factor, and overexpression of *WRI1* resulted in an increased triacylglycerol level in both seeds and leaves (49). A more recent study revealed that LEC2, a plant-specific B3 transcription factor, directly regulated WRI1, which, in turn, controlled the expression of a subset of genes involved in fatty acid biosynthesis and seed maturation (50, 51).

The shrunken seed phenotype of *str1* mutant is similar to that of *wri1* but more severe. The embryo defect appears earlier before the seed-filling stage, resulting in the abortion of the majority of the developing embryos and empty seeds. Mutation of *STR2* in *str1* background enhances the embryo/seed phenotype, indicating that both are involved in embryo/seed development. At this stage, the exact biochemical function of STR1/STR2 in embryo and seed development is unknown. It is possible that they are either directly or indirectly involved in a metabolic pathway that is essential to supply/convert the essential nutrients to support the normal embryogenesis and seed development, based on the putative function of sulfurtransferase in sulfur metabolism (5, 6). Alternatively, they could be involved in the removal of a toxic substance produced during the seed-setting stage. In the absence of STR1/STR2, embryos either are in a nutrient-deficient state or suffer from toxicity. Both can result in the delay of embryo growth/development and/or aborted embryos.

Plant sulfurtransferases have been speculated to function in the detoxification of cyanide, a co-product of ethylene biosynthesis (11, 16, 52). *In vitro*, sulfurtransferases catalyze the transfer of a sulfur atom from a donor such as thiosulfate and 3-mercaptopyruvate to cyanide, leading to the formation of the less toxic thiocyanate (18). However, *in vivo* evidence supporting a role of STR1/STR2 in cyanide removal in plants is still lacking. Plants produce high levels of ethylene during flowering and seed setting (53, 54). In *Arabidopsis*, flowers/siliques produce ~100 times more ethylene than leaves (supplemental Fig. 5, Supplemental Methods). In this study, the flowers were staged according to Smyth *et al.* (55), and flowers at stage 16 contain embryos at the torpedo stage. The average rate of ethylene production in flowers from stage 16 and younger was ~600 pmol/h/g of fresh weight. Cyanide should be produced at the same rate. Unlike ethylene, which can diffuse out of the cells, cyanide will accumulate in the cells if not biochemically removed. A ~600 pmol/h/g of fresh weight production rate is equivalent to an increase of 0.6 $\mu$M/h in cellular cyanide concentration if 1 g of fresh weight is equal to 1 ml. This clearly imposes a threat to normal cellular activity if cyanide is not removed. Cytochrome oxidase and Rubisco have $K_d$ or $IC_{50}$ values of 1 and 6 $\mu$M, respectively. A number of other redox-related enzymes are also sensitive to HCN at the micromolar range (56).

STR1 is localized in mitochondria, based on immunodetection of isolated mitochondria and transient expression of *STR1*-GFP fusion in *Arabidopsis* protoplasts (19). Furthermore, 3-mercaptopyruvate exists in mitochondria (17), suggesting that the biochemical function of STR1 may indeed involve the utilization of 3-mercaptopyruvate as a substrate in the detoxification of cyanide in mitochondria. As a co-product of ethylene biosynthesis, cyanide is produced outside of mitochondria. However, at cellular pH, the majority of cyanide is in the protonated form, which is membrane-permeable. It has been reported that cyanide can move into mitochondria by both facilitated transport and passive diffusion (57). Due to the high sensitivity of cytochrome oxidase to cyanide ($K_d$ of 1 $\mu$M) (56), protection of normal mitochondrial activity in the presence of cyanide is imperative to the survival of the cells and normal cellular activities. It is tempting to speculate that STR1 may play a role in the protection of developing embryos from cyanide toxicity, based on its mitochondrial localization and *in vitro* biochemical activity in cyanide detoxification. As a cytoplasmic protein, STR2 may be involved in a similar detoxification process. The lack of phenotype in other tissues/organs (*i.e.* leaves) of the *str1* mutant plants could be a result of low level biosynthesis of ethylene (supplemental Fig. 5) and its co-product cyanide. In summary, we demonstrate in this report that STR1/STR2 sulfurtransferases play an important role in plant embryo/seed development, which highlights the important biological function of this group of ubiquitous enzymes in plants.

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