The Arabidopsis Embryo Mutant *schlepperless* Has a Defect in the *Chaperonin-60α* Gene

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We identified a T-DNA-generated mutation in the *chaperonin-60α* gene of Arabidopsis that produces a defect in embryo development. The mutation, termed *schlepperless* (*slp*), causes retardation of embryo development before the heart stage, even though embryo morphology remains normal. Beyond the heart stage, the *slp* mutation results in defective embryos with highly reduced cotyledons. *slp* embryos exhibit a normal apical-basal pattern and radial tissue organization, but they are morphologically retarded. Even though *slp* embryos are competent to transcribe two late-maturation gene markers, this competence is acquired more slowly as compared with wild-type embryos. *slp* embryos also exhibit a defect in plastid development–they remain white during maturation in planta and in culture. Hence, the overall developmental phenotype of the *slp* mutant reflects a lesion in the chloroplast that affects embryo development. The *slp* phenotype highlights the importance of the chaperonin-60α protein for chloroplast development and subsequently for the proper development of the plant embryo and seedling.

Plant embryogenesis is a complex developmental process that can be divided into four conceptual phases (West and Harada, 1993; Goldberg et al., 1994). During the first phase, the body plan of the mature embryo is established and specific groups of cells give rise to the shoot meristem, cotyledons (embryonic leaves), axis (hypocotyl), radicle (embryonic root), and the root meristem. The embryo then undergoes maturation during the second phase of development, which is characterized by the deposition of storage materials (Goldberg et al., 1989). The third phase involves desiccation leading to seed dormancy. When proper conditions allow, germination follows seed dormancy leading to the development of a seedling. The cotyledons serve as a source of food reserves during germination. Each of the four phases requires coordinated expression of specific and overlapping genetic programs involving cell division, cell differentiation, and other housekeeping, cellular functions (Goldberg et al., 1989).

The molecular and cellular mechanisms that dictate the early events of embryogenesis are not yet known. One approach to address this question is the isolation and characterization of mutants that are impaired in embryo development. Several embryo-defective mutants have been isolated in Arabidopsis (Mayer et al., 1991; Meinke, 1991; Yadegari et al., 1994; Meinke, 1995), petunia (Souer et al., 1996), and corn (Clark and Sheridan, 1991). Some of the mutated genes affecting Arabidopsis embryogenesis have been isolated, including *GNOM* (Shevell et al., 1994), *SHOOT MERISTEMLESS* (*STM*; Long et al., 1996), *MONOPTEROS* (*MP*; Hardtke and Berleth, 1998), *KNOLLE* (Lukowitz et al., 1996), and *EMBRYO-DEFECTIVE DEVELOPMENT1* (*EDD1*; Uwer et al., 1998). Despite the cloning of these genes, their specific roles in the embryogenic process remain to be elucidated. Nevertheless, the functional identities of the encoded proteins indicate that some may play roles in cellular processes that are important for controlling plant embryogenesis as well as other aspects of plant development. For instance, the gene product of *MP* is involved not only in the establishment of embryo axis formation (Berleth and Jürgens, 1993), but also in vascular development beyond the embryonic stage (Hardtke and Berleth, 1998). Likewise, aside from being involved in specifying the apical and basal regions of the embryos (Mayer et al., 1993; Shevell et al., 1994), *GNOM* may also be involved later in plant development because its gene product is essential for establishing cell polarity required for normal cell division and expansion, (Shevell et al., 1994; Grebe et al., 2000; Shevell et al., 2000). Genes that affect the function and/or development of organelles may also be playing similarly significant roles in embryogenesis. For example, a mutation in *EDD1* leads to embryo arrest between globular and heart stages (Uwer et al., 1998).

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Because *EDD1* encodes a plastidic form of glycyl-tRNA synthetase (Uwer et al., 1998), it is essential for carrying out the metabolic processes that occur within the chloroplast, which can be important for maintaining normal development of the adult plant.

In this paper, we describe the characterization of an embryo-defective mutant allele that was isolated from a population of T-DNA-mutagenized Arabidopsis lines (Yadegari et al., 1994). The embryonic cotyledons of this mutant, termed *schlepperless* (*slp*), are highly reduced and the entire embryo remains white during maturation even though the wild-type (WT) embryos turn green. The coding sequence interrupted by the T-DNA insertion corresponds to the nuclear-encoded plastid *chaperonin-60α* subunit gene. We named this mutant *schlepperless*, because it is derived from the Yiddish term "schlepper" (meaning "to carry"). Our analysis indicates that the absence of a functional chaperonin-60α protein has adverse consequences on the development of the chloroplast, and subsequently, the development of the embryo. We conclude that the chaperonin-60α protein is essential for the proper development of Arabidopsis plants and, most likely, other eukaryotic organisms.

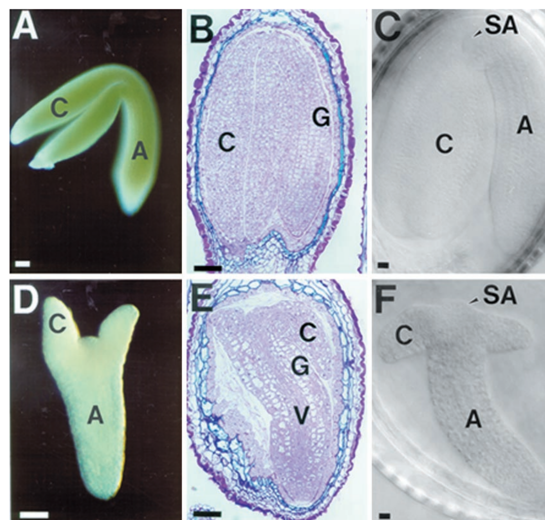

Figure 1. Morphology of *schlepperless* embryos. Mature embryos from WT (A, B, and C) and *schlepperless* (D, E, and F). Whole mount photographs of embryos dissected out of the same mature silique (A and D). Sections of embryos from mature silique embedded in LR White plastic resin (B and E). Nomarski photographs of embryos taken from the same mature silique (C and F). C, Cotyledon; Ep, epidermis; G, ground tissue; H, hypocotyl; SA, shoot apical meristem; V, vascular tissue. Bars = 50 μm.

RESULTS

schlepperless Embryos Develop Abnormally

We characterized WT embryo development to establish a base line to which *slp* embryo development could be compared. The greening of a WT embryo started at the heart stage of embryogenesis (data not shown), which was indicative of chloroplast development from proplastid progenitors (Schultz and Jensen, 1968; Mansfield and Briarty, 1991). As the embryo matured, the hypocotyl and cotyledons of WT embryo turned green (Fig. 1A). At this stage, the cotyledons were fully expanded and the whole embryo occupied most of the space taken previously by the endosperm within the maturing seed (Fig. 1, B and C). The corresponding *slp* embryo (taken from the same heterozygous silique as the WT embryo) was white and did not turn green even at a mature stage (Fig. 1D).

In contrast, the *slp* embryo was not as large as the WT embryo (Fig. 1, A–C) and its cotyledons were very reduced in size (Fig. 1, D–F). However, the tissue organization in advanced stage mutant embryos was apparently normal (Fig. 1E). The vascular tissue, ground tissue, and epidermal cells were present in their proper positions relative to each other as in the WT embryos (compare Fig. 1B with 1E). The radicle and the shoot apical meristem (which appears as a dome shape) were also present and appeared normal in the *slp* embryo (Figs. 1, C and F).

To determine at what stage of embryogenesis the *schlepperless* phenotype was first observed, a developmental series of *slp* embryo sections were obtained and were compared with those of the segregating WT embryos from a heterozygous *SLP/slp* plant (see "Materials and Methods"). Figure 2, A through E, show sections of WT embryos up to the early curled stage (Jürgens and Mayer, 1994) from a heterozygous plant. The morphology of the *slp* embryo appeared to be normal up to the heart stage of embryogenesis (Fig. 2, F–I). However, development of the mutant embryos was considerably retarded compared with WT embryos (compare Fig. 2, A–E, with Fig. 2, F–I). The WT embryos were already at the early curled stage (Fig. 2E), whereas the *slp* embryos were still morphologically at the heart stage (Fig. 2I). The tissue organization of the *slp* embryo appeared to be normal at this early stage of embryogenesis. The developing dermal, ground, and vascular tissues that were present in the WT were also apparent in the *slp* mutant (compare Fig. 2, A–C, with Fig. 2, F–H). Together, these data indicate that the *slp* mutant embryos develop more slowly than WT embryos during the early stages of seed development and have an abnormal morphology by maturity.

schlepperless Embryos Germinate in Culture

To determine if the *slp* mutant phenotype can be rescued by tissue culture, mature mutant embryos were dissected from seeds and germinated on media containing Suc, vitamins, and salts (see "Materials and Methods"). The *slp* embryos were able to germinate in the culture media, although their development was slower and abnormal compared with that of WT embryos. After 12 d in culture, WT seedlings usually possessed four green rosette leaves and a

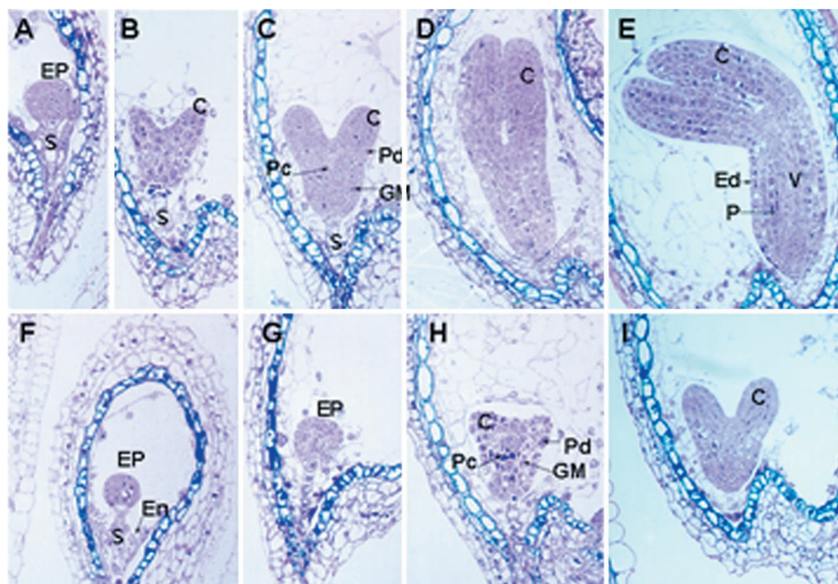

Figure 2. Developmental analysis of *schlepperless* embryos. A developmental series of sections for Arabidopsis WT (A–E) and *schlepperless* (F–I) embryos embedded in LR White plastic resin. The mutant embryos were from the same silique from which the corresponding WT embryos were taken. A, Axis; C, cotyledon; EP, embryo proper; Ep, epidermis; En, endosperm; GM, ground meristem; P, storage parenchyma; Pc, procambium; Pd, protoderm; S, suspensor; V, vascular tissue.

well-developed root system (Fig. 3A). The cotyledons of the seedlings expanded fully and became green (Fig. 3A). In contrast, the seedlings that developed from *slp* embryos were white (Fig. 3B). The undeveloped embryonic cotyledons emerged from the *slp* seeds but did not develop any further. No further greening was observed in the *slp* seedlings, even after remaining in culture for 72 d (Fig. 3C). The aerial portions of the *slp* seedlings were stunted and only exhibited callus-like structures after extended periods of culture (data not shown), even though the mutant seedlings were able to form normal-looking roots and root hairs (Fig. 3C). Some of the *slp* seedlings developed leaf-like structures that were translucent and contained trichomes, originating from the apical dome (Figs. 3, B and C).

Germination of desiccated (dried) mutant seeds was also tested on the same germination medium (GM). The seedlings that developed resembled those obtained from non-mature and un-desiccated embryos (data not shown), suggesting that *slp* mutant embryos are able to undergo a normal dessication and maturation program. Together, these results indicate that even though *slp* embryos are capable of germinating, they are unable to form normal seedlings and, ultimately, mature plants. In addition, our results indicate that the *slp* phenotype could not be rescued by components present in the tissue culture medium.

schlepperless Embryos Are Competent to Transcribe Late-Maturation Genes

To determine if *slp* embryos were capable of transcribing genes that are normally expressed during the maturation phase in WT embryos, we crossed *SLP/slp* heterozygous plants to an Arabidopsis line transgenic for a *7S::GUS* construct (Hirai et al., 1994). This construct contained 0.9 kb of the 5′ region of the β-conglycinin α-subunit gene fused to the β-glucuronidase (*GUS*) reporter gene (Hirai et al., 1994). Histochemical localization of GUS enzyme activity was monitored in WT and *slp* embryos during seed development (see "Materials and Methods"). In WT embryos, initial expression of the *7S::GUS* transgene occurred at the early bent cotyledon stage (Fig. 4B). *7S::GUS* expression at this stage was limited to the upper hypocotyl region and the cotyledons. In mature WT embryos, the transgene was expressed in the cotyledons and in the hypocotyl (Fig. 4E).

In contrast, the initial expression of the *7S::GUS* transgene was delayed in *slp* mutant embryos. Developing mutant embryos taken from the same siliques as WT embryos (Fig. 4, A–E) did not show any GUS staining at first (compare Fig. 4B with Fig. 4G). GUS activity was initially detected in the hypocotyl region of mutant embryos (Fig. 4, H and I). Only later, in more mature *slp* embryos, was the expression pattern extended into the short cotyledons (Fig. 4J).

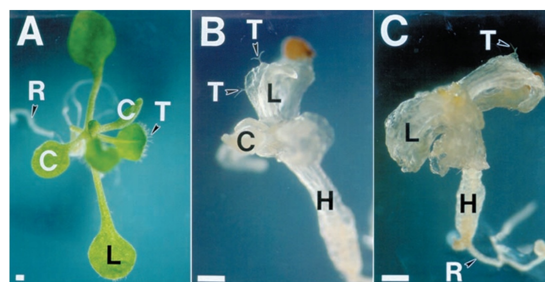

Figure 3. Germination of WT and *schlepperless* seeds in vitro. A, WT seedling after 12 d in culture; B and C, *schlepperless* seedling after 42 (B) and after 70 (C) d in culture. The mutant seedling shown in C was the same seedling shown in B. C, Cotyledon; H, hypocotyl; L, leaf; R, root; T, trichome. Bars = 50 μm.

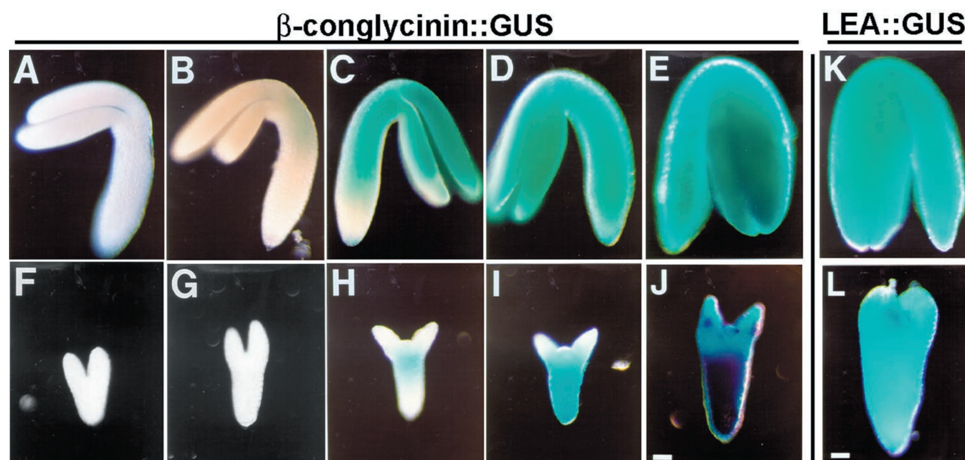

Figure 4. *schlepperless* embryo is competent to transcribe late-maturation genes. Expression pattern of β-conglycinin::GUS transgene in WT (A–E) and *schlepperless* (F–J) embryos at different developmental stages. Expression pattern of LEA::GUS transgene in mature WT (K) and *schlepperless* (L) embryos. Corresponding WT and mutant embryos were taken from the same silique. Blue staining indicates activity of GUS. Bars = 50 μm.

Mutant *slp* embryo development was also analyzed using a late embryogenesis abundant (*LEA*) gene fusion construct (*LEA::GUS*; Goupil et al., 1992). In WT plants, *LEA::GUS* is expressed late in embryogenesis and has an expression pattern similar to the *7S::GUS* gene (Goupil et al., 1992; Hirai et al., 1994; West et al., 1994; Fig. 4K). Mutant *slp* embryos containing the *LEA::GUS* construct showed a similar pattern of GUS activity as was obtained with the *7S::GUS* construct (data not shown; Fig. 4L). In early *slp* embryos, *LEA::GUS* expression was limited to the hypocotyl region (data not shown). During embryo maturation, *LEA::GUS* gene expression extended into the highly reduced cotyledon region of the mutant *slp* embryos (Fig. 4L). Taken together, these data indicate that the mutant embryos are competent to transcribe genes that are normally expressed late in embryogenesis. However, the acquisition of this competence is dependent upon the developmental state of the embryos. The delay of maturation gene expression in *slp* embryos most likely reflects the inherent delay of *slp* morphological development.

schlepperless Is Tagged with T-DNA

A single *SLP/slp* mutant line was obtained in a screen of T-DNA-mutagenized lines of Arabidopsis (see "Materials and Methods"). Two lines of evidence led us to conclude that this mutant allele was most likely interrupted at one locus, and that this T-DNA interruption is the cause of the *slp* phenotype. First, the results of DNA blot analysis of 50 WT and heterozygous F_1 segregants, where both the left and right borders of the T-DNA were used as probes, indicated that the T-DNA cosegregated only with the heterozygous plants and not with the WT segregants (data not shown). Second, an analysis of nearly 150 kanamycin-resistant (Kan-R) F_1 progeny (indicative of the presence of *neomycin phosphotransferase II* gene contained within the T-DNA) showed 100% cosegregation of Kan-R and the *slp* phenotype (data not shown).

Using plasmid rescue cloning of T-DNA-flanking genomic sequences, and by constructing a genomic library of *SLP/slp* heterozygous plants (see "Materials and Methods"), a map of the *slp* mutant locus was obtained indicating that the *SLP* gene was interrupted in one locus with three T-DNAs arranged in a concatemer (Fig. 5A). Genomic clones corresponding to all four T-DNA junctions (left border/left border junction, the right border/right border junction, the left border/plant sequence junction, and the right border/plant sequence junction) were isolated and they were confirmed using genomic DNA-blot analysis (Fig. 5, A and B; data not shown). For example, when a blot containing digested genomic DNAs from heterozygous *SLP/slp* (HZ) and WT plants was hybridized with a right border T-DNA sequence, two *Hin*dIII fragments were detected (2.3 and 4.5 kb in Fig. 5B). These fragments represent the right border/plant sequence junction (2.3 kb) and the right border/right border junction (4.5 kb).

We used the plant genomic sequences flanking the T-DNA insert to isolate the corresponding WT genomic clones from an Arabidopsis λ-library. One of the WT genomic clones isolated was λ101 with an insert size of 11 kb (Fig. 5A). Sequence analysis of the genomic clone suggested the presence of an open reading frame. A partial cDNA clone, pC31, was subsequently isolated using a 6.6-kb *Eco*RI fragment of λ101 as a probe (data not shown).

To determine whether the open reading frame identified represented the gene mutated in the *SLP/slp* line, we rescued the mutation by complementation. An 11-kb *Not*I fragment from the λ101 genomic clone (Fig. 5A) was sub-cloned into pGSH166N vec-

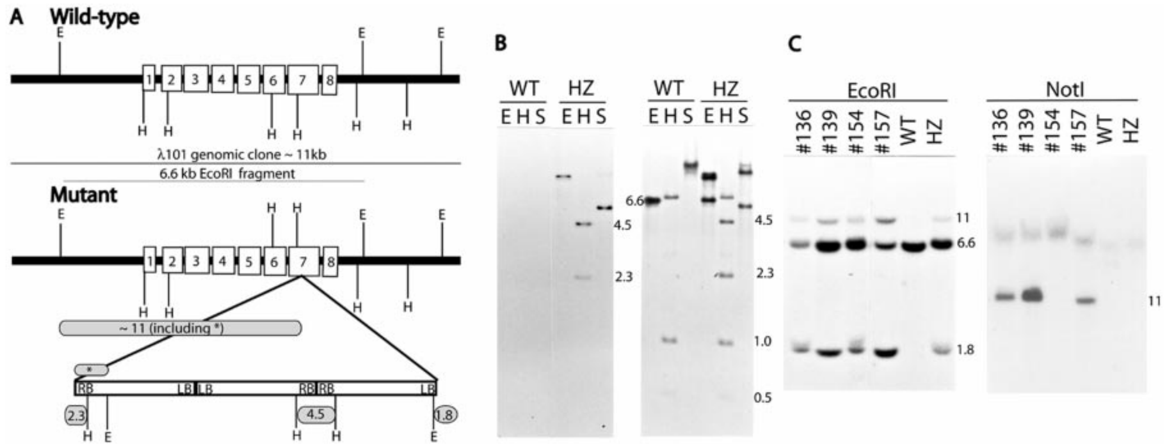

Figure 5. The *Chaperonin-60α* gene is interrupted by T-DNA in *slp* mutant. A, Diagrammatic representation of the WT *chaperonin-60α* locus and the concatemer of T-DNAs inserted in *slp* line (not drawn to scale). Exons are shown as unshaded boxes superimposed on the heavy line. The 11-kb WT genomic clone and the 6.6-kb *Eco*RI sub-clone are indicated by thin lines. The insertion point of the T-DNA concatemer is indicated by two lines below the seventh exon. Left border (LB) and right border (RB) regions are indicated in the adjacent rectangles that represent the T-DNA concatemer (see Errampalli et al., 1991, for the T-DNA map). Numbers in shaded rounded rectangles indicate the expected size of some polymorphic fragments as shown in the genomic blots (B and C). Not all of the restriction sites within the T-DNA are shown. E, *Eco*RI; H, *Hin*dIII, S, *Sal*I. B, DNA gel-blot analysis of WT and heterozygous (HZ) individuals using right border (first) or a *Bam*HI fragment representing a portion of the RB-plant-flanking region (second). Relevant fragments are indicated by fragment size corresponding to the map in (A). C, Genomic DNA-blot analysis of randomly selected F_2 plants used in the genetic analysis presented in Table I. The probe used was the partial pC31 cDNA clone. Genomic DNAs digested with *Eco*RI were electrophoresed in 1% (w/v) agarose gel, whereas those digested with *Not*I were electrophoresed in 0.5% (w/v) agarose gel. Samples from genotypically WT (WT) and heterozygous (HZ) individuals were included as controls.

tor (see "Materials and Methods"). This fragment contained the whole chaperonin-60α gene (approximately 5.7 kb) and no other genes were present on this fragment (see Lin et al., 1999). The vector contains a hygromycin-resistant (Hyg-R) marker and, therefore, could be differentiated from the T-DNA that contained Kan-R marker used in the initial mutagenesis (Errampalli et al., 1991; Feldmann, 1991). A complementation analysis was performed (see "Materials and Methods" for details) after crossing the heterozygous mutant line (*slp/SLP*; Kan-R) with the lines transgenic for the genomic clone (i.e. *tCpn*; Hyg-R). If the complementation was successful, we expected that we would observe a new class of heterozygous individuals producing mutant seeds at a 6.25% frequency. We found this new class of F_2 segregants as shown in Table I (Class III). This new class would not have been found in a non-complemented heterozygous line (*slp/SLP*; Kan-R) that produced mutant seeds at 25% frequency. Crosses using other independent transformants (i.e. *tCpn*; Hyg-R) produced similar results (data not shown).

Table I. *Summary of F_2 analysis in one of the complementation crosses*

Chi-square analysis: calculated $\chi^2 = 3.21 < $ tabular $\chi^2 = 5.99$ (at 0.05 level and degrees of freedom = 2).

F_2 Class (Description)	Expected F_2 If There Is No Complementation		Expected F_2 If There Is Complementation		Observed F_2	
	Genotypes[a]	%	Genotypes[a]	%	%	No. (Total = 270)
Class I (produce all WT seeds)	*SLP/SLP* (1) *SLP/SLP; tCpn* (2) *SLP/SLP; tCpn/tCpn* (1)	33.3	*SLP/SLP* (1) *SLP/SLP; tCpn* (2) *SLP/SLP; tCpn/tCpn* (1) *SLP/slp; tCpn/tCpn* (2) *slp/slp; tCpn/tCpn* (1)	46.6	50	135
Class II (produce mutant seeds at 25% frequency)	*SLP/slp* (2) *SLP/slp; tCpn* (4) *SLP/slp; tCpn/tCpn* (2)	66.7	*SLP/slp* (2) *slp/slp; tCpn* (2)	26.7	28	76
Class III (produce mutant seeds at 6.25% frequency)	–	–	*SLP/slp; tCpn* (4)	26.7	22	59

[a] Nos. in parentheses indicate the no. of individuals expected to have the particular genotype out of 12 (if there is no complementation) or 15 (if there is complementation) surviving F_2 individuals.

To establish cosegregation of the complementing transgene with the phenotype, genomic DNAs from randomly selected F_2 plants were analyzed using DNA-blot analysis (see "Materials and Methods"). The phenotypic WT F_2 segregants identified as *SLP/slp* heterozygotes, based on the presence of the polymorphic *Eco*RI fragments (11 and 1.8 kb, Fig. 5, A and C), were determined to contain the WT version of *SLP* on the 11-kb *Not*I-containing transgene inherited from the parent transformant (*tCpn*, Hyg-R; Fig. 5C). F_2 individuals nos. 136 and 139 were examples of such WT segregants (Fig. 5C). We also identified some F_2 segregants that were genotypically *slp/slp* homozygotes, based on testcross analysis (data not shown). F_2 individual number 157 (Fig. 5C) was an example of an *slp/slp* homozygous segregant that should have been dead in a non-complementing background. It produced mutant seeds at about 25% frequency because it contained one copy of the transgene (*tCpn*, data not shown). These data indicated that an 11-kb *Not*I fragment containing the transgene with a WT copy of *SLP* was responsible for the complementation of the *slp* phenotype.

The *slp* mutation was also mapped (see "Materials and Methods") and determined to be located between positions 40.6 and 56.1 of chromosome 2 (data not shown). This is consistent with the published sequences for chromosome 2 in which *chaperonin-60α* gene is located within the same physical region (Lin et al., 1999). Taken together, the genetic and molecular evidence indicated that the interruption of the *SLP* locus by T-DNA insertion is responsible for the embryonic abnormalities observed in the *slp/slp* mutant individuals.

SCHLEPPERLESS Locus Encodes a Chaperonin-60α Protein

Sequencing of both the λ101 genomic clone and the partial cDNA pC31 clone showed that the interrupted open reading frame corresponded to a plastid *chaperonin-60α* subunit gene (Hemmingsen et al., 1988; Martel et al., 1990; Cloney et al., 1994). The exons and introns were deduced from a comparison of the genomic clone sequence to the partial cDNA sequences (data not shown; Martel et al., 1990) and to the previously published sequence of *chaperonin-60α* from *Brassica napus* (Martel et al., 1990; Cole et al., 1994). The deduced sequence of the chaperonin-60α protein of Arabidopsis has 586 amino acid residues, and has a predicted molecular mass of approximately 62 kD. The first 46 amino acids appears to constitute a transit peptide that, if cleaved at an Asn (N) residue (amino acid no. 47, Fig. 6), would yield the mature form of the protein (Martel et al., 1990).

The Arabidopsis chaperonin-60α protein shows a very high degree of conservation when compared with plastid chaperonin-60 α-subunit from other species. For example, it has 95% similarity to *B. napus* (Cole et al., 1994) and castor bean (Hemmingsen et al., 1988). However, it is quite divergent from other chaperonin proteins produced by Arabidopsis (e.g. the β-subunit of chaperonin-60 and chaperonin-60 mitochondrial proteins). It shows only 70% similarity to the β-subunit form (Zabaleta et al., 1992) and about 90% similarity to the chaperonin-60 protein localized in the mitochondria (Prasad and Stewart, 1992; see also accession no. AP001297 of chromosome 3 of the Arabidopsis genome). It has 70% and 72% similarity to the groEL proteins from *E. coli* (Hemmingsen et al., 1988) and Brucella (Roop et al., 1992), respectively. GroEL is the prokaryotic equivalent of chaperonin-60α protein. Figure 6 shows the similarity alignment of the Arabidopsis chaperonin-60α protein with other chaperonin proteins. Portions of the rescued plasmids and mutant phage clones were also sequenced to determine the exact nucleotide where T-DNA insertion interrupted the gene. This analysis showed that the concatemer of three T-DNAs was inserted in the seventh exon, thus interrupting the protein-coding sequence at amino acid 449 (Figs. 5A and 6). Therefore, an active chaperonin-60α protein product would not be present in the *slp/slp* embryos.

The *Chaperonin-60α* mRNA Is Present in Several Organs

To begin to analyze the expression pattern of the *chaperonin-60α* gene in Arabidopsis, an RNA blot containing poly(A^+) mRNA from leaf, silique, stem, and inflorescence tissues was hybridized with the 6.6-kb *Eco*RI fragment from the λ101 genomic clone (Fig. 5A; see "Materials and Methods"). As shown in Figure 7, the chaperonin-60α mRNA accumulates in all stages of development examined. The 1.8-kb mRNA that was detected in the four types of tissues is consistent with the expected size of the mRNA that would be encoded by the *SLP* gene.

Plastids Are Undeveloped in *slp/slp* Embryos

Because it is known that chaperonin-60α protein is involved in the folding and assembly of proteins (e.g. Rubisco; Goloubinoff et al., 1989a, 1989b) that are imported into the chloroplast, we determined if chloroplast development was affected in the *slp/slp* embryos. WT (green) and mutant (white) seeds from siliques of a heterozygous *SLP/slp* plant were fixed, embedded, sectioned, and analyzed for the presence and morphology of embryonic plastids using transmission electron microscopy (see "Materials and Methods"). As shown in Figure 8, the plastids in WT embryos developed into chloroplasts with well-stacked, membrane-appressed grana (Fig. 8A). In contrast, no well-developed plastids were detected in mutant *slp/slp* embryos–the mutant plastids contained unstacked or seemingly collapsed membrane structures (Fig. 8B). These results indicated that the

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      At Chaperonin-60a    (1) -------MASANALSSASVLCSSRQSKLGGGNQQQGQRVSYNKRTI-RRF
      At Chaperonin-60b    (1) MASTFTATSSIGSMVAPNGHKSDKKLISKLSSSSFGRRQSVCPRPRRSSS
      Bn Chaperonin-60a    (1) -------MATANALSSPSVLCSSRQGKLSGGSQQKGQRVSYRKAN--RRF
                  GroEL    (1) --------------------------------------------------
At Mitochondrial Cpn60    (1) -----------------------MYRFASNLASKARIAQNARQVSSRMSW

      At Chaperonin-60a   (43) SVRANVKEIAF--DQHSRAALQAGIDKLADCVGLTLGPRGRNVVLD-EFG
      At Chaperonin-60b   (51) AIVCAAKELHFNKDGTTIRRLQAGVNKLADLVGVTLGPKGRNVVLESKYG
      Bn Chaperonin-60a   (42) SLRANVKEIAF--DQSSRAALQAGIDKLADAVGLTLGPRGRNVVLD-EFG
                  GroEL    (1) ----AAKDVKF--GNDARVKMLRGVNVLADAVKVTLGPKGRNVVLDKSFG
At Mitochondrial Cpn60   (28) SRNYAAKEIKF--GVEARALMLKGVEDLADAVKVTMGPKGRNVVIEQSWG

      At Chaperonin-60a   (90) SPKVVNDGVTIARAIELPNAMENAGAALIREVASKTNDSAGDGTTTASIL
      At Chaperonin-60b  (101) SPRIVNDGVTVAREVELEDPVENIGAKLVRQAAAKTNDLAGDGTTTSVVL
      Bn Chaperonin-60a   (89) SPKVVNDGVTIARAIELPDAMENAGAALIREVASKTNDSAGDGTTTASVL
                  GroEL   (45) APTITKDGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTTTATVL
At Mitochondrial Cpn60   (76) APKVTKDGVTVAKSIEFKDKIKNVGASLVKQVANATNDVAGDGTTCATVL

      At Chaperonin-60a  (140) AREIIKHGLLSVTSGANPVSLKRGIDKTVQGLIEELQKKARPVKGRDDIR
      At Chaperonin-60b  (151) AQGFIAEGVKVVAAGRNPVLITRGIEKTAKALVTELKKMSKEVEDS-ELA
      Bn Chaperonin-60a  (139) AREIIKHGLLSVTSGANPVSLKRGIDKTVQALIEELEKRSRPVKGGRDIK
                  GroEL   (95) AQAIITEGLKAVAAGMNPMDLKRGIDKAVTAAVEELKALSVPCSDSKAIA
At Mitochondrial Cpn60  (126) TRAIFAEGCKSVAAGMNAMDLRRGISMAVDAVVTNLKSKARMISTSEEIA

      At Chaperonin-60a  (190) AVASISAGNDDLIGSMIADAIDKVGPDGVLSIESSSSFETTVEVEEGMEI
      At Chaperonin-60b  (200) DVAAVSAGNNDEIGNMIAEAMSKVGRKGVVTLEEGKSAENNLYVVEGMQF
      Bn Chaperonin-60a  (189) AVATISAGNDELIGAMIADAIDKVGPDGVSPIESSSSFETTVEVEEGMEI
                  GroEL  (145) QVGTISANSDETVGKLIAEAMDKVGKEGVITVEDGTGLQDELDVVEGMQF
At Mitochondrial Cpn60  (176) QVGTISANGEREIGELIAKAMEKVGKEGVITIQDGKTLFNELEVVEGMKL

      At Chaperonin-60a  (240) DRGYISPQFVTNPEKLLAEFENARVLITDQKITAIKDIIPILEKTTQLRA
      At Chaperonin-60b  (250) DRGYISPYFVTDSEKMSVEFDNCKLLLVDKKITNARDLVGVLEDAIRGGY
      Bn Chaperonin-60a  (239) DRGYISPQFVTNPEKLLVEFENARVLITDQKITAIKDIIPILEKTTQLRA
                  GroEL  (195) DRGYLSPYFINKPETGAVELESPFILLADKKISNIREMLPVLEAVAKAGK
At Mitochondrial Cpn60  (226) DRGYTSPYFITNQKTQKCELDDPLILIHEKKISSINSIVKVLELALKRQR

      At Chaperonin-60a  (290) PLLIIAEDVTGEALATLVVNKLRGVLNVVAVKAPGFGERRKAMLQDIAIL
      At Chaperonin-60b  (300) PILIIAEDIEQEALATLVVNKLRGTLKIAALRAPGFGERKSQYLDDIAIL
      Bn Chaperonin-60a  (289) PLLIIAEDVTGEALATLVVNKLRGVLNVVAVKAPGFGERRKAMLQDIAIL
                  GroEL  (245) PLLIIAEDVEGEALATAVVNTIRGIVKVAAVKAPGFGDRRKAMLQDIATL
At Mitochondrial Cpn60  (276) PLLIVSEDVESDALATLILNKLRAGIKVCAIKAPGFGENRKANLQDLAAL

      At Chaperonin-60a  (340) TGAEYLAMDMSLLVENATIDQLGIARKVTISKDSTTLIADAASKDELQAR
      At Chaperonin-60b  (350) TGATVIREEVGLSLDKAGKEVLGNASKVVLTKETSTIVGDGSTQDAVKKR
      Bn Chaperonin-60a  (339) TEPS-TALDMGLLVENTTIDQLGIARKVTISKDSTTLIADAASKAELQAR
                  GroEL  (295) TGGTVISEEIGMELEKATLEDLGQAKRVVINKDTTTIIDGVGEEAAIQGR
At Mitochondrial Cpn60  (326) TGGEVITDELGMNLEKVDLSMLGTCKKVTVSKDDTVILDGAGDKKGIEER

      At Chaperonin-60a  (390) IAQLKKELFETDSVYDSEKLAERIAKLSGGVAVIKVGAATETELEDRKLR
      At Chaperonin-60b  (400) VTQIKNLIEQADEDYEKEKLNERIAKLSGGVAVIQVGAQTETELKEKKLR
      Bn Chaperonin-60a  (388) ISQLKKESFETDSVYDSEKLAERIAKLSGGVAVIKVGAATETELEDRKLR
                  GroEL  (345) VAQIRQQIEEATSDYDREKLQERVAKLAGGVAVIKVGAATEVEMKEKKAR
At Mitochondrial Cpn60  (376) CEQIRSAIELSTSDYDKEKLQERLAKLSGGVAVLKIGGASEAEVGEKKDR

      At Chaperonin-60a  (440) IEDAKNATFAAIEEGIVPGGGAALVHLSTVIPAIKETFEDADERLGADIV
      At Chaperonin-60b  (450) VEDALNATKAAVEEGIVVGGGCTLLRLASKVDAIKATLDNDEEKVGADIV
      Bn Chaperonin-60a  (438) IEDAKNATFAAIEEGIVPGGGATLVHLSTVIPAIKETFEDADVRLGADIV
                  GroEL  (395) VEDALHATRAAVEEGVVAGGGVALIRVASKLADLRG--QNEDQNVGIKVA
At Mitochondrial Cpn60  (426) VTDALNATKAAVEEGILPGGGVALLYAARELEKLPT--ANFDQKIGVQII

      At Chaperonin-60a  (490) QKALLSPAALIAQNAGVEGEVVVEKIMFSD-WENGYNAMTDTYENLFEAG
      At Chaperonin-60b  (500) KRALSYPLKLIAKNAGVNGSVVSEKVLSNDNVKFGYNAATGKYEDLMAAG
      Bn Chaperonin-60a  (488) QKALVAQS-LIAQNAGIEGEVVVEKIMFSE-WELGYNAMTDTYENLLEAG
                  GroEL  (443) LRAMEAPLRQIVLNCGEEPSVVANTVKGGD-GNYGYNAATEEYGNMIDMG
At Mitochondrial Cpn60  (474) QNALKTPVYTIASNAGVEGAVIVGKLLEQDNPDLGYDAAKGEYVDMVKAG

      At Chaperonin-60a  (539) VIDPAKVTRCALQNAASVAGMVLTTQAIVVDKPKPKAPAAAAPEGLMV--
      At Chaperonin-60b  (550) IIDPTKVVRCCLEHAASVAKTFLMSDCVVVEIKEPEPVPVGNPMDNSGLG
      Bn Chaperonin-60a  (536) VIDPAKVTRCALQNAASVAGMVLTTQAIVVDKPKPKAPAAAAPEGLMV--
                  GroEL  (492) ILDPTKVTRSALQYAASVAGLMITTECMVTDLPKNDAADLGAAGGMGGMG
At Mitochondrial Cpn60  (524) IIDPLKVIRTALVDAASVSSLLTTTEAVVVDLPKDESESGAAGGGMGGMV
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Figure 6. Alignment of the Arabidopsis chaperonin-60α protein with other related proteins. The Arabidopsis chaperonin-60α protein was aligned with chaperonin-60α protein from *B. napus* (95% similarity), Arabidopsis chaperonin-60β protein (70% similarity), the Arabidopsis mitochondrial chaperonin-60 (90% similarity), and GroEL protein from *Escherichia coli* (70% similarity) using the AlignX program of VectorNTI software. Amino acids that are identical in all five proteins are presented in yellow blocks with red letters. Conservative amino acids are presented in gray blocks with black letters. Sources of these proteins are cited in the text. The genomic sequence of the Arabidopsis *Chaperonin-60α* has the GenBank accession no. U49357.

differentiation of plastids into chloroplasts during Arabidopsis embryo development requires a functional chaperonin-60α protein.

DISCUSSION

The role of the chloroplast in plant embryogenesis has not been explored extensively. For the most part, the role of chloroplast has been studied only in relation to postembryonic stages of plant development. The results from several nuclear gene mutations that have detrimental effects on chloroplast biogenesis and metabolism (Tsugeki et al., 1996; Uwer et al., 1998) indicate that this organelle can be playing a significant role in plant embryogenesis. Our analysis of the *SCHLEPPERLESS* gene supports this conclusion.

The Arabidopsis *slp* mutant is defective in embryo development. The *slp/slp* mutant cotyledons are very short and the whole embryo is white, even at the mature stage (Fig. 1D). Mutant embryos up to the heart stage appear to be morphologically normal,

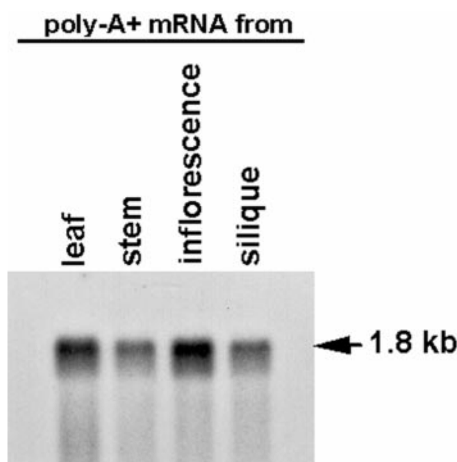

Figure 7. RNA-blot analysis of *chaperonin-60α*. Each lane contains 0.5 μg of poly(A^+) mRNA from each organ. The RNA blot was hybridized with the 6.6-kb *Eco*RI fragment from λ101 genomic clone (see Fig. 5A).

although embryo development is temporally retarded in comparison with WT development (Fig. 2). The morphological defect of *slp/slp* embryos is manifested after the heart stage of embryogenesis (Fig. 2). In addition, the acquisition of competence to transcribe late-maturation genes is also delayed in mutant embryo and appears to depend on the developmental state of the embryo (Fig. 4). This result is in contrast with the transcriptional competence of *raspberry*, *leafy cotyledon1*, and other embryo-defective (*emb*) mutants that are capable of transcribing late-maturation genes in a temporally appropriate manner as WT embryos (West et al., 1994; Yadegari et al., 1994; Devic et al., 1996).

The morphology of *slp/slp* embryos is similar to that of *edd1* mutants (Uwer et al., 1998). Both *edd1* and *slp* seeds are able to germinate and form white plantlets when cultured in vitro (Fig. 3; Uwer et al., 1998). In contrast to some embryo-defective mutants whose chloroplasts are not affected by the mutation and are able to turn green when cultured (Franzmann et al., 1989; Patton et al., 1998), *slp/slp* embryos could not be rescued by the tissue culture medium (Fig. 3).

The development of chloroplast is affected in the *slp/slp* mutant, resulting in aberrant plastids in mature mutant embryos (Fig. 8). The fact that the embryos remain white (Fig. 1D) indicates that *slp/slp* mutant is most likely photosynthetic incompetent. However, in contrast with mutants that lack the photosynthetic capacity but remain morphologically normal (e.g. albino mutants), *slp/slp* embryos have abnormal morphology. The albino mutants have defects in chloroplast ultrastructure and are not photosynthetically competent, but are able to form morphologically normal mature adult plants (Hudson et al., 1993; Long et al., 1993; Sundberg et al., 1997). Therefore, the defect in *slp* mutant is more complex than just not being able to effectively carry out photosynthesis. The defect can be explained by the nature of the gene mutated in *schlepperless,* which appears to affect not only photosynthesis but also other processes that occur in the chloroplast and are required for proper chloroplast development.

SCHLEPPERLESS Locus Encodes Chaperonin-60α Subunit Protein

We cloned the WT *SLP* gene corresponding to the gene interrupted by T-DNA in the *slp* mutant (Fig. 5) and demonstrated that this genomic clone is capable of complementing the *slp* mutation (Table I and Fig. 5D). The gene encodes the plastid chaperonin-60α-subunit protein that shows significant homology with other chaperonin proteins, both from prokaryotes and eukaryotes (Hemmingsen et al., 1988; Zabaleta et al., 1992; Cole et al., 1994). The phenotypes (including white color and undeveloped plastid) that we observed for *slp* mutant are all consistent with the *chaperonin-60α* gene being mutated in this embryo-defective line.

The chaperonin-60α-subunit is considered to be the equivalent of the prokaryotic groEL protein (Hemmingsen et al., 1988). Mutation in the G*roEL* gene is lethal and the protein is considered essential for bacterial growth (Fayet et al., 1989). The heptameric ring structure of groEL proteins, in conjunction with heptameric ring of groES (another form of chaperonin), are involved in the folding and assembly of proteins to attain their proper and functional conformations (for review, see Ellis and van der Vies, 1991). Some details of the molecular mechanisms for this chaperonin-mediated protein folding and assembly are already known and elucidated using in vitro analysis (Weissman et al., 1994, 1996; for example, see Weissman et al., 1994, 1996; Mayhew et al., 1996; Ma and Karplus, 1998; Wang et al., 1998). In fact, Rubisco (one of the chloroplast-localized proteins) is one of the substrates used for this in vitro analysis (Goloubinoff et al., 1989a, 1989b; Gutteridge and Gatenby, 1995).

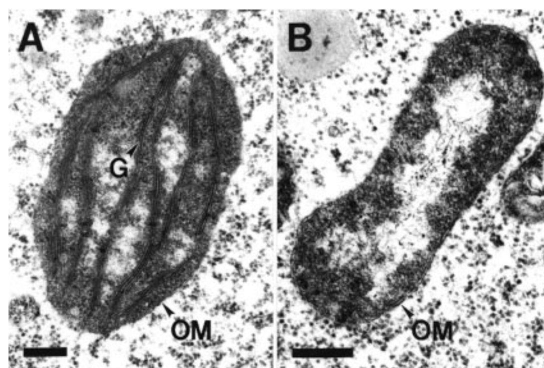

Figure 8. Transmission electron microscopy analysis of plastids. Chloroplast (A) from a WT embryo and undeveloped plastid (B) from *slp* embryo. G, Grana; OM, outer membrane. Bars = 0.15 μm.

Chaperonin-60α Protein Is Required for Chloroplast Development

During chloroplast biogenesis, plastid volume and composition change as a consequence of acquisition of photosynthetic competence and activation of other biosynthetic processes (Mullet, 1988). The acquisition of this competence and the activation of the biosynthetic processes require the activation of both nuclear and chloroplast genes (Mullet, 1988). The nuclear-encoded chloroplast-localized proteins must be expressed at the proper temporal and developmental state of the chloroplast. In addition, some of these nuclear-encoded components have to be assembled inside the chloroplasts (e.g. photosystem II components that are normally assembled in the grana; Hermann et al., 1985), and the assembly requires that these components be in their proper conformation. We propose that chaperonin-60α protein is important for folding a variety of proteins essential for chloroplast development and functions.

In the *slp* embryos, we showed that the plastids are not fully developed and that there is no evidence for stacked or organized thylakoid membrane (Fig. 8). This indicates that functional chaperonin-60α protein is necessary for chloroplast development. Therefore, it is possible that, in the absence of functional chaperonin-60α-subunit, proteins that are imported into the chloroplasts are not folded properly and thus not functional. As such, the plastids are deprived of the necessary components for their biogenesis and/or function. These components may include proteins that are important not only for photosynthetic activity (e.g. light-harvesting chloroplast a/b protein), but other proteins (e.g. Gln synthetase) that are important for other functions of the chloroplasts (i.e. amino acid and fatty acid biosyntheses; Lubben et al., 1989). The products that are synthesized within the chloroplast (e.g. sugars, fatty acids, terpenoids, and amino acids) are not only utilized by the chloroplast organelle itself for its own development and function (for example, see Jarvis et al., 2000), but also by the cell for other metabolic processes. Fatty acids biosynthesis is considered essential for growth and its absence is lethal (Ohlrogge and Browse, 1995).

Embryo Development Is Dependent on Functional Chloroplasts

Chloroplasts in embryos are derived from undifferentiated proplastids that are normally inherited maternally by the plant zygote (Kirk and Tilney-Bassett, 1978). Greening starts in the early heart-shaped embryos and the initiation of greening is always associated with the development of granal stacks within the plastids. The plastids in globular embryos contain single lamellae, but in heart-shaped embryos, they begin to form rudimentary stacks of membrane that develop further into full grana by the torpedo stage (Schultz and Jensen, 1968; Mansfield and Briarty, 1991). There are indications that non-green proembryos could be actively synthesizing proteins that are eventually localized in the plastids. For example, mRNAs corresponding to chloroplast *psbA* gene (encodes the D1 protein of photosystem II) and nuclear *rbcS* gene (encodes the small subunit of Rubisco) begin to accumulate in the proembryo (Degenhardt et al., 1991). Because the folding of proteins encoded by these mRNAs is chaperonin mediated, functional chaperonin-60α protein is necessary at the very early stages of embryogenesis, even before the embryo becomes green.

As shown in Figure 2, we demonstrated that *slp* embryo is morphologically normal up to the heart stage but its development is slower than that of WT embryos. Whatever accounts for this slow development may be related to the state of competence of the plastids. It is possible that competent proplastids contribute to the normal rate of embryonic process. It is interesting to note that cell division is extremely rapid as embryo develops from globular to heart stage and as the cotyledon initials are formed quickly (Mansfield and Briarty, 1991). It is possible that an early developed plastid starts to synthesize products that are nutritionally important and become readily available to the embryo as it develops. Otherwise, the amount of these biosynthetic products may be limited and insufficient for the *slp* embryo to undergo a normal rate of development. This limited amount of products may come from the proplastids probably rendered competent to some extent, albeit for a limited period during early embryogenesis, by the presence of a limited supply of chaperonin-60α protein contained within the maternally inherited proplastids. It is also possible that the folding of the chloroplast-localized proteins in the proplastids at the early stage of embryogenesis may be mediated by other chaperonins (e.g. hsp70 or chaperonin-60β; Madueño et al., 1993; Tsugeki and Nishimura, 1993, Zabaleta et al., 1994). This, perhaps, renders the proplastids functional to a limited degree at this stage of embryo development.

The data presented in this paper do not preclude the possibility that the folding of some non-chloroplastic proteins (e.g. cytoplasmic proteins) can be mediated by chaperonin-60α, similar to the groEL-mediated folding of citrate synthase, polynucleotide phosphorylase, and ketoglutarate dehydrogenase (Horwich et al., 1993) or to the chaperonin-mediated folding of actin (Gao et al., 1992; Siegers et al., 1999; Thulasiraman et al., 1999) and tubulin (Yaffe et al., 1992). If chaperonin-60α is involved in the folding of some cytoplasmic proteins in plant cells, then this particular defect may be reflected in the overall phenotype of *schlepperless* and is indistinguishable from the consequent defects in the chloroplast. The *schlepperless* mutant should allow studies to be carried out that distinguish between the effect of chaperonin-60α on chloroplast and cytoplasmic proteins.

In the developing embryo, the demand for biosynthetic products is very high, especially during the late maturation phase when macromolecules (proteins, lipids, and/or carbohydrates) are stored in the cotyledons (Goldberg et al., 1989; Shotwell and Larkins, 1989), leading to the expansion of the whole organ. Thus, it may not be surprising that in *slp* embryos, where plastids are undeveloped and nonfunctional as a result of mutation in the *chaperonin-60α* gene (Fig. 8) cotyledons do not expand fully. In a similar manner, the loss of function in other nuclear genes whose products are chloroplast localized and/or may be needed for early plastid biogenesis and competence lead also to defects in embryo development. Mutations in these genes either prevent embryo to undergo morphogenesis as in the case of *raspberry* mutants (Yadegari et al., 1994; N.R. Apuya, R. Yadegari, R.L. Fischer, J.J. Harada, and R.B. Goldberg, unpublished data) or prevent embryo to develop cotyledons as in the case of *edd1* mutant (defective in plastidic form of glycyl-tRNA synthetase; Uwer et al., 1998). It is important to realize that mutations in these genes are important for understanding the molecular, biochemical, and cellular basis of plant embryogenesis. In this respect, the role of chloroplast in embryonic development should be explored in more detail. Characterizing other nuclear-encoded gene products that are not necessarily involved directly in photosynthesis but are important for chloroplast function during embryogenesis can elucidate this role.

In conclusion, the results of our analyses indicate that chaperonin-60α protein is essential for the growth and development in plants and the absence of this protein leads to severe defects in embryo and seedling development.

MATERIALS AND METHODS

Mutant Isolation and Genetic Analysis

The line characterized in this study was A2137, one of the 5,822 T-DNA-mutagenized lines of Arabidopsis ecotype Wassilewskija that were screened at the DuPont Experimental Station (Wilmington, DE, in November 1990) and at the University of Arizona (Tucson, AZ, in November 1991; Feldmann and Marks, 1987; Errampalli et al., 1991; Feldmann, 1991; Castle et al., 1993; Yadegari et al., 1994). The recessive embryo-defective mutation was maintained in heterozygous plants (*slp/SLP*) that produced WT and mutant seeds at a 3:1 ratio. The cosegregation of T-DNA and the embryo defective phenotype was analyzed by a Kan-R assay and by genomic DNA-blot analysis using the T-DNA right and left border sequences as probes. To map the chromosomal location of the mutation, heterozygous plants were crossed to the mapping lines obtained from the Arabidopsis Biological Resource Center (Columbus, OH). Initial cross was made to the mapping line CS3078 (a mapping line containing one marker in every chromosome) to narrow down the chromosomal location of the mutation. A subsequent cross was made to mapping line W6 that has *cp2*, *cer8*, and *as* markers in chromosome 2. Linkage analysis to the phenotypic markers was performed by characterizing 500 segregants from the F_2 population and estimates of recombination were done using the RECF2 program (Koornneef and Stam, 1992).

Seed Germination in Tissue Culture

Seeds were sterilized in a commercial bleach solution for about 10 min and rinsed four times with sterile water. Sterilized seeds were subsequently plated in germination medium (GM) containing 1× Murashige and Skoog salt, 1% (w/v) Suc, 100 mg L^{-1} inositol, 1 mg L^{-1} thiamine, 0.5 mg L^{-1} nicotinic acid, 0.5 g L^{-1} MES (pH 7), and 0.5% (w/v) phytagar (Gibco, Gaithersburg, MD). Germinating mutant seeds were transferred to fresh GM plates every 10 d until termination of the experiment. For Kan-R and/or Hyg-R assay, seeds were germinated in GM containing 50 μg mL^{-1} kanamycin sulfate and/or 20 μg mL^{-1} hygromycin.

GUS Detection

Embryos resulting from the cross between the heterozygous plants and the homozygous transgenic lines containing either β-conglycinin-α′-promoter::GUS or LEA::GUS fusion constructs were assayed for GUS activity using the procedures of Jefferson et al. (1987) with some modifications. The GUS staining solution consisted of 50 mM sodium phosphate buffer (pH 7), 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 1 mM 5-bromo-4-chloro-3-indoyl glucuronide. Dissected embryos were incubated in this solution at 37°C for either 2 h (LEA::GUS) or 5 h (β-conglycinin-α′::GUS). Embryos were destained for 5 h in 70% (v/v) ethanol and processed for dark-field microscopy.

Microscopy

Bright-Field Microscopy

Siliques from heterozygous plants were collected, cut into 2-mm pieces, fixed, and embedded in LR White plastic resin (Polysciences, Inc., Warrington, PA). Sections (1 μm thick) were made using a microtome (LKB Ultratome V; LKB, Bromma, Sweden) and stained for about 10 min with 0.5% (w/v) toluidine blue in 0.1% (w/v) borate solution. Bright-field photographs were taken with Gold 100 film (ISO 100/21°, Kodak, Rochester, NY) using a compound microscope (Olympus BH-2; Olympus Corporation, Lake Success, NY).

Nomarski Microscopy

Mutant and WT seeds were fixed in ethanol:acetic acid (9:1) solution overnight, and successively washed in 90% and 70% (v/v) ethanol for at least 30 min each. Seeds were cleared with chloral hydrate:glycerol:water solution (8:1:2, w:v:v) for at least 2 h prior to microscopy (Berleth and

Jürgens, 1993). Embryos were visualized using Nomarski optics on a Zeiss Axiophot (Carl Zeiss, Inc., Oberkochlen, Germany). Photographs were taken using Kodak TMAX 100 (E.I. 100/21°) film.

Transmission Electron Microscopy

The procedures cited by Yadegari et al. (1994) were followed except LR White plastic resin was used as the embedding medium.

Whole Mount Photography

Dark-field photographs of germinating seedlings in culture were taken using a dissecting microscope (Olympus SZH, Olympus Corp.). Dark-field photographs of embryos assayed for GUS were taken using a compound microscope (Olympus BH2) using Kodak Gold 100 film (ISO 100/21°).

Genomic DNA Isolation, Restriction Analysis, DNA Blotting, and Labeling

Genomic DNA was isolated according to the procedures established by Dellaporta et al. (1983). Digestion of genomic DNA with restriction enzymes was done overnight following the conditions recommended by the manufacturers. Digested DNA was size fractionated by electrophoresis in agarose gels and then transferred to Nytran nylon membrane (Schleicher and Schuell, Keene, NH) following the recommended protocol by the manufacturer. Prehybridization and hybridization of DNA blots were done following the procedures recommended by Ausubel et al. (1992). Labeled DNA probes were synthesized using the random priming technique (Feinberg and Vogelstein, 1983).

Isolation of Mutant and WT Genomic Clones

Plasmid rescue was done following the protocol of Behringer and Medford (1992). The procedures recommended by Sambrook et al. (1989) for colony lifts and hybridization were followed. A genomic library of the heterozygous line was also constructed using the λGEM-12 vector and following the protocol recommended by Promega (Madison, WI). Screening of the library and the plasmid transformants was done using the right and left border sequences of T-DNA as probes. The procedures established by Ausubel et al. (1992) for plating and transferring bacteriophage library and for pretreatment of filters for hybridization were followed. The isolation of WT genomic clones was done following the same protocol but using the plant flanking sequences obtained from the mutant clones as probes. The *Escherichia coli* strain KW251 was used for all genomic phage experiments. Sub-cloning of certain fragments from rescued plasmids and phage clones was done using pGEM-3Z(f-) as vector and following the standard cloning procedures recommended by Sambrook et al. (1989).

Isolation of cDNA Clones

A cDNA library (in λZAP vector) constructed from poly(A^+) mRNA from WT Arabidopsis siliques was used to isolate cDNA clones. The protocol for the isolation of phage genomic clones was essentially followed except for using XL1-Blue strain as the host bacteria. The plasmid forms of the cDNA clones were isolated from the corresponding phage cDNA clones following the in vivo excision protocol recommended by Stratagene (La Jolla, CA). The 6.6-kb *Eco*RI fragment from WT genomic clone λ101 (see Fig. 5A) was used as probe for the cDNA library screening.

DNA Sequencing

The sequencing of cDNA clones, and sub-clones of rescued plasmids and phage genomic clones, was done following the dideoxy-sequencing procedures recommended by United States Biochemicals (Cleveland). The sequencing of the 6.6-kb *Eco*RI fragment of genomic clone λ101 was done at Plant Genetics Systems using the PCR with *Taq* polymerase and an automated sequencer (Applied Biosystems, Inc., Foster City, CA). Analysis of sequences was done using the Genetics Computer Group software and the National Center for Biotechnology Information BLAST e-mail server.

RNA Techniques

Polysomal RNAs were isolated according to the procedures described by Cox and Goldberg (1988). Poly(A^+) mRNAs were isolated using the Poly-AT Tract mRNA Isolation System (Promega) and following the recommended protocol by the manufacturer. The isolated mRNAs were electrophoresed through a formaldehyde-agarose gel, transferred to a Nytran membrane (Schleicher and Schuell), and hybridized with ^{32}P-labeled DNA according to the procedures recommended by Ausubel et al. (1992).

Plant Transformation and Complementation Analysis

An 11-kb *Not*I fragment from λ101 genomic clone (see Fig. 5A) was sub-cloned into the *Not*I site of pGSH166N vector (courtesy of Plant Genetic Systems, Gent, Belgium). The resulting recombinant was subsequently transferred to *Agrobacterium tumefaciens* to be used for root transformation. The original procedures established by Valvekens et al. (1988) for Agrobacterium-mediated transformation of Arabidopsis root explants were followed. WT Arabidopsis (ecotypes C24 and Nossen) were used as recipients.

Complementation analysis was done by crossing heterozygous plants (*slp/SLP*; Kan-R) to transgenic lines containing the WT *chaperonin-60α* gene (designated as *tCpn*; Hyg-R). The Hyg-R and Kan-R F_1 progenies from this cross were selected and allowed to grow in the greenhouse. The F_2 seeds collected from the F_1 plants were germinated without selection. The resulting F_2 plants were phenotyped by dissecting two to three siliques from each plant and the

number of mutant and WT seeds were counted for genetic analysis as presented in Table I. Depending on the percentage of mutant seeds contained within the two or three siliques, the F_2 plants were grouped into three classes as listed in Table I. Segregants in class I were individuals that produced only WT embryos, whereas those in class II and class III produced mutant embryos at different frequencies. If the complementation were successful, the segregants that are either heterozygous (*SLP/slp*) or homozygous (*slp/slp*) would be included in class I as long as they are homozygous to the transgene (*tCpn*). The frequency of mutant embryos produced by individuals in either class II or class III depends on their genotypes as well (see Table I). For instance, if homozygous (*slp/slp*) segregants contain only one copy of the transgene, then we expect them to be classified in class II (i.e. producing mutant seeds at 25% frequency). However, if the heterozygous segregants (*SLP/slp*) contain only one copy of the transgene, then they would be classified in class III (i.e. producing mutant seeds at about 6.25% frequency), which is a class that is produced only if the complementation was successful (see Table I). Genomic DNAs from randomly selected F_2 plants were isolated for DNA-blot analysis shown in Figure 5C.

The genotypes of some randomly selected F_2 segregants representative of each class were determined by germinating their progeny seeds under kanamycin and hygromycin selection. Kan-R allowed us to follow the segregation of the *slp* mutation, whereas Hyg-R allowed us to follow the segregation of the transgene (*tCpn*). At the same time, testcross analysis was also done by crossing the selected individuals to WT (*SLP/SLP*) to determine the homozygosity or heterozygosity of the *slp* mutation. The seed products of the testcross were germinated under selection for the two antibiotics and the resulting plants were analyzed for an embryo-defective phenotype.

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