Inactivation of a Glycyl-tRNA Synthetase Leads to an Arrest in Plant Embryo Development

Ursula Uwer,[a,1] Lothar Willmitzer,[b] and Thomas Altmann[b,2]

[a] Institut für Genbiologische Forschung, Ihnestrasse 63, 14195 Berlin, Germany
[b] Max-Planck-Institut für Molekulare Pflanzenphysiologie, Karl-Liebknecht-Strasse 25, 14476 Golm, Germany

Embryo formation is the first patterning process during vegetative plant growth. Using transposons as insertional mutagens in Arabidopsis, we identified the mutant *edd1* that shows <u>e</u>mbryo-<u>d</u>efective <u>d</u>evelopment. The insertion mutation is lethal, arresting embryo growth between the globular and heart stages of embryonic development. The mutant phenotype cosegregates with a transposed *Dissociation* element. Sequences flanking the transposed element were isolated and used to isolate a full-length cDNA clone representing the wild-type *EDD1* gene. Complementation of the mutant through Agrobacterium-mediated gene transfer of an *EDD1* wild-type copy as well as loss of the transposon concomitant with phenotypic reversion demonstrated that the transposon had caused the mutation. Based on homology to *Escherichia coli*, the *EDD1* gene is predicted to encode a novel glycyl-tRNA synthetase (GlyRS) that has not been identified previously in higher plants. An N-terminal portion of the plant protein is able to direct a marker protein into pea chloroplasts. Thus, the gene identified by the embryo-defective insertion mutation encodes a GlyRS homolog, probably acting within the plastidic compartment.

INTRODUCTION

Pattern formation presents one of the most challenging aspects of plant development. It addresses the organizational aspect of development, namely, the origin of diverse cell types, tissues, and organs at specific locations to form a structurally and functionally meaningful context. In flowering plants, most pattern-forming events occur in the postembryonic sporophyte after seed germination. However, the primary plant architecture, which is fairly uniform among flowering plants, is generated during early embryogenesis (reviewed in Jürgens et al., 1991; Jürgens and Mayer, 1994).

Formation of the embryo body can be divided conceptually into three overlapping phases (West and Harada, 1993; Jürgens and Mayer, 1994). During the first morphogenetic phase, an apical–basal axis is established throughout the embryo body, with the root apical meristem at one end and the shoot apical meristem at the other. A radial pattern is created by the formation of three primordial tissue layers, that is, protoderm, procambium, and ground meristem, and bilateral symmetry occurs throughout the development of the cotyledons (Goldberg et al., 1994; Meinke, 1995).

The second phase is characterized by embryo growth and the accumulation of storage reserves (Souèges, 1919; Müller, 1963; Goldberg et al., 1989). During the final phase, the embryo prepares for desiccation, dehydrates, and enters a period of developmental arrest. The ovule develops into the seed, which is surrounded by an elaborated coat that enables the differentiated embryo to survive harsh environmental conditions until germination and sporophytic development are favorable.

Higher plant embryogenesis was investigated extensively during the past century (Hanstein, 1870; Vandendries, 1909; Schnarf, 1929; Maheshwari, 1950). Studies using light and electron microscopy have provided detailed descriptions of the morphological and anatomical changes that characterize embryonic development (Schulz and Jensen, 1969; Tykarska, 1979; Mansfield and Briarty, 1990a, 1990b, 1991a, 1991b; West and Harada, 1993; Goldberg et al., 1994; Jürgens and Mayer, 1994).

Cellular differentiation has been studied largely in relation to the biosynthesis and accumulation of storage proteins, lipids, and starch macromolecules, which ultimately serve as nutrients for the postgerminative seedling (Jenner, 1982; Slack and Browse, 1984; Shotwell and Larkins, 1989; Galili et al., 1993). The development of somatic embryo systems (De Jong et al., 1993; Liu et al., 1993; Zimmerman, 1993) as well as studies on hormone action (Schiavone and Cooke, 1987; Liu et al., 1993; Fischer and Neuhaus, 1996) have revealed at least some of the signals that direct proper embryo development.

Genetic analysis provides a complementary approach to the study of plant embryo development. It allows genes with essential functions during this critical stage of the plant life
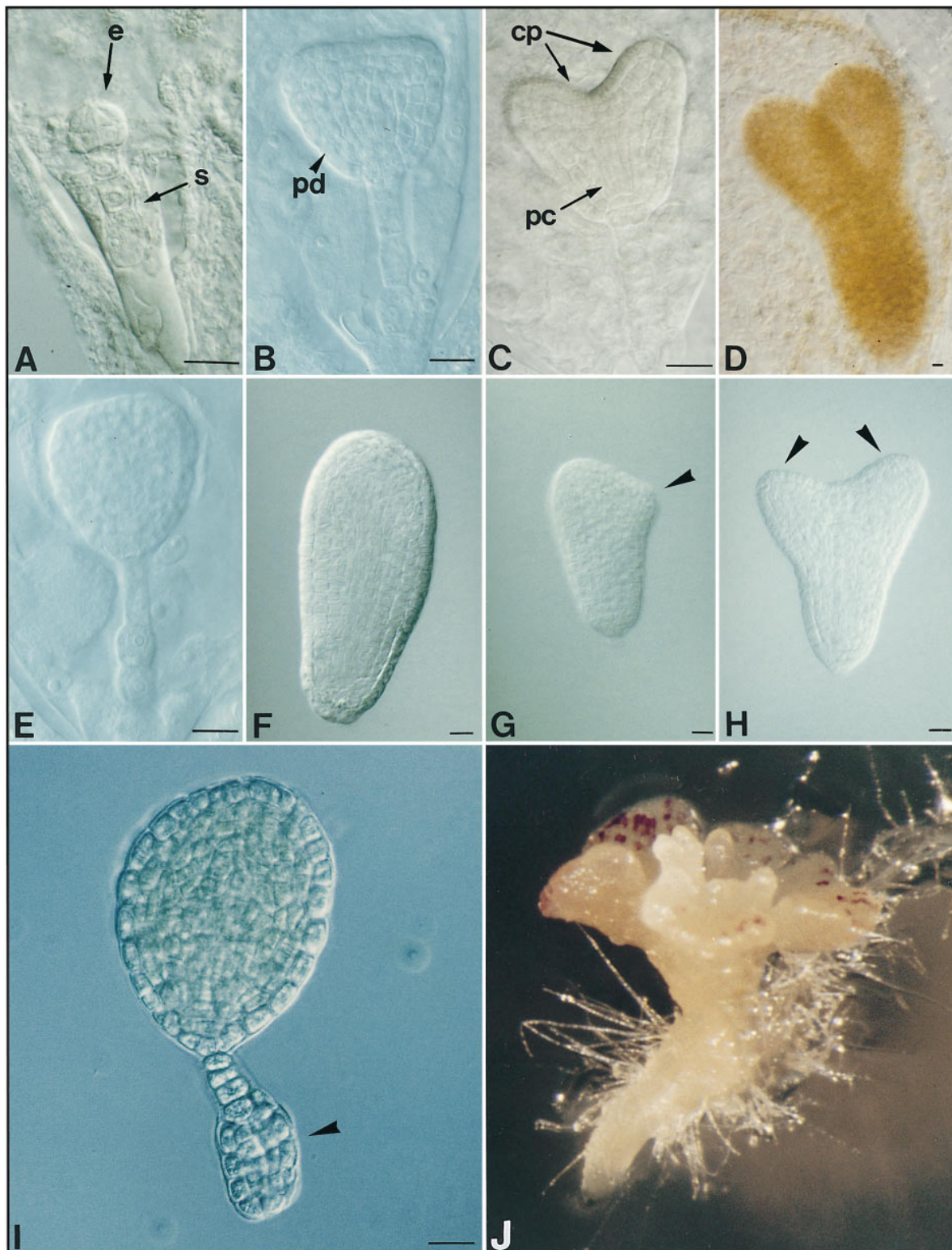
Figure 1. Phenotypes of Wild-Type and *edd1* Embryos.

cycle to be identified. To date, only a few genes have been isolated that are expressed specifically during early embryogenesis, and very little is known about genes acting on embryo pattern. One way to identify "embryonic" genes is to disrupt normal embryo development by mutation. Embryonic mutations have been identified in a wide range of flowering plants, including Arabidopsis (Müller, 1963; Jürgens et al., 1991; Meinke, 1991; Franzmann et al., 1995), maize (Sheridan and Neuffer, 1982; Clark and Sheridan, 1991), barley (Bosnes et al., 1987; Felker et al., 1987), carrot (Lo Schiavo et al., 1988), rice (Nagato et al., 1989), and pea (Bhattacharyya et al., 1990). Many of these mutations have been induced with chemical mutagens, x-irradiation, or T-DNA from Agrobacterium.

In this report, we describe the isolation and characterization of a recessive embryo-defective mutant of Arabidopsis, *edd1* (for *embryo-defective development*), caused by insertion of a *Dissociation (Ds)* transposon. Development of mutant embryos diverges from normal embryo formation, because *edd1* embryos do not establish normal bilateral symmetry and fail to complete their development. The *EDD1* gene was isolated and shown to encode a protein with significant homology to the bacterial glycyl-tRNA synthetase (GlyRS) from *Escherichia coli.*

RESULTS

Isolation of the *edd1* Mutant

A two-component transposon system was used to induce insertional mutants in Arabidopsis (ecotype C24) by using an Ac_{cl}/Ds_A transposition line. As described earlier (Altmann et al., 1992, 1995), this system is based on the maize transposable element *Activator (Ac)* and consists of an immobilized *Ac* element, Ac_{cl}, and a nonautonomous *Ds* element, Ds_A. In the presence of Ac_{cl}, Ds_A is mobilized. Descendants of the transposition line were screened for novel phenotypes. One mutant line that was identified showed a recessive seed-

defective phenotype. This line harbored 25% aborted seeds randomly distributed throughout the siliques. Abnormal seeds are readily distinguishable as white seeds from wild-type green seeds in immature siliques. In mature siliques, affected seeds had a severly shrunken brownish appearance and dried out and died. Germination of mutant seeds was never observed. This type of embryo-defective mutant was named *edd1*, for *embryo-defective development.* In wild-type plants, seeds containing defective embryos were observed at much lower frequency (<1%).

Developmental Profiles of *edd1* Embryos

We compared the phenotypes of wild-type and mutant embryos from siliques of *edd1* plants at various stages of embryo development. As shown in Figure 1A, a wild-type globular-stage embryo contains a spherical embryo proper attached to a linear suspensor of seven to nine cells, including the hyphophysis at the base of the embryo proper. Due to local cell divisions within the apical embryo region, a triangular embryo proper is formed (Figure 1B). At this stage of development, the embryo proper displays three primary tissue layers—protoderm, ground meristem, and procambium. Cotyledon primordia arise at the flanks of the upper tier, producing a heart-shaped embryo (Figure 1C). At the same time, the hypophysis divides to form a region of cells from which the root apical system will develop. During the torpedo stage (Figure 1D), the cotyledons and hypocotyl elongate, and the suspensor begins to degenerate. Chlorophyll accumulation becomes visible throughout the embryo and the embryo sac. The mature embryo proper consists of two cotyledons adjacent to the hypocotyl, and the embryo occupies nearly the whole seed (data not shown).

To understand the role of the *EDD1* gene, we tried to elucidate the gene's primary site and time of action during embryo development. *edd1* embryos appeared normal through the early-globular embryo stage, and we could not distinguish between mutant and wild-type embryos. By contrast, *edd1* embryos were readily identified within siliques that

---

Figure 1. (continued).

Seeds isolated from immature siliques of *edd1* were cleared, as described in Methods, and photographed by using Nomarski optics, with the exception of **(J).**
(A) to **(D)** Wild-type embryos at the globular stage **(A)**, transition stage **(B)**, heart stage **(C)**, and torpedo stage **(D)**.
(E) to **(I)** *edd1* embryos at the same developmental stage as shown in **(D)** for wild-type embryos. Most mutant embryos develop an enlarged apical region without cotyledon formation **(E)**, whereas a smaller percentage develop small apical bulges (indicated by arrowheads), possibly representing cotyledon primordia (**[F]** to **[H]**). Abnormal development was also detected in the suspensor region, where an enlarged multicellular suspensor region (indicated by an arrowhead) developed instead of a single linear cell file **(I)**.
(J) Isolated *edd1* embryo cultured for 1 month on a hormone-free nutrition medium. The seedling, which measured ∼1 cm, developed leaf projections and an elaborate root system.
cp, cotyledon primordia; e, embryo proper; pc, procambium; pd, protoderm; s, suspensor. Bars = 25 μm in **(A)** to **(I)**.

contained wild-type embryos at heart stage. At this time of silique development, *edd1* embryos are retarded in their growth and do not display the bilateral symmetry of wild-type embryos (Figure 1E).

Microscopic analysis of isolated *edd1* embryos from siliques that contained wild-type embryos at the torpedo stage revealed that enlargement of mutant embryos occurred but never reached wild-type levels and was not accompanied by normal cotyledon or axis differentiation. Individual mutants displayed variable phenotypes. Most *edd1* embryos failed to develop a pair of cotyledons but showed an enlarged apical region of the embryo proper due to abnormal cell divisions within the embryo (Figure 1F). In rare cases, formation of one or two small apical bulges could be observed (Figures 1G and 1H). Chlorophyll accumulation within mutant embryos or the surrounding embryo sac never became visible. The radial arrangement of tissues seemed to be unaffected: mutant embryos were bordered by a normally formed protoderm, and central vascular strands could be distinguished. Abnormal development was also detected within the suspensor region of mutant embryos. This region was enlarged in contrast to the simple linear cell file of wild-type embryos (Figure 1I, indicated by an arrowhead).

To determine the growth and differentiation potential of mutant embryos cultured in vitro, we removed *edd1* embryos from immature seeds at various developmental stages and transferred them to hormone-free synthetic nutrient medium. Under these conditions, mutant embryos enlarged and formed small plantlets (Figure 1J). None of the cultured mutants was green, but some produced purple sectors. Despite a high frequency of regeneration, cultured plantlets failed to continue their growth and turned into calli. Fertile plants were not obtained. From this observation, we conclude that either the culture conditions were inappropriate for complete rescue of mutant embryos or the *EDD1* gene product is essential for normal vegetative growth.

Isolation of the *EDD1* Gene

In plants segregating for the *edd1* phenotype, the presence of a novel Ds_A insertion not detectable in the original Ac_{cl}/Ds_A line could be monitored using DNA gel blot analysis (data not shown). The transposed Ds_A element was separated from the Ac_{cl} element by crossing the *edd1* line to the untransformed wild type, and progeny plants were selected that lacked the Ac_{cl} element (Figure 2A). Descendants of plants that carried the stabilized Ds_A element (Ds_A^*) were tested for linkage between the transposed element and the *edd1* mutation (Figure 2B). Among the 110 plants tested (76 *edd1*/+ plants and 34 wild-type +/+ plants), no recombination between the mutant locus and the inserted Ds_A element was observed. This strict linkage between the Ds_A insertion and the mutant locus strongly indicated that the Ds_A insertion caused the embryo-defective phenotype.
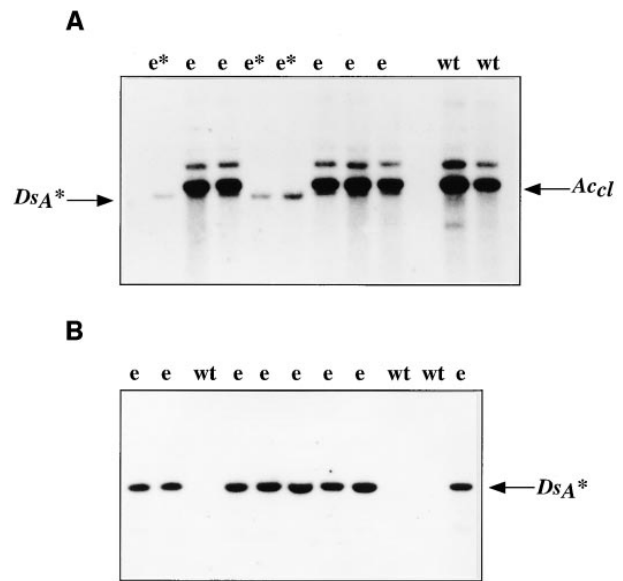

Figure 2. Genomic DNA Gel Blot Analysis.

Total DNA from *edd1* segregants displaying either the mutant (e) or wild-type (wt) phenotype was digested with EcoRI-HindIII and hybridized with a ^{32}P-labeled 3′ HindIII-BamHI fragment of *Ac*. Arrows indicate the positions of the corresponding 3′ fragments of Ac_{cl} and the transposed Ds_A^* at the new locus.

(A) The *edd1* mutant was crossed to the untransformed wild type, and F$_1$ plants were tested for separation of Ac_{cl} and Ds_A. *edd1* segregants marked with an asterisk (e*) only contained the transposed Ds_A^* element; the Ac_{cl} as well as the Ds_A element in the original locus were no longer present in these plants.

(B) To demonstrate linkage between the novel Ds_A^* locus and the mutant phenotype, we analyzed progeny plants of the *edd1* mutant with the stabilized Ds_A^* element for recombination events. Plants showing the mutant phenotype (e) all contained the Ds_A^* element, whereas plants displaying wild-type characteristics (wt) did not.

To study the molecular and biochemical basis of the *edd1* phenotype, we used the transposon sequence to screen a genomic library made from DNA isolated from heterozygous *edd1*/+ plants to isolate DNA adjacent to both termini of the Ds_A element in the mutant. A 3.5-kb EcoRI-SalI chromosomal fragment was subcloned; it contained the complete Ds_A element and 0.5 and 1.0 kb of flanking genomic sequences, respectively. The sequence of the flanking regions was determined from both directions, and the cloned fragments were used to screen wild-type Arabidopsis cDNA libraries, resulting in the isolation of several cDNA clones. The complete nucleotide sequence of the longest cDNA clone (p7/9) was determined. It comprises 3438 bp and has an open reading frame (ORF) extending from nucleotide 32 to nucleotide 3235, with the stop codon TGA and a putative polyadenylation signal, AATAAA (Joshi, 1987), downstream of the translational stop. The nucleotide sequence was submitted to the EMBL database (accession number AJ003069).

Expression of the *EDD1* gene was examined using RNA blot analysis of poly(A)-enriched RNA isolated from various Arabidopsis tissues. Isolated mRNA from leaves, buds, flowers, stems, and roots was hybridized using the complete Arabidopsis *EDD1* cDNA as a probe. *EDD1* transcripts are of low abundance (Figure 3A), and no tissue-specific expression of the examined gene could be monitored compared with the *ACTIN1* gene control (Figure 3B; Nairn et al., 1988). Interestingly, a second transcript of ∼0.9 kb that cross-hybridized with the *EDD1* probe could be detected. This transcript was detected only when using the full-length cDNA clone as a hybridization probe. When we used the *Ds* flanking regions or a 5′ cDNA fragment as a probe, this mRNA was not detected.

The *EDD1* Gene Encodes GlyRS

The predicted *EDD1*-encoded protein is 1068 amino acids long, with an apparent molecular mass of ∼117 kD. Homology searches using the BLAST service of the National Center for Biotechnology Information (Bethseda, MD) revealed that the Arabidopsis protein shares no significant similarity to any known eukaryotic protein. As shown in Figure 4A, significant homology was found between the *EDD1*-encoded protein and bacterial GlyRS from *E. coli* (Keng et al., 1982; Webster et al., 1983). GlyRS belongs to the family of aminoacyl-tRNA synthetases (AARSs), an essential but structurally diverse class of enzymes that catalyze the addition of amino acids to specific tRNA molecules. GlyRS specifically ligates the amino acid glycine to its cognate tRNA species. The *E. coli* enzyme is a tetramer of two subunits, $\alpha_2\beta_2$ (Keng et al., 1982). A single mRNA encodes both subunits of the enzyme with the termination site for the α ORF in frame with an initiation site for the β ORF located nine nucleotides downstream of the translational stop codon. In contrast, only a single ORF was found for the plant homolog described in this study, where both subunits (named domain a and b for the *EDD1*-encoded protein) are translated into a single polypeptide chain.

Sequence comparison between the full-length cDNA clone and the subcloned genomic fragment enabled us to discriminate coding and noncoding regions within the subcloned region and to determine the exact integration site of the transposed Ds_A element. As shown in Figure 4B, the Ds_A element integrated into an exon region of domain a. Upon integration of the Ds_A element, a duplication of 8 bp of plant DNA (GGTATTGA) at the target locus took place. The 8-bp duplication is a characteristic typical of transposable elements belonging to the *Ac/Ds* family (Pohlmann et al., 1984). The target site itself contains parts of the 3′ consensus splice site of the neighboring intron region (Figure 4C). The insertion site indicates that *edd1* is a loss-of-function mutation and that this allele does not produce a functional protein.

For the isolation of the *EDD1* promoter, we subcloned and analyzed a 4-kb BglII genomic fragment that cross-hybridized
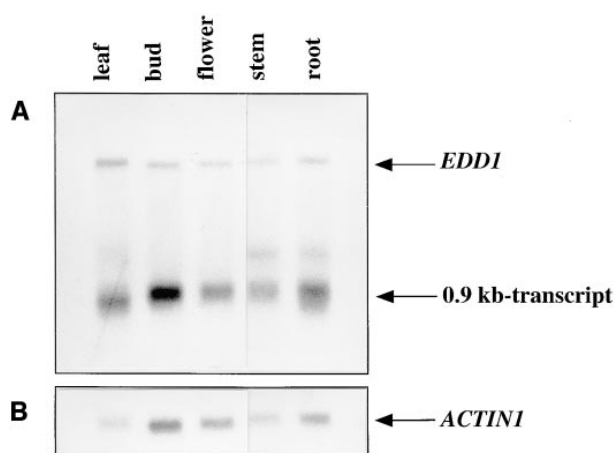

Figure 3. Analysis of *EDD1* Expression in Different Organs of Arabidopsis Wild-Type Plants.

RNA gel blot analysis of *EDD1* expression is shown. Approximately 3 µg of poly(A)-enriched RNA from leaf, bud, open flower, stem, and root was loaded per lane. The blot was hybridized successively with the Arabidopsis *EDD1* cDNA and the Arabidopsis *ACTIN1* gene (Nairn et al., 1988). The arrows at right indicate the detected transcripts.

(A) RNA gel blot hybridized with the *EDD1* cDNA. In addition to the *EDD1* transcripts, a second transcript of ∼0.9 kb that cross-hybridizes with the *EDD1* probe was detected.

(B) Same blot as shown in **(A)** hybridized with the Arabidopsis *ACTIN1* gene.

with a 5′-terminal probe of the isolated plant cDNA. This fragment contained 1294 bp of the promoter region and was shown to be extremely AT rich (Figure 5A). A putative TATA box could be located 107 or 135 nucleotides upstream of the cDNA start site. Homology searches comparing the Arabidopsis *EDD1* promoter region with eukaryotic promoter databases revealed short stretches of homology to other plant promoters, such as the α-phaseolin promoter from bean (Anthony et al., 1990) and the legumin *legA* promoter from pea (Lycett et al., 1984; see also Figure 5B). Both genes are predominantly expressed during embryogenesis in the cotyledons of these plants.

The *EDD1*-Encoded GlyRS Is Targeted to Chloroplasts

The similarity of the EDD1 protein to the bacterial enzymes extends almost throughout the whole ORF (except from the 61 amino acids at the extreme N terminus of the plant protein) and is most prominent in the N-terminal half of the protein, ranging from 60 to 70% identical amino acids. The C-terminal part of the protein shows a slightly lower degree of similarity (50% identical amino acids). The N-terminal end of the protein (amino acids 1 to 61) is characterized by a positive net charge with a pI of 12.48, a high percentage of

A
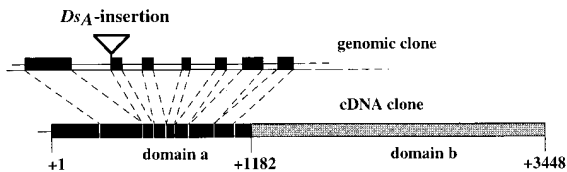
```
EDD1    176 H D I R F V E D N W E S P V L G A W G L G W E I W M D G M E I T Q F T Y F Q Q A G S L P L S P V S  224
            *   *   *   *   *   *   *   *   *       *     *   *   *   *   *   *   *   *       *     *   *   *   *   *   *   *   *   *   *       *       *         *   *
GlyRS   112 H D I R F V E D N W E N P T L G A W G L G W E V W L N G M E V T Q F T Y F Q Q V G G L E C K P V T  160
```

```
EDD1    377 E K V S E D P R S F I I E I G T E E M P P Q D V I N A S E Q L R V R V L E L L E N Q R L R H G A V  435
                  *       *   *   *   *   *   *     *   *                         *                     *     *       *     *   *     *
GlyRS   303 - E A A M Q E K T F L V E I G T E E L P P K A L R S L A E S F A A N F T A E L D N A G L A H G T V  351
```
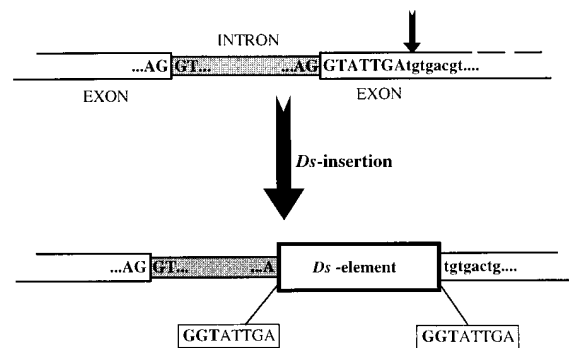
B

C

Figure 4. Molecular Analysis of the *edd1* Locus.

(A) A comparison of two segments of the Arabidopsis EDD1 protein with the corresponding protein sequence from the *E. coli* GlyRS is shown. Asterisks indicate identical amino acids in both sequences. The dash indicates a stop codon within the bacterial sequence. The homology between EDD1 and the *E. coli* protein is most prominent in the N-terminal protein half (see upper alignment), ranging from 60 to 70% identical amino acids. Homology is lower in the C-terminal half, which corresponds to the bacterial β subunit (see lower alignment).
(B) Insertion site of the *Ds$_A$* element. The structure of the *Ds$_A$*-disrupted gene is shown in comparison with the *edd1* cDNA. Black boxes represent exons, dashed lines show their position within the cDNA, and introns are indicated by white regions between exons. The *Ds$_A$* element inserted into an exon region that encodes parts of the N-terminal protein half with homology to the bacterial α subunit.
(C) Integration of *Ds$_A$* took place close to an exon/intron border region, as indicated by arrows. The 8-bp duplication is depicted in boxes. The duplicated sequence contains parts of the 3′ consensus splice sequence of the neighboring intron region.

serine (18.3%) and arginine (13.3%) residues, and a complete lack of the amino acids glutamate, aspartate, and tyrosine. These characteristics are indicative of a potential function of this N-terminal region as a transit peptide for import into plastids (von Heijne et al., 1989; von Heijne and Nishikawa, 1991).

We were interested to determine whether the EDD1 protein is post-translationally imported into plastids and tested the import capacity of the in vitro–synthesized EDD1 protein into isolated pea chloroplasts. Coupled in vitro transcription and translation of the full-length *EDD1* cDNA yielded only low levels of the 110-kD EDD1 protein, and no chloroplast import studies could be performed with the complete pro-

tein. To circumvent the problem of an insufficient amount of the EDD1 protein, we fused parts of the cDNA encoding the putative transit peptide to the *uidA* gene, encoding the 68-kD β-glucuronidase (GUS) protein of *E. coli* (Jefferson, 1987).

Translation of the chimeric gene yielded a single fusion protein of ∼75 kD (Figure 6, lane 1). In the presence of intact pea chloroplasts under the appropriate import conditions, 70 to 80% of the added chimeric preprotein was found to be processed into a protein of ∼65 to 70 kD (Figure 6, lane 2)— a size to be expected if the putative transit peptide were cleaved. The addition of the protease thermolysin to the reaction mixture digested the preprotein in the supernatant, whereas the mature protein imported into the chloroplasts

was protected and remained intact (Figure 6, lane 3). The addition of a detergent destroyed the integrity of the plastid membrane. The truncated protein was no longer protected by the chloroplast membrane and was digested by the protease (Figure 6, lane 4). We conclude from these data that the N-terminal part of the EDD1 protein comprising the amino acids 1 to 61 is capable of mediating transport of the GUS protein into pea chloroplasts in vitro and that the isolated plant gene most probably encodes a plastidic GlyRS isoform.

Somatic Reversion of the *edd1* Mutation

One characteristic of transposon insertion mutants is their instability in the presence of a transposase (TPase) source. Under such conditions, wild-type revertants can occur (Bancroft et al., 1993; Chuck et al., 1993; Tacke et al., 1995). To induce excision of the Ds_A element from the *edd1* locus and thus to generate wild-type sectors, we cross-fertilized the mutant line to a transgenic Arabidopsis line that contained a TPase gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter. This TPase gene fusion was shown earlier to drive high frequencies of somatic and germinal Ds_A excision in Arabidopsis (Swinburne et al., 1992; Long et al., 1993). If the *edd1* mutation had been caused by the insertion of Ds_A, instability of the mutant phenotype could occur as the result of Ds_A transactivation through the introduced TPase. We screened 240 F_1 plants (heterozygous for the *edd1* mutation) from this cross for somatic reversion events monitored as wild-type side shoots branching from a mutant main stem. Such a situation is expected to occur at low frequency because a fairly large revertant sector that does not cover the main stem must be generated. Furthermore, restoration of gene function most probably requires precise excision, with removal of the duplicated plant DNA sequences due to the position of Ds_A close to an exon/intron border. Accordingly, only a single variegated plant that showed a revertant sector could be recovered. Seeds from the revertant branch all grew into plants that did not segregate embryo-defective seeds.

Revertant plants were analyzed for the presence of the Ds_A element by DNA gel blot analysis. As shown in Figure 7, the Ds_A* present in *edd1* was no longer detectable in the revertant progeny (R1 to R10). In these plants, the introduced TPase (*Ac*) as well as additional fragments (Ds_A^+) potentially representing novel Ds_A insertion loci could be monitored. To confirm that the *edd1* mutation was caused by the Ds_A insertion, the genomic regions around the termini of Ds_A* were amplified from genomic DNA of 30 individual progeny plants of the revertant sector and subsequently were analyzed. Polymerase chain reaction (PCR) amplicons that corresponded to the *EDD1* gene were subcloned, and 15 of these PCR clones were sequenced. All of the 15 sequenced fragments showed the original wild-type sequence, indicating that Ds_A excision led to an exact restoration of the *EDD1*
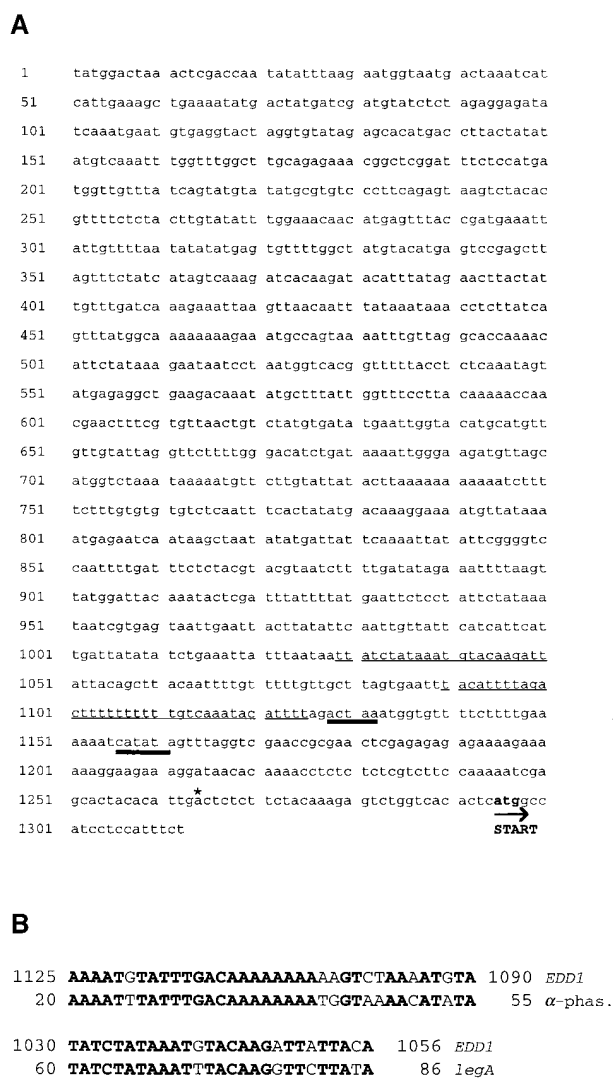
A

```
   1    tatggactaa actcgaccaa tatatttaag aatggtaatg actaaatcat
  51    cattgaaagc tgaaaatatg actatgatcg atgtatctct agaggagata
 101    tcaaatgaat gtgaggtact aggtgtatag agcacatgac cttactatat
 151    atgtcaaatt tggtttggct tgcagagaaa cggctcggat ttctccatga
 201    tggttgttta tcagtatgta tatgcgtgtc ccttcagagt aagtctacac
 251    gttttctcta cttgtatatt tggaaacaac atgagtttac cgatgaaatt
 301    attgttttaa tatatatgag tgttttggct atgtacatga gtccgagctt
 351    agtttctatc atagtcaaag atcacaagat acatttatag aacttactat
 401    tgtttgatca aagaaattaa gttaacaatt tataaataaa cctcttatca
 451    gtttatggca aaaaaaagaa atgccagtaa aatttgttag gcaccaaaac
 501    attctataaa gaataatcct aatggtcacg gtttttacct ctcaaatagt
 551    atgagaggct gaagacaaat atgctttatt ggtttcctta caaaaaccaa
 601    cgaactttcg tgttaactgt ctatgtgata tgaattggta catgcatgtt
 651    gttgtattag gttcttttgg gacatctgat aaaattggga agatgttagc
 701    atggtctaaa taaaaatgtt cttgtattat acttaaaaaa aaaaatcttt
 751    tctttgtgtg tgtctcaatt tcactatatg acaaaggaaa atgttataaa
 801    atgagaatca ataagctaat atatgattat tcaaaattat attcggggtc
 851    caatttgat ttctctacgt acgtaatctt ttgatataga aattttaagt
 901    tatggattac aaatactcga tttattttat gaattctcct attctataaa
 951    taatcgtgag taattgaatt acttatattc aattgttatt catcattcat
1001    tgattatata tctgaaatta tttaataatt atctataaat gtacaagatt
1051    attacagctt acaattttgt ttttgttgct tagtgaattt acatttttaga
1101    ctttttttttt tgtcaaatac atttttagact aaatggtgtt ttcttttgaa
1151    aaaatcatat agtttaggtc gaaccgcgaa ctcgagagag agaaaagaaa
1201    aaaggaagaa aggataacac aaaaccctctc tctcgtcttc caaaaatcga
1251    gcactacaca ttgactctct tctacaaaga gtctggtcac actcatggcc
1301    atcctccatttct
```
                                    →
                                  START

B

```
1125  AAAATGTATTTGACAAAAAAAAAAGTCTAAAATGTA  1090  EDD1
  20  AAAATTTATTTGACAAAAAAAATGGTAAAACATATA    55  α-phas.

1030  TATCTATAAATGTACAAGATTATTACA  1056  EDD1
  60  TATCTATAAATTTACAAGGTTCTTATA    86  legA
```

Figure 5. The *EDD1* Promoter.

(A) All 1263 bp of the *EDD1* promoter region are shown. The regions with homology to the α-phaseolin promoter from bean (residues 1125 to 1090, minus strand) and the legumin *legA* promoter from pea (residues 1030 to 1056, plus strand) are underlined with thin lines. The putative TATA boxes are indicated with boldface lines. The transcription start is marked with an asterisk; the translation start (atg) is indicated by an arrow.

(B) Homology of the *EDD1* promoter to other plant promoters is shown. Short homology stretches depicting the sequence similiarity between the *EDD1* promoter and promoter elements from the α-phaseolin (α-phas.) promoter of bean and the legumin (*legA*) promoter from pea are shown. Boldface letters indicate conserved bases.
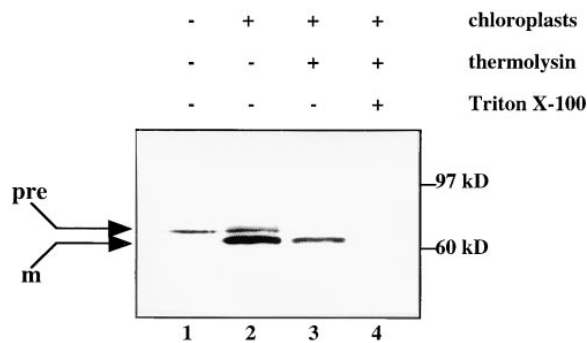
Figure 6. Plastidic Targeting of the Chimeric GUS Protein.

Pea chloroplasts were incubated with the ^{35}S-labeled chimeric 75-kD GUS protein containing an N-terminal portion of the Arabidopsis GlyRS protein synthesized in a cell-free reticulocyte lysate. Lane 1 contains the in vitro–translated preprotein used in this import assay. Lane 2 contains the protein isolated from chloroplasts recovered after incubation with the radioactively labeled in vitro translation product. Lane 3 contains protein from chloroplasts incubated with the labeled preprotein and post-treated with the protease thermolysin. Lane 4 is as given for lane 3, except that chloroplasts were post-treated with thermolysin and the detergent Triton X-100. Protein molecular mass markers in kilodaltons are given at right. (+), presence; (−), absence; pre, preprotein; m, mature protein.

gene. In addition to the revertant locus, a second PCR product was also amplified. Its sequence showed extensive homologies to the *EDD1* gene, indicating the possible presence of a second related gene encoding another GlyRS isoform (data not shown).

The chromosomal locations of *edd1* and the homologous locus were determined by hybridization to the CIC yeast artificial chromosome (YAC) library (Creusot et al., 1995). The map position of the identified YACs was deduced (D. Bouchez, unpublished data), and *edd1* was positioned on the lower arm of chromosome 3, close to the markers m409 and csr (*ALS* gene), at a map position that was ~82 centimorgans (cM), whereas the homologous locus was located on chromosome 5, close to the marker mi125, at a map position that was ~85 cM. Allelism tests were performed with the mutants *emb69*, *emb117*, *emb149*, *emb154*, and *emb172* (kindly provided by D. Meinke, Oklahoma State University, Stillwater), which map to the same chromosomal region on chromosome 3 as does *edd1*. Our results demonstrate that the *EDD1* locus had not been identified earlier by mutation and belongs to a separate complementation group.

Genetic Complementation of the *edd1* Mutation

To demonstrate genetic complementation of the mutant *edd1* by the isolated gene, we generated transgenic Arabidopsis lines that harbored the plant *EDD1* cDNA under the control of the CaMV 35S promoter (Franck et al., 1980) or

the *USP* promoter (for <u>u</u>nknown <u>s</u>eed <u>p</u>rotein) from fava bean (Bäumlein et al., 1991; Fiedler et al., 1993), respectively. Both promoters differ in their stage and tissue specificity and should drive different expression levels of the cloned cDNA. The two transgenic lines, named 35S–*EDD1* and *USP–EDD1*, were used separately to cross-fertilize heterozygous *edd1* plants. In the case of genetic complementation, a reduction of aborted seeds (from 1 of 4 to 1 of 16) within siliques of F$_1$ plants that inherited the *edd1* mutation and the transgene was expected.

Introduction of the *EDD1* cDNA into the *edd1* genetic background under the control of the 35S promoter did not decrease the percentage of defective seeds. Progeny plants from the cross between heterozygous *edd1* plants and the 35S–*EDD1* transgenic line contained 100% normal seeds, 25% defective seeds, or >25% defective seeds, with seeds being aborted at different developmental stages in the latter case.

In contrast, a reduction in defective seeds was observed in the progeny of two independent lines that resulted from a cross between heteozygous *edd1* plants and the *USP–EDD1* transgenic line (see Table 1). The fraction of defective seeds varied during silique development. At an early developmental stage, approximately one of 16 of the seeds was defective, and only these aborted seeds contained mutant *edd1* embryos (Figure 8B, left). Of the remaining green seeds, approximately three of 16 showed retarded embryo growth. Whereas most of the normal seeds contained bent cotyledon-stage embryos at that time of silique development (Figure 8B, center), such retarded embryos contained torpedo-stage embryos (Figure 8B, right). The segregation ratio is indicative of a situation in which the retarded growth behavior could represent homozygous *edd1* embryos com-
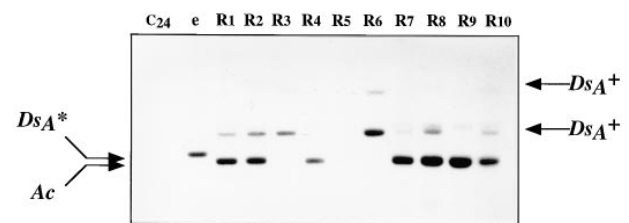

Figure 7. Somatic Reversion and Complementation of the *edd1* Mutation.

Revertants were generated by crossing the mutant *edd1* line to a transgenic line that contains a TPase gene under the control of the CaMV 35S RNA promoter (Swinburne et al., 1992; Long et al., 1993). One progeny plant of this cross contained a revertant (wild-type) sector. Seeds from that branch all grew into wild-type plants. Genomic DNA from those revertant plants was digested with EcoRI and HindIII and hybridized with a ^{32}P-labeled 3′ HindIII-BamHI fragment of *Ac*. Revertant plants (R1 to R10) do not contain the Ds_A* element at the position observed for the mutant line *edd1* (e) but segregate for the introduced TPase gene (*Ac*) and for Ds_A at new locations (Ds_A+).

Table 1. Segregation of Embryo-Defective Seeds in F_1 Plants Derived from Crosses between Heterozygous *edd1* Plants and the *USP–EDD1* Transgenic Line

F_1 Plants	*edd1*/Total[a]	% *edd1*[b]	$f_{edd1} = 1/4$[c]	$f_{edd1} = 1/16$[c]
1	26/114	22.8	0.6	$<10^{-10}$
2	15/274	5.5	$<10^{-10}$	0.62
3	23/92	25	1	$<10^{-6}$
4	23/127	18.1	0.75	$<10^{-7}$
5	31/129	24	0.8	$<10^{-10}$
6	11/135	8.1	$<10^{-5}$	0.35
7	6/85	7.1	0.0004	0.76
8	6/126	4.7	$<10^{-6}$	0.5

[a]The fraction of embryo-defective seeds (*edd1*) compared with the total seed number was determined in the siliques of F_1 plants segregating for wild-type and abnormal seeds.
[b]The percentage of defective seeds.
[c]For each plant, the fraction of embryo-defective seeds was analyzed by χ^2 test of goodness of fit.

plemented by the introduced *EDD1* cDNA copy. At a later developmental stage, an increase of defective seeds was observed. The percentage of seeds that contained mutant *edd1* embryos still represented approximately one of 16 of the total seed number. In addition to this class of defective seeds, aborted seeds containing torpedo-stage embryos also appeared. They possibly represent homozygous (*edd1/edd1*) mutants with the transgene present.

Limited DNA gel blot analysis of progeny plants from the cross between the *USP–EDD1* transgenic line and *edd1* plants was performed using an *edd1*-specific probe for hybridization (Figure 8A). Arabidopsis wild-type plants were homozygous for the *EDD1* allele, whereas *edd1* plants (heterozygous plants giving rise to 25% aborted seeds) contained the wild-type as well as the mutant allele. Several progeny plants from the above-mentioned cross contained an additional locus corresponding to the introduced cDNA (Figure 8A, lanes 1 to 4). Plants that were heterozygous for the *EDD1* locus (*edd1/EDD1*) but contained the introduced transgene (numbers 1, 3, and 4) showed a reduced percentage of aborted seeds. We conclude from these data that the introduced *USP–EDD1* gene fusion is capable of partially complementing the *edd1* mutation. The effect seems to be dependent on the developmental stage of the embryo and probably corresponds to the expression pattern of the *USP* promoter.

DISCUSSION

In this article, we describe the recessive embryo-defective mutant *edd1* of Arabidopsis. This mutant was isolated by using transposon mutagenesis. Genomic plant sequences flanking the transposon in mutant plants have been rescued

and used to isolate a full-length cDNA clone representing the wild-type *EDD1* gene.

Conclusive evidence that the *EDD1* gene had been cloned was obtained by genetic reversion and complementation experiments. In the mutant, a gene encoding a GlyRS has been disrupted upon integration of the transposable element Ds_A. Reversion of the *edd1* phenotype through mobilization of the transposon was shown to occur at low frequency after cross-fertilizing the mutant line to a transgenic Arabidopsis line that contains an active TPase gene fusion. In our experiments, only a single revertant for the Ds_A-induced *edd1* mutant could be rescued, showing a precise excision of the transposon. Precise excision in the *Ac/Ds* system is rare but has been reported for a revertant of the *Ds*-induced alcohol dehydrogenase *Adh1* mutant (Dennis et al., 1986) and the *Ds*-induced *iojap* mutant (Han et al., 1992). The low reversion frequency indicates that a precise restoration of the original wild-type sequence may be necessary to rebuild protein function. Genetic complementation of the mutant phenotype was achieved by introducing the Arabidopsis *EDD1* cDNA under the control of the *USP* promoter from fava bean into the *edd1* genetic background.

Analysis of the EDD1 predicted amino acid sequence revealed a significant homology of the plant protein to GlyRS of *E. coli*, which belongs to the AARS family (Keng et al., 1982; Webster et al., 1983). AARSs catalyze the first step in protein synthesis by attaching amino acids to the 3′ end of the cognate tRNA in a two-step energy-dependent reaction sequence. At least 20 such enzymes, with one enzyme being specific for each amino acid found in proteins, constitute a minimum set for protein biosynthesis. The sequence homology between the deduced Arabidopsis protein and the protein from *E. coli* is extremely high, showing up to 70% identical amino acids. A similiar degree of sequence homology between bacterial and eukaryotic synthetase enzymes had not previously been documented because homologies between yeast and bacterial enzymes normally range from 20 to 50% in the portions that can be aligned (Schimmel, 1987). This high level of similiarity leaves little doubt that the identified gene encodes a plant GlyRS. No significant homology of the plant protein to other so-far characterized eukaryotic GlyRSs from yeast (Kern et al., 1981), silkworm (Nada et al., 1993), or humans (Ge et al., 1994; Shiba et al., 1994) has been detected, indicating that the plant enzyme corresponding to the isolated gene shares a common evolutionary history with the enzyme from *E. coli*, which is distinct from that of eukaryotes.

Results derived from protein uptake experiments show that an N-terminal portion of the Arabidopsis GlyRS is capable of directing a marker protein into pea chloroplasts. We conclude from these data that the plant enzyme described here most probably acts within the plastidic compartment. Nevertheless, additional functions of the GlyRS in other cellular compartments cannot be entirely excluded. However, there is only little evidence for mistargeting or cotargeting of preproteins to different organelles (Hurt et al., 1986; Huang
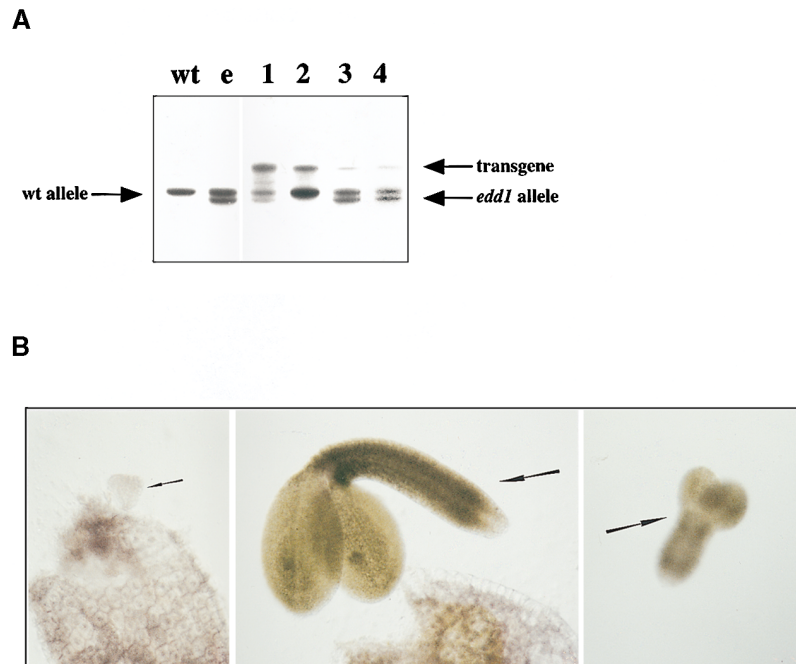
A

B

Figure 8. Genetic Complementation of the *edd1* Mutation.

edd1 plants were cross-fertilized to the transgenic line *USP–EDD1* containing the wild-type *EDD1* gene under the control of the *USP* promoter from fava bean.

(A) DNA gel blot analysis of wild-type and complemented plants. Total Arabidopsis DNA was digested with EcoRI and HindIII and hybridized with a ^{32}P-labeled *EDD1*-specific probe. Several F$_1$ progeny plants of the above-mentioned cross contained the introduced transgene (lanes 1 to 4). wt, wild-type plants; e, *edd1* heterozygous plants.

(B) Seeds derived from the above-described cross were dissected, and embryo development was studied. Approximately one of 16 of the total seed number contained defective embryos (left). Whereas most of the seeds contained bent cotyledon-stage embryos at that time of silique development (center), approximately three of 16 showed retarded embryo growth, with immature seeds containing torpedo-stage embryos (right). Embryos are indicated by arrows.

et al., 1990; Creissen et al., 1995). AARSs are essential components of the protein-synthesizing machinery and are found in each cellular compartment, which harbors genetic information and is able to express it. Plant cells contain three such compartments that perform protein synthesis, namely, the nucleoplasm, the mitochondria, and the plastids. Because none of the AARS enzymes has been shown to be encoded by the plant organellar genomes, they probably are nuclear encoded and imported from the cytosol.

Mutation of the *edd1* locus does not impair early embryonic growth, and development of mutant embryos proceeds normally until the end of the globular embryo stage. Embryo growth is disturbed during transition to the heart-shaped stage. Limited elongation and enlargement of mutant embryos occur but are not accompanied by normal cotyledon and shoot meristem formation. Mutant embryos display variable phenotypes that are independent of their developmental stage, suggesting that penetrance of the mutation is incomplete. In most cases, cotyledons are completely missing, and only seldom can small apical bulges, possibly rep-

resenting strongly reduced cotyledons, be detected around the apical region. Microscopic examination of mutant embryos suggested that formation of the radial tissue pattern comprising protoderm, ground meristem, and vascular tissue proceeds normally, indicating that cellular differentiation within the embryo proper occurs in the absence of normal morphogenesis. Growth of the suspensor is deregulated, yielding an enlarged suspensor instead of a single cell file. Abnormal suspensor development has been reported in earlier studies and seems to result directly from aberrant growth of the embryo proper (Akhundova et al., 1978; Clark and Sheridan, 1991; Yeung and Meinke, 1993; Schwartz et al., 1994; Vernon and Meinke, 1994; Yadegari et al., 1994).

From the obtained sequence information, we deduced that the protein encoded by the *EDD1* locus probably acts as a plastidic GlyRS. However, its function during plastidic translation does not provide a direct hint to its role during embryogenesis. The most simple explanation is that the EDD1 protein (GlyRS) is one of the components necessary for the biosynthesis of metabolic products that are of gen-

eral importance for cellular functions. It cannot be excluded that a simple metabolite, such as glucose or a group of substances normally produced by the plastids, becomes limiting during embryogenesis due to the loss of GlyRS function, resulting in an arrest of embryo growth. The observation that mutant embryos can be partially rescued if grown in vitro on nutritive media may suggest that the defect is due in part to such a basic lack of nutrients. However, the medium cannot completely rescue the *edd1* mutant. This rather contradicts a pure metabolic deficiency causing the developmental arrest of *edd1* embryos and indicates that additional mechanisms are probably affected in the mutant. A model that we propose implies that the *edd1* mutation interferes with the exchange of signals between the plastid and the nucleus, which are necessary for normal embryogenesis.

Biogenesis of plastids is tightly coupled with temporal and spatial stages of plant development. Thus, a key component of plastid development is the coordination of gene expression between the nuclear and plastidic genomes that are separated by several membranes and are vastly different in complexity, structure, gene organization, and regulatory mechanisms. Several lines of evidence indicate that the developmental status of the plastid is determined by the same developmental program that controls the cell in which it resides (Taylor, 1989). Genetic information in the nucleus therefore ultimately regulates the expression of the plastid genome as well as the expression of nuclear genes encoding plastid proteins. However, little is known about the molecular nature of the genes that regulate chloroplast development or the way this regulation is achieved. Only a few mutations in nuclear genes that regulate chloroplast development have been subjected to molecular analysis. These mutants are affected in a variety of chloroplast functions, including protein targeting to the thylakoid (*hcf106* of maize; Martienssen et al., 1989; Voelker and Barkan, 1995), maintenance of ribosomes within the plastid (*iojap* of maize; Walbot and Coe, 1979; Han et al., 1992), chloroplast division (*arc* mutants of Arabidopsis; Pyke and Leech, 1992, 1994; Robertson et al., 1995), and communication between chloroplast and mitochondrion (*chm* of Arabidopsis; Martínez-Zapater et al., 1992).

Furthermore, the developmental stage of the chloroplast itself appears to regulate the expression of nuclear genes coding for chloroplast-destined proteins (Harpster et al., 1984; Mayfield and Taylor, 1984; Taylor, 1989). For example, in photooxidative mutants of maize or in a variety of plants in which chloroplast development is arrested with an inhibitor, light-regulated nuclear genes such as *CAB* (encoding the chlorophyll *a/b* binding proteins of photosystem II) or *RBCS* (encoding the small subunit of ribulose 1,5-bisphosphate carboxylase) are not expressed. Thus, it has been hypothesized that signals from chloroplasts regulate nuclear gene expression (Oelmüller, 1989; Taylor, 1989). A signal from the plastid, known as the plastid factor, has long been postulated to provide the nuclear genome with information about the developmental status of the plastid in each cell (Bradbeer

et al., 1979; reviewed in Taylor, 1989). The plastid factor is proposed to coordinate gene expression in the nucleus with the needs of the chloroplast.

A block of protein synthesis within the plastids resulting from a lack of GlyRS activity is expected to interfere with this complex signal exchange program. The dependence of the signal generation on plastid mRNA translation could be based either on a malfunction within the plastid sensed by the signal generating machinery or on the block of biosynthetic pathways that involve plastid-encoded activities. The latter may affect the synthesis of the hypothesized plastid signal itself.

Plastid differentiation in plant embryos has been investigated by Schulz and Jensen (1969). They demonstrated that plastids within the ground meristem and protoderm tend to form internal lamellae, which is reminiscent of developing thylakoids, early during embryogenesis. These morphological alterations coincide with the appearance of transcripts for two chloroplast proteins: immature Capsella seeds already express the nuclear-encoded *RBCS* gene as well as the plastid-encoded *PSBA* gene (Q_B protein of photosystem II). Transcripts are uniformly distributed throughout the torpedo-stage embryo, with the exception of procambial cells (Fiebig et al., 1990). The expression of the plastidic AARS enzymes and their tRNA substrates is linked to plastid differentiation and is partly under developmental and light-regulated control. Exposure of dark-grown Euglena cells to light results in a marked increase in the level of plastidic AARS and parallels chloroplast biogenesis (Reger et al., 1970; Parthier et al., 1972; Reinbothe and Parthier, 1990). In cotton, AARSs show a four- to 16-fold increase of expression in cotyledons during germination (Brantner and Dure, 1975). In contrast to the Euglena system, this increase does not require an exposure to light in cotton but appears to be a preprogrammed aspect of the germination process.

The effect of the *edd1* mutation first becomes visible during the transition phase. Mutant embryos do not display normal cotyledon initials but develop an abnormal embryo structure without chlorophyll accumulation. This suggests that *edd1* acts at an early stage of chloroplast development, before chlorophyll biosynthesis is activated. Because the *edd1*-encoded GlyRS is a plastid-localized protein and chloroplast function is obviously impaired in the mutant, we hypothesize that the developing chloroplast creates a signal required for cotyledon morphogenesis and that *edd1* plants are defective in the production or transmission of that signal. A different mutation that most probably also affects protein synthesis in plastids and leads to an arrest of embryogenesis has been described by Tsugeki et al. (1996). In this mutant, a transposon insertion in the Arabidopsis *SSR16* gene, which is highly homologous to the gene encoding the S16 ribosomal protein from *Neurospora crassa*, causes an embryo-defective lethal mutation.

Coupling between plastidic and cellular differentiation has been demonstrated through mutations in several loci that are involved in both chloroplast development and palisade

parenchyme morphogenesis, for example, in bean (Zaumeyer, 1938, 1942; Wade, 1941) and Arabidopsis (Kirk and Tilney-Basset, 1978; Wetzel et al., 1994). Furthermore, mutation of *DCL* (for <u>d</u>efective <u>c</u>hloroplasts and <u>l</u>eaves) or *DAG* (for <u>dif</u>-ferentiation <u>a</u>nd <u>g</u>reening) loci of Antirrhinum blocks the development of chloroplasts and interferes with palisade cell formation (Chatterjee et al., 1996; Keddie et al., 1996).

The *DAG* as well as the *DCL* loci encode proteins with unknown function. These proteins are targeted to the plastids. In the affected tissue, the palisade layer is missing and replaced with smaller spherical-shaped cells similiar to those observed in spongy mesophyll. Plastids from the mutant tissue are small, contain very few or no thylakoids, and resemble proplastids. Mesophyll cells of the Arabidopsis chloroplast division mutant *arc6* contain two to six enlarged chloroplasts instead of ~100 normal-sized chloroplasts (Robertson et al., 1995). Although the internal structures of the enlarged *arc6* chloroplasts are not radically different from normal granal and thylakoid structures, the shape of mesophyll cells is different in *arc6* leaves, leading to an altered leaf morphology in mutant plants. The *arc6* mutation probably affects proplastid division.

The effect of *dag*, *dcl*, and *arc6* on leaf morphology is different from that of many other genes affecting chloroplast development, such as *olive* of Antirrhinum (Hudson et al., 1993), *hcf106* of maize (Miles, 1994), and *albina* and *xantha* of barley (Henningsen et al., 1993). These mutants have partially developed but defective chloroplasts without having any morphogenetic effects on palisade development. This could imply that only signals generated early in the development of chloroplasts are necessary for stimulating the final divisions and expansions of cells of the palisade mesophyll.

In our model (see Figure 9), *EDD1* is involved in the production of a very early signal that is required to initiate the first step in the differentiation of proplastids, which in turn triggers cotyledon morphogenesis. *DCL*, *DAG*, and *ARC6* are required at a later time for the development of the mesophyll tissue. In contrast, *OLIVE*, *HCF106*, *ALBINA*, and *XANTHA* act late during chloroplast development without affecting cell and tissue morphogenesis.

Molecular studies of RNAs present in developing seeds indicate that 20,000 genes are expressed during embryogenesis in higher plants (Goldberg et al., 1989). Although it is reasonable to assume that many of these genes encode ba-
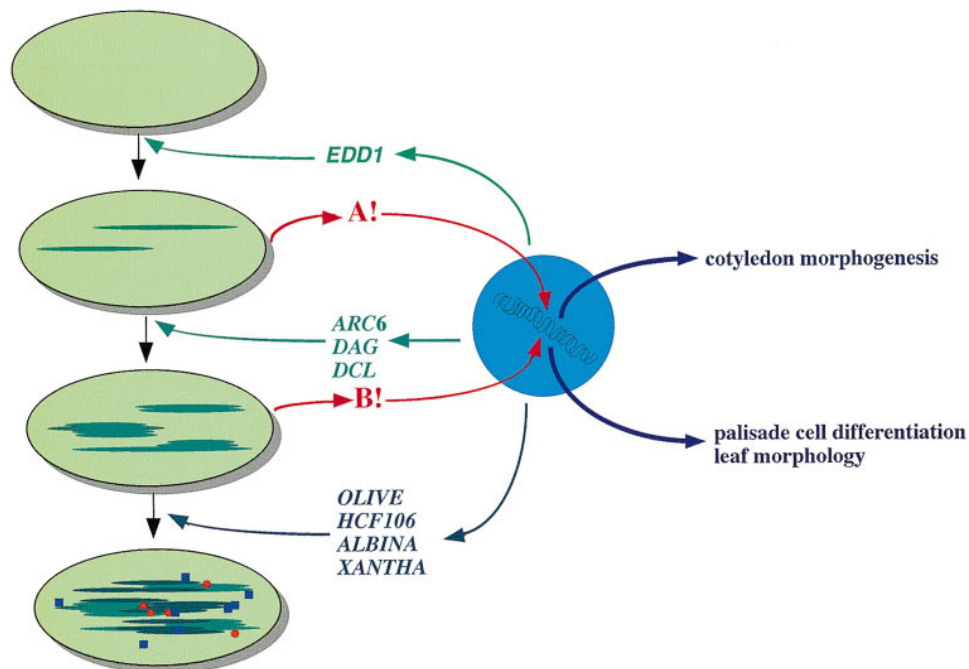

Figure 9. Model of *EDD1* Gene Action during Embryogenesis.

EDD1 function is required early during chloroplast biogenesis for the expression of plastidic genes. The *edd1* mutation affects the synthesis of the chloroplast signal A!. In this model, A! (e.g., a plastid-derived metabolite) serves as the primary signal in a simple signal transduction pathway that activates the expression of nuclear genes involved in cotyledon formation. *ARC6*, *DAG*, and *DCL* act downstream of *EDD1*. Their activities are required for the development of the thylakoid membrane system and for the synthesis of signal B!, which in turn triggers palisade cell differentiation and controls leaf morphology. *OLIVE*, *HCF106*, *ALBINA*, and *XANTHA* are also involved in chloroplast development. A mutation in any of these genes leads to defective chloroplasts, but there is no morphogenetic effect. Therefore, we assume that they act downstream of *EDD1*, *ARC6*, *DAG*, and *DCL* and do not take part in the above-described signal transduction pathway that connects the nucleus and the plastids.

sic cellular functions, it was a surprising result that even in the case of mutants classified on the basis of histological analysis as "pattern mutants," the corresponding genes encode rather general functions (Shevell et al., 1994; Meinke, 1995; Busch et al., 1996; Lukowitz et al., 1996).

The results obtained from the analysis of *edd1* as described here represent another example in this respect and emphasize the fact that the function of genes such as *EDD1* cannot be dismissed as being merely housekeeping but should be considered essential to the whole process of embryogenesis.

METHODS

Plants and Growth Conditions

The embryo-defective *Arabidopsis thaliana* mutant *edd1* (for embryo-defective development; former designation No. 240) described in this report was initially identified through *Activator/Dissociation* (*Ac/Ds)* transposon mutagenesis (Altmann et al., 1995). Recessive embryo mutants were maintained as heterozygotes that produced 25% mutant embryos upon self-pollination. All Arabidopsis plants were grown in soil under a 16-hr-day (3000 lux fluorescent light, 20°C) and 8-hr-night (17°C) regime at a relative humidity of 70%. For crosses, flowers of the female parent were emasculated ∼2 days before anthesis and cross-pollinated 2 or 3 days later.

Transformation of Arabidopsis and Growth in Aseptic Culture

The Arabidopsis ecotype C24 was used for *Agrobacterium tumefaciens*–mediated gene transfer. Agrobacterium strain C58C1 with the plasmid pGV2260 (Deblaere et al., 1985) was used for transformation. Cotyledon infection, selection, regeneration of transformed plants, and growth of the plants to maturity were performed as described by Schmidt and Willmitzer (1988).

Nomarski Optics of Cleared Seeds

Seeds were removed from siliques and cleared for 30 min to 24 hr in a lactophenol solution (glycerol–lactate–phenol–H_2O, 1:1:2:1 [v/v]) on a microscope slide. Seeds of later developmental stages and defective seeds of *edd1* plants required extended clearing periods. Cleared seeds were examined using a Zeiss Axiophot (Carl Zeiss, Inc., Oberkochen, Germany) microscope equipped with Nomarski optics. Photographs were taken using Kodak Ektachrome 320T film.

Tissue Culture of Mutant Embryos

Siliques from heterozygous *edd1* plants were surface-sterilized by soaking in 1.5% sodium hypochlorite and 0.1% Tween 80 for 10 min. After several rinses in sterile water, embryos were removed from mutant seeds by using fine forceps and cultured on Gamborg's B5 basal medium (Gamborg et al., 1968), supplemented with 10% su-crose and 0.8% agarose. Sucrose concentration was decreased stepwise to 3% during the cultivation procedure.

Construction of *Escherichia coli* and Agrobacterium Plasmids

DNA manipulations of plasmid DNA were performed according to Sambrook et al. (1989). Transformation of bacterial cells was performed using the Bio-Rad gene pulser, according to the manufacturer's protocol. To obtain the plasmid pUU1, a 3.5-kb EcoRI-SalI chromosomal fragment was ligated into an EcoRI-SalI predigested pBluescript SK+ vector (Stratagene, La Jolla, CA). For construction of the chimeric *uidA* gene for the chloroplast import assay, the 5′ end of the *EDD1* cDNA was amplified by polymerase chain reaction (PCR), using the primers SP5/1N (5′-CCGGCAGCTGGTCACACT-CATGGC-3′) and SP3/2N (5′-CCGGCAGCTGGAGGCGTTGAA-TCG-3′), corresponding to the 5′ and 3′ ends of the putative transit peptide, respectively. PCR products of the reaction were cloned into the PCR II vector (Invitrogen, San Diego, CA). The transit sequence was excised using PvuII and cloned upstream of the *uidA* gene in the vector pBGUS(3) (T. Mozo, unpublished data), which had been pre-digested with SmaI, to obtain plasmid pSP-BGUS3. pSP-BGUS3 was used for in vitro transcription and translation.

For the construction of the vectors p35S–*EDD1* and p*USP*–*EDD1*, which were used for complementation of the *edd1* mutant, the *EDD1* cDNA was cloned behind the two promoters as follows. For the construction of the vector p35S–*EDD1*, the *EDD1* cDNA was excised from the cDNA clone p7/9 as an Asp718-SmaI fragment, filled in with the Klenow fragment of DNA polymerase I, and ligated in the sense orientation downstream of the 35S RNA promoter in a modified version of the binary vector pBinAR (Höfgen and Willmitzer, 1990), which had been predigested with XbaI and filled in with the Klenow fragment. For the generation of the plant transformation vector p*USP*–*EDD1*, the cDNA insert was treated in the same way but li-gated in the sense orientation downstream of the *USP* promoter in the plant transformation vector pBinUSP (C. Benning, unpublished results) predigested with XbaI and filled in with the Klenow fragment.

Isolation and Analysis of Plant DNA and RNA

Genomic DNA was isolated as described by Dellaporta et al. (1983) by the addition of a cetyl trimethylammonium bromide precipitation and digested with restriction enzymes. After electrophoretic separation on 1% agarose gels, depurination, and denaturation (in 0.5 M NaOH and 1.5 M NaCl), the DNA was blotted onto BiodyneB membranes (Pall, Portsmouth, UK) by using capillary transfer with 20 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate; Sambrook et al., 1989). Hybridization with radioactively labeled DNA fragments (Feinberg and Vogelstein, 1983) was performed at 65°C in 0.25 M so-dium phosphate buffer, pH 7.2, 7% SDS, 1% BSA, and 1 mM EDTA. Total RNA was obtained from frozen plant tissue by using the proce-dure of Logemann et al. (1987). Poly(A)+ RNA was isolated from total RNA, according the manufacturer's protocol, using the Oligotex sus-pension (Qiagen, Hilden, Germany). Approximately 3 μg of poly(A)+ RNA was fractionated in a 1% formaldehyde–agarose gel. Hybridiza-tions and washings were done at high stringency, according to stan-dard procedures, using ^{32}P-radiolabeled probes (Church and Gilbert, 1984). For the control hybridization, the *ACTIN1* gene was radioac-tively labeled (Nairn et al., 1988).

Isolation of the *EDD1* Gene

Genomic DNA was isolated from soil-grown *edd1* plants, partially digested with Sau3A, and ligated with the EMBL3 λ vector predigested with BamHI (Stratagene). The ligated DNA was packaged in vitro using GigaPack Gold II (Stratagene). Bacterial strains K803 or SRB (Stratagene) infected with the recombinant phages were plated on NZCYM media (Sambrook et al., 1989). Approximately 250,000 λ phages were screened under stringent hybridization conditions, according to Sambrook et al. (1989). The 1.2-kb HindIII-BamHI fragment of pKU-3 (Baker et al., 1987), representing the 3′ *Ac* fragment, was used as hybridization probe. Probes were radiolabeled using the random primed labeling kit (Boehringer Mannheim, Germany). A 3.5-kb EcoRI-SalI chromosomal fragment of one positive clone that hybridized to the 3′ *Ac* probe was subcloned into pBluescript SK+ (Stratagene; clone pUU1) and used for sequence analysis and for subsequent hybridizations.

For PCR detection of the transposon footprint, DNA was extracted from leaves of revertants, as described by Edwards et al. (1991). The PCR reaction was performed with synthetic primers, TrapS3 (5′-CAGTTGTCTTCTACAAACCG-3′) and TrapS5 (5′-GACACACTC-AATTTCAAGTC-3′) (TIB MOLBIOL, Berlin, Germany), corresponding to the 5′ and 3′ flanking regions of the Ds_A. Amplified fragments were separated on an agarose gel, cut out, purified using the QIAquick gel extraction kit (Qiagen), and ligated directly into the cloning vector PCR II for sequence analysis.

EDD1 cDNA Cloning

A 3.5-kb EcoRI-SalI fragment from pUU1 was used as a probe to screen an Arabidospis C24 λ ZAP cDNA library. This library was prepared from bud, flower, and silique mRNA by using the λ ZAP II cDNA synthesis kit (Stratagene). Plaques (5 × 10⁵ plaque-forming units) were screened under stringent conditions. Positive cDNAs were isolated and subcloned into pBluescript SK+ by in vivo excision, according to the manufacturer's protocol. The longest cDNA clone (clone p7/9), comprising 3448 bp, was used for sequencing. The genomic fragments described earlier as well as the full-length cDNA clone were sequenced on both strands by using synthetic oligonucleotides (TIB MOLBIOL). Sequence reactions were performed using ^{35}S-dATP and the T7 sequencing kit (Pharmacia, Bromma, Sweden), according to the manufacturer's protocol. Sequence analyses were performed using the GCG sequence analysis software package (Genetics Computer Group Inc., Madison, WI) and the BLAST Network service of the National Center of Biotechnology Information (Bethesda, MD). Comparison between the EDD1 protein from Arabidopsis and the homologous protein from *E. coli* was done with the Pile-up and Pretty box programs (Genetics Computer Group Inc.).

Mapping the *edd1* Mutation

To determine the genetic position of the *edd1* locus on the Arabidopsis genome, we screened the CIC yeast artificial chromosome (YAC) library (Creusot et al., 1995) by using the complete *edd1* cDNA clone as a hybridization probe. Several YAC clones falling into two classes cross-hybridized with the probe. The clones CIC3B10, CIC3D2, CIC11D12, and CIC11G6 showed strong hybridization signal,

whereas the YAC clones CIC3A4, CIC6E4, CIC5F5, CIC9D5, CIC10B9, and CIC12F8 cross-hybridized only weakly. All of these YAC clones were previously mapped to two different regions of the Arabidopsis genome. The clones CIC3B10, CIC3D2, CIC11D12, and CIC11G6 are positioned on chromosome 3, close to the markers m409 and csr (*ALS* gene), at a map position that is ~82 centimorgans (cM). All of the other clones are anchored on chromosome 5, close to the marker mi125, at a map position that is ~85 cM (Schmidt et al., 1997; Camilleri et al., 1998). To characterize both loci, we amplified isolated YAC DNA from the above-mentioned clones by using the primers utilized earlier to distinguish the *EDD1* gene and the additional homolog. PCR amplification of the clones CIC3B10, CIC3D2, CIC11D12, and CIC11G6 yielded a 360-bp fragment representing the *EDD1* locus, whereas PCR amplification of CIC3A4, CIC10B9, CIC6E4, CIC5F5, CIC12F8, and CIC9D5 produced a 380-bp band representing the locus homologous to *EDD1* mentioned earlier. From these data, we conclude that *edd1* is positioned on the lower arm of chromosome 3.

In Vitro Transcription and Translation

In vitro transcription and translation were performed according to the supplier's instruction by using either the (TNT)-coupled reticulocyte lysate system or the TNT-coupled wheat germ extract system (Promega, Madison, WI) in the presence of ^{35}S-methionine (Amersham, Braunschweig, Germany).

Import into Isolated Pea Chloroplasts

Intact pea chloroplasts were isolated from pea seedling (7 to 10 days old), as described by Bartlett et al. (1982). Import reactions were performed in the light for 30 min at 25°C in a total volume of 240 μL containing 20 μL of the in vitro translation product, chloroplasts corresponding to 80 μg of chlorophyll, 12 μL of 1 M Hepes/KOH, pH 8.0, 32 μL of 2 M sorbitol, 18 μL of 250 mM methionine, and 2.4 μL of 0.1 M Mg-ATP. Chloroplasts were pelleted and resuspended in 600 μL of 50 mM Hepes and 0.33 M sorbitol, pH 8.0. One-third of the chloroplast fraction was treated with 2.5 μL of thermolysin (10 mg/mL) and 10 μL of 0.1 M $CaCl_2$ for 20 min on ice, whereas the remaining chloroplasts were incubated on ice for the same period of time. Protease treatment was stopped by the addition of 10 μL of 0.5 M EDTA. Chloroplasts of a second fraction that were preincubated on ice were disrupted by adjusting the reaction mixture to 1% Triton X-100. Unbroken chloroplasts were reisolated through a 45% Percoll cushion by centrifugation at 4500*g* for 8 min, washed in 50 mM Hepes and 0.33 M sorbitol, pH 8.0, and resuspended in 1 × SDS sample buffer. The proteins were analyzed by electrophoresis (Laemmli, 1970) followed by fluorography with Amplify (Amersham).

REFERENCES

Akhundova, G.G., Grinikh, L.I., and Shevchenko, V.V. (1978). Development of *Arabidopsis thaliana* embryos after gamma irradiation of plants in the generative phase. Ontogenez **9,** 514–519.

Altmann, T., Schmidt, R., and Willmitzer, L. (1992). Establishment of a gene tagging system in *Arabidopsis thaliana* based on the maize transposable element *Ac*. Theor. Appl. Genet. **84,** 371–383.

Altmann, T., Felix, G., Jessop, A., Kauschmann, A., Uwer, U., Pena-Cortés, H., and Willmitzer, L. (1995). *Ac/Ds* transposon mutagenesis in *Arabidopsis thaliana*: Mutant spectrum and frequency of *Ds* insertion mutants. Mol. Gen. Genet. **247,** 646–652.

Anthony, J.L., Vonder Haar, R.A., and Hall, T.C. (1990). Nucleotide sequence of an α-phaseolin gene from *Phaseolus vulgaris*. Nucleic Acids Res. **18,** 3396.

Baker, B., Coupland, G., Fedoroff, N., Starlinger, P., and Schell, J. (1987). Phenotypic assay for excision of the maize controlling element *Ac* in tobacco. EMBO J. **6,** 1547–1554.

Bancroft, I., Jones, J.D.G., and Dean, C. (1993). Heterologous transposon tagging of the *DRL1* locus in Arabidopsis. Plant Cell **5,** 631–638.

Bartlett, S.G., Grossman, A.R., and Chua, N.-H. (1982). In vitro synthesis and uptake of cytoplasmatically-synthesized chloroplast proteins. In Methods in Chloroplasts Molecular Biology, M. Edelmann, R.B. Hallick, and N.-H. Chua, eds (Amsterdam, The Netherlands: Elsevier Biochemical Press), pp. 1081–1091.

Bäumlein, H., Boerjan, W., Nagy, I., Bassüner, R., Van Montagu, M., Inzé, D., and Wobus, U. (1991). A novel seed protein gene from *Vicia faba* is developmentally regulated in transgenic tobacco and *Arabidopsis* plants. Mol. Gen. Genet. **225,** 459–467.

Bhattacharyya, M.K., Smith, A.M., Ellis, T.H.N., Hedley, C., and Martin, C. (1990). The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch-branching enzyme. Cell **60,** 115–122.

Bosnes, M., Harris, E., Aigeltinger, L., and Olsen, O.A. (1987). Morphology and ultrastructure of 11 barley shrunken endosperm mutants. Theor. Appl. Genet. **74,** 177–187.

Bradbeer, J.W., Atkinson, Y.E., Börner, T., and Hagemann, R. (1979). Cytoplasmic synthesis of plastid polypeptides may be controlled by plastid synthesized RNA. Nature **279,** 816–817.

Brantner, J.H., and Dure, L.S. (1975). The developmental biochemistry of cotton seed embryogenesis and germination. VI. Levels of cytosol and chloroplast aminoacyl-tRNA synthetases during cotyledon development. Biochim. Biophys. Acta **414,** 99–114.

Busch, M., Mayer, U., and Jürgens, G. (1996). Molecular analysis of the *Arabidopsis* pattern formation gene *GNOM*: Gene structure and intragenic complementation. Mol. Gen. Genet. **250,** 681–691.

Camilleri, M., Lafleuriel, J., Macadré, C., Varoquaux, F., Parmentier, Y., Picard, G., Caboche, M., and Bouchez, D. (1998). A YAC contig map of *Arabidopsis thaliana* chromosome 3. Plant J., in press.

Chatterjee, M., Sparvoli, S., Edmunds, C., Garosi, P., Findlay, K., and Martin, C. (1996). *DAG*, a gene required for chloroplast differentiation and palisade development in *Antirrhinum majus*. EMBO J. **15,** 4194–4207.

Chuck, G., Robbins, T., Nijar, C., Ralston, E., Courtney-Gutterson, N., and Dooner, H.K. (1993). Tagging and cloning of a petunia flower color gene with the maize transposable element *Activator*. Plant Cell **5,** 371–378.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA **81,** 1991–1995.

Clark, J.K., and Sheridan, W.F. (1991). Isolation and characterization of 51 *embryo-specific* mutations of maize. Plant Cell **3,** 935–951.

Creissen, G., Reynold, H., Yongbiao, X., and Mullineaux, P. (1995). Simultaneous targeting of pea gluthatione reductase and of a bacterial fusion protein to chloroplasts and mitochondria in transgenic tobacco. Plant J. **8,** 167–175.

Creusot, F., Fouilloux, E., Dron, M., Lafleuriel, J., Picard, G., Billault, A., Le Paslier, D., Cohen, D., Chabouté, M.-E., Durr, A., Fleck, J., Gigot, C., Camilleri, C., Bellini, C., Caboche, M., and Bouchez, D. (1995). The CIC library: A large insert YAC library for genome mapping in *Arabidopsis thaliana*. Plant J. **8,** 763–770.

Deblaere, R., Bytebier, D., De Greve, H., Deboek, F., Schell, J., Van Montagu, M., and Leemans, J. (1985). Efficient octopine Ti-plasmid derived for *Agrobacterium*-mediated gene transfer. Nucleic Acids Res. **13,** 4777–4780.

De Jong, A.J., Heidstra, R., Spaink, H.P., Hartog, M.V., Meijer, E.A., Hendricks, T., Lo Schiavo, F., Terzi, M., Bisseling, T., Van Kammen, A., and De Vries, S.C. (1993). *Rhizobium* lipooligosaccharides rescue a carrot somatic embryo mutant. Plant Cell **5,** 615–620.

Dellaporta, S.L., Wood, J., and Hicks, J.B. (1983). A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. **1,** 19–21.

Dennis, E.S., Gerlach, W.L., Peacock, W.J., and Schwartz, D. (1986). Excision of the *Ds* controlling element from the *Adh1* gene of maize. Maydica **23,** 47–57.

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. **19,** 1349.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132,** 6–13.

Felker, F.C., Peterson, D.M., and Nelson, O.E. (1987). Early grain development of the *seg2* maternal-effect shrunken endosperm mutant of barley. Can. J. Bot. **65,** 943–948.

Fiebig, C., Neuhaus, H., Teichert, J., Röcher, W., Degenhardt, J., and Link, G. (1990). Temporal and spatial pattern of plastid gene expression during crucifer seedling development and embryogenesis. Planta **181,** 191–198.

Fiedler, U., Filistein, R., Wobus, U., and Bäumlein, H. (1993). A complex ensemble of *cis*-regulatory elements controls the expression of a *Vicia faba* non-storage seed protein gene. Plant Mol. Biol. **22,** 669–679.

Fischer, C., and Neuhaus, G. (1996). Influence of auxin on the establishment of bilateral symmetry in monocots. Plant J. **9,** 659–669.

Franck, A., Guilley, H., Jonard, G., Richards, K., and Hirth, L. (1980). Nucleotide sequence of cauliflower mosaic virus. Cell **21,** 285–294.

Franzmann, L.H., Yoon, E.S., and Meinke, D.W. (1995). Saturating the genetic map of *Arabidopsis thaliana* with embryonic mutants. Plant J. **7,** 341–350.

Galili, G., Altschuler, Y., and Levanony, H. (1993). Assembly and transport of seed storage proteins. Trends Cell Biol. **3,** 437–442.

Gamborg, O.L., Miller, R.A., and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50,** 151–158.

Ge, G., Trieu, E.P., and Targoff, I.N. (1994). Primary structure and functional expression of human glycyl-tRNA synthetase, an autoantigen in myositis. J. Biol. Chem. **269,** 28790–28797.

Goldberg, R.B., Barker, S.J., and Perez-Grau, L. (1989). Regulation of gene expression during plant embryogenesis. Cell **56,** 149–160.

Goldberg, R.B., de Paiva, G., and Yadegari, R. (1994). Plant embryogenesis: Zygote to seed. Science **266,** 605–614.

Han, C.-D., Coe, E.H., and Martienssen, R.A. (1992). Molecular cloning and characterization of *iojap* (*ij*), a pattern striping gene of maize. EMBO J. **11,** 4037–4046.

Hanstein, J. (1870). Entwicklungsgeschichte der Keime der Monokotyle und Dikotyle. Bot. Abhandl. Bonn. **1,** 1–112.

Harpster, M.H., Mayfield, S.P., and Taylor, W.C. (1984). Effects of pigment-deficient mutants on the accumulation of photosynthetic proteins in maize. Plant Mol. Biol. **3,** 59–71.

Henningsen, K.W., Boynton, J.E., and von Wettstein, D. (1993). Mutants at *XANTHA* and *ALBINA* Loci in Relation to Chloroplast Biogenesis in Barley (*Hordeum vulgare* L.). (Copenhagen, Denmark: Royal Danish Academy of Sciences and Letters).

Höfgen, R., and Willmitzer, L. (1990). Biochemical and genetic analysis of different patatin isoforms expressed in various organs of potato (*Solanum tuberosum*). Plant Sci. **66,** 221–239.

Huang, J., Hack, E., Thornburg, R.W., and Myers, A.M. (1990). A yeast mitochondrial leader peptide functions in vivo as a dual targeting signal for both chloroplasts and mitochondria. Plant Cell **2,** 1249–1260.

Hudson, A., Carpenter, R., Doyle, S., and Coen, E.S. (1993). *OLIVE*: A key gene required for chlorophyll biosynthesis in *Antirrhinum majus.* EMBO J. **12,** 3711–3719.

Hurt, E.C., Soltanifar, N., Goldschmidt-Clermont, M., Rochaix, J.-D., and Schatz, G. (1986). The cleavable presequence of an imported chloroplast protein directs attached polypeptides into yeast mitochondria. EMBO J. **5,** 1343–1350.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. **5,** 387–405.

Jenner, C.F. (1982). Storage of starch. In Encyclopedia of Plant Physiology (NS): Plant Carbohydrates, Vol. 13A, F.A. Loewus and W. Tanner, eds (Berlin: Springer-Verlag), pp. 700–747.

Joshi, C.P. (1987). Putative polyadenylation signals in nuclear genes of higher plants: A compilation and analysis. Nucleic Acids Res. **15,** 9627–9644.

Jürgens, G., and Mayer, U. (1994). *Arabidopsis.* In Embryos—Color Atlas of Development, J.B.L. Bard, ed (Edinburgh, Scotland: Genetics Unit of Western General Hospital), pp. 7–21.

Jürgens, G., Mayer, U., Torres Ruiz, R.A., Berleth, T., and Misera, S. (1991). Genetic analysis of pattern formation in the *Arabidopsis* embryo. Development **1** (suppl.), 27–38.

Keddie, J.S., Carroll, B., Jones, J.D.G., and Gruissem, W. (1996). The *DCL* gene of tomato is required for chloroplast development and palisade cell morphogenesis in leaves. EMBO J. **15,** 4208–4217.

Keng, T., Webster, T.A., Sauer, R.T., and Schimmel, P. (1982). Gene for *Escherichia coli* glycyl-tRNA synthetase has tandem subunits coding regions in the same reading frame. J. Biol. Chem. **257,** 12503–12508.

Kern, D., Giegé, R., and Ebel, J.-P. (1981). Glycyl-tRNA synthetase from baker's yeast. Interconversion between active and inactive form of the enzyme. Biochemistry **20,** 122–131.

Kirk, J.T.O., and Tilney-Basset, R.A.E. (1978). The Plastids: Their Chemistry, Structure, Growth, and Inheritance, 2nd ed. (Amsterdam, The Netherlands: Elsevier).

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Liu, C.-m., Xu, Z.-h., and Chua, N.-H. (1993). Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. Plant Cell **5,** 621–630.

Lo Schiavo, F., Giuliano, G., and Sung, Z.R. (1988). Characterization of a temperature-sensitive carrot cell mutant impaired in somatic embryogenesis. Plant Sci. **54,** 157–164.

Logemann, J., Schell, J., and Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissue. Anal. Biochem. **163,** 16–20.

Long, D., Swinburne, J., Martin, M., Wilson, K., Sundberg, E., Lee, K., and Coupland, G. (1993). Analysis of the frequency of inheritance of transposase *Ds* elements in *Arabidopsis* after activation by 35S CaMV promoter fusion to the *Ac* transposase gene. Mol. Gen. Genet. **241,** 627–636.

Lukowitz, W., Mayer, U., and Jürgens, G. (1996). Cytokinesis in the *Arabidopsis* embryo involves the syntaxin-related *KNOLLE* gene product. Cell **84,** 61–71.

Lycett, G.W., Croy, R.R.D., Shirsat, A.H., and Boulter, D. (1984). The complete nucleotide sequence of a legumin gene from pea (*Pisum sativum* L.). Nucleic Acids Res. **12,** 4493–4506.

Maheshwari, P. (1950). An Introduction to the Embryology of Angiosperms. (New York: McGraw-Hill).

Mansfield, S.G., and Briarty, L.G. (1990a). Development of the free-nuclear endosperm in *Arabidopsis thaliana* (L.). Arabidopsis Inf. Serv. **27,** 53–64.

Mansfield, S.G., and Briarty, L.G. (1990b). Endosperm cellularization in *Arabidopsis thaliana* (L.). Arabidopsis Inf. Serv. **27,** 65–72.

Mansfield, S.G., and Briarty, L.G. (1991a). Early embryogenesis in *Arabidopsis thaliana.* I. The mature embryo sac. Can. J. Bot. **69,** 447–460.

Mansfield, S.G., and Briarty, L.G. (1991b). Early embryogenesis in *Arabidopsis thaliana.* II. The developing embryo. Can. J. Bot. **69,** 461–476.

Martienssen, R.A., Barkan, A., Freeling, M., and Taylor, W.C. (1989). Molecular cloning of a maize gene involved in photosynthetic membrane organisation that is regulated by Robertson's *Mutator.* EMBO J. **8,** 1633–1639.

Martínez-Zapater, J.M., Gil, P., Capel, J., and Somerville, C.R. (1992). Mutations at the Arabidopsis *CHM* locus promote rearrangements of the mitochondrial genome. Plant Cell **4,** 889–899.

Mayfield, S.P., and Taylor, W.C. (1984). Carotenoid-deficient maize seedlings fail to accumulate light-harvesting chlorophyll *a/b*–binding protein (LHCP) mRNA. Eur. J. Biochem. **144,** 79–84.

Meinke, D.W. (1991). Embryonic mutants of *Arabidopsis thaliana.* Dev. Genet. **12,** 382–392.

Meinke, D.W. (1995). Molecular genetics of plant embryogenesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46,** 369–394.

Miles, D. (1994). The role of high chlorophyll fluorescence photosynthesis mutants in the analysis of chloroplast thylakoid membrane assembly and function. Maydica **39,** 35–45.

Müller, A.J. (1963). Embryonentest zum Nachweis rezessiver Letalfaktoren bei *Arabidopsis thaliana.* Biol. Zentbl. **82,** 133–163.

Nada, S., Chang, P.K., and Dignam, J.D. (1993). Primary structure of the gene for glycyl-tRNA synthetase from *Brombyx mori.* J. Biol. Chem. **268,** 7600–7667.

Nagato, Y., Kitano, H., Kamijima, O., Kikuchi, S., and Satoh, H. (1989). Development mutants showing abnormal organ differentiation in rice embryos. Theor. Appl. Genet. **78,** 11–15.

Nairn, C.J., Winesett, L., and Ferl, R.J. (1988). Nucleotide sequence of an actin gene from *Arabidopsis thaliana.* Gene **65,** 247–257.

Oelmüller, R. (1989). Photooxidative destruction of chloroplasts and its effects on nuclear gene expression and extraplastidic enzyme levels. Photochem. Photobiol. **49,** 229–239.

Parthier, B., Kauspe, R., and Samtleben, S. (1972). Light-stimulated synthesis of aminoacyl-tRNA synthetases in greening *Euglena gracilis.* Biochim. Biophys. Acta **277,** 335–341.

Pohlmann, R.F., Fedoroff, N.V., and Messing, J. (1984). The nucleotide sequence of the maize controlling element *Activator.* Cell **37,** 635–643.

Pyke, K.A., and Leech, R.M. (1992). Chloroplast division and expansion is radically altered by nuclear mutations in *Arabidopsis thaliana.* Plant Physiol. **99,** 1005–1008.

Pyke, K.A., and Leech, R.M. (1994). A genetic analysis of chloroplast division and expansion in *Arabidopsis thaliana.* Plant Physiol. **104,** 201–207.

Reger, B.J., Fairfiel, S.A., Epler, J.L., and Barnett, W.E. (1970). Identification and origin of some chloroplast aminoacyl-tRNA synthetases and tRNAs. Proc. Natl. Acad. Sci. USA **67,** 1297–1302.

Reinbothe, S., and Parthier, B. (1990). Translational regulation of plastid gene expression in *Euglena gracilis.* FEBS Lett. **265,** 7–11.

Robertson, E.J., Pyke, K.A., and Leech, R.M. (1995). *arc6,* an extreme chloroplast division mutant of *Arabidopsis* also alters proplastid proliferation and morphology in shoot and root apices. J. Cell Sci. **108,** 2937–2944.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Schiavone, F.M., and Cooke, T.J. (1987). Unusual pattern of somatic embryogenesis in domesticated carrot: Developmental effects of exogenous auxins and auxin transport inhibitors. Cell Differ. **21,** 53–62.

Schimmel, P. (1987). Aminoacyl-tRNA synthetases: General scheme of structure–function relationship in the polypeptides and recognition of transfer RNAs. Annu. Rev. Biochem. **56,** 125–158.

Schmidt, R., and Willmitzer, L. (1988). High efficiency *Agrobacterium tumefaciens*–mediated transformation of *Arabidopsis thaliana* leaf and cotyledon explants. Plant Cell Rep. **7,** 583–586.

Schmidt, R., Love, K., West, J., Lenehan, Z., and Dean, C. (1997). Detailed description of 31 YAC contigs spanning the majority of *Arabidopsis thaliana* chromosome 5. Plant J. **11,** 563–573.

Schnarf, K. (1929). Embryologie der Angiospermen. (Berlin: Borntraeger).

Schulz, P., and Jensen, W.A. (1969). *Capsella* embryogenesis: The suspensor and the basal cell. Protoplasma **67,** 139–163.

Schwartz, B.W., Yeung, E.C., and Meinke, D.W. (1994). Disruption of morphogenesis and transformation of the suspensor in abnormal suspensor mutants of *Arabidopsis.* Development **120,** 3235–3245.

Sheridan, W.F., and Neuffer, M.G. (1982). Maize developmental mutants. J. Hered. **73,** 318–329.

Shevell, D.E., Leu, W.-M., Gillmor, C.S., Xia, G., Feldmann, K.A., and Chua, N.-H. (1994). *EMB30* is essential for normal cell division, cell expansion, and cell adhesion in Arabidopsis and encodes a protein that is similiar to Sec7. Cell **77,** 1051–1062.

Shiba, K., Schimmel, P., Motegi, H., and Noda, T. (1994). Human glycyl-tRNA synthetase. Wide divergence of primary structure from bacterial counterpart and species-specific aminoacylation. J. Biol. Chem. **269,** 30049–30055.

Shotwell, M.A., and Larkins, B.A. (1989). The molecular biology and biochemistry of seed storage proteins. In The Biochemistry of Plants, Vol. 15, A. Marcus, ed (San Diego, CA: Academic Press), pp. 297–345.

Slack, C.R., and Browse, J.A. (1984). Synthesis of storage lipids in developing seeds. In Seed Physiology, D.R. Murray, ed (Sydney, Australia: Academic Press), pp. 209–244.

Souèges, E.C.R. (1919). Les premières divisions de l'oeuf et les differenciations du suspenseur chez le *Capsella bursa-pastoris* Moench. Ann. Sci. Nat. Bot. **10** (sér.), 1–28.

Swinburne, J., Balcells, L., Scofield, S.R., Jones, J.D.G., and Coupland, G. (1992). Elevated levels of *Activator* transposase mRNA are associated with high frequencies of *Dissociation* excision in Arabidopsis. Plant Cell **4,** 583–595.

Tacke, E., Korfhage, C., Michel, D., Maddaloni, M., Moto, M., Lanzini, S., Salamini, F., and Döring, H.-P. (1995). Transposon tagging of the maize *Glossy2 locus* with the transposable element *En/Spm.* Plant J. **8,** 907–917.

Taylor, W.C. (1989). Regulatory interactions between nuclear and plastid genomes. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40,** 211–233.

Tsugeki, R., Kochieva, E.Z., and Fedoroff, N.V. (1996). A transposon insertion in the *Arabidopsis SSR16* gene causes an embryo-defective lethal mutation. Plant J. **10,** 479–489.

Tykarska, T. (1979). Rape embryogenesis. II. Development of embryo proper. Acta Soc. Bot. Pol. **48,** 391–421.

Vandendries, R. (1909). Contribution à l'histoire du dévelopement des crucifères. Cellule **25,** 412–459.

Vernon, D.M., and Meinke, D.W. (1994). Embryogenic transformation of the suspensor in *twin,* a polyembryonic mutant of *Arabidopsis.* Dev. Biol. **164,** 566–573.

Voelker, R., and Barkan, A. (1995). Two nuclear mutations disrupt distinct pathways for targeting proteins to the chloroplast thylakoid. EMBO J. **14,** 3905–3914.

von Heijne, G., and Nishikawa, K. (1991). Chloroplast transit peptides: The perfect random coil. FEBS Lett. **278,** 1–3.

von Heijne, G., Steppuhn, J., and Herrmann, R.G. (1989). Domain structure of mitochondrial and chloroplast targeting peptides. Eur. J. Biochem. **180,** 535–545.

Wade, B.L. (1941). Genetic studies on variegation in snap beans. J. Agric. Res. **63,** 661–667.

Walbot, V., and Coe, E.H. (1979). Nuclear gene conditions a programmed change to ribosome-less plastids in *Zea mays.* Proc. Natl. Acad. Sci. USA **76,** 2760–2764.

Webster, T., Gibson, B.W., Keng, T., Biemann, K., and Schimmel, P. (1983). Primary structures of both subunits of *Escherichia coli* glycyl-tRNA synthetase. J. Biol. Chem. **258,** 10637–10641.

West, M.A.L., and Harada, J.J. (1993). Embryogenesis in higher plants. An overview. Plant Cell **5,** 1361–1369.

Wetzel, C.M., Jiang, C.-Z., Meehan, L.J., Voytas, D.F., and Rodermel, S.R. (1994). Nuclear-organelle interactions: The *immutans* variegation mutant of *Arabidopsis* is plastid autonomous and impaired in carotenoid biosynthesis. Plant J. **6,** 161–175.

Yadegari, R., de Paiva, G.R., Laux, T., Koltunow, A.M., Apuya, N., Zimmerman, J.L., Fischer, R.L., Harada, J.J., and Goldberg, R.B. (1994). Cell differentiation and morphogenesis are uncoupled in Arabidopsis *raspberry* embryos. Plant Cell **6,** 1713–1729.

Yeung, E.C., and Meinke, D.W. (1993). Embryogenesis in angiosperms: Development of the suspensor. Plant Cell **5,** 1371–1381.

Zaumeyer, W.J. (1938). A heritable abnormality of beans resembling mosaic. Phytopathology **28,** 520–522.

Zaumeyer, W.J. (1942). Inheritance of leaf variegation in beans. J. Agric. Res. **64,** 119–127.

Zimmerman, J.L. (1993). Somatic embryogenesis. A model for early development in higher plants. Plant Cell **5,** 1411–1423.