The Plant Cell, Vol. 3, 149–157, February 1991

Embryonic Lethals and T-DNA Insertional Mutagenesis in *Arabidopsis*

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T-DNA insertional mutagenesis represents a promising approach to the molecular isolation of genes with essential functions during plant embryo development. We describe in this report the isolation and characterization of 18 mutants of *Arabidopsis thaliana* defective in embryo development following seed transformation with *Agrobacterium tumefaciens*. Random T-DNA insertion was expected to result in a high frequency of recessive embryonic lethals because many target genes are required for embryogenesis. The cointegrate Ti plasmid used in these experiments contained the nopaline synthase and neomycin phosphotransferase gene markers. Nopaline assays and resistance to kanamycin were used to estimate the number of functional inserts present in segregating families. Nine families appeared to contain a T-DNA insert either within or adjacent to the mutant gene. Eight families were clearly not tagged with a functional insert and appeared instead to contain mutations induced during the transformation process. DNA gel blot hybridization with internal and right border probes revealed a variety of rearrangements associated with T-DNA insertion. A general strategy is presented to simplify the identification of tagged embryonic mutants and facilitate the molecular isolation of genes required for plant embryogenesis.

INTRODUCTION

Several methods have been proposed to facilitate the molecular isolation of mutant genes in *Arabidopsis thaliana* (Meyerowitz, 1987). Chromosome walking from flanking restriction fragment length polymorphism markers represents the most promising long-term approach but still requires considerable effort despite the small genome size, the availability of restriction fragment length polymorphism maps (Chang et al., 1988; Nam et al., 1989), and the construction of yeast artificial chromosomes containing large inserts of *Arabidopsis* DNA (Ward and Jen, 1990). Insertional mutagenesis provides an alternative approach to gene isolation in higher plants. Three types of insertional agents have been examined: endogenous transposable elements, foreign transposons introduced through transformation, and T-DNA from *Agrobacterium tumefaciens*. Although endogenous transposable elements have been identified in *Arabidopsis* (Voytas and Ausubel, 1988), and movement of maize transposons in dicots has been demonstrated (Hehl and Baker, 1990; Jones et al., 1990; Yoder, 1990), the feasibility of transposon tagging in *Arabidopsis* remains to be established (Schmidt and Willmitzer, 1989).

In contrast, T-DNA insertional mutagenesis has already been utilized to isolate genes in *Arabidopsis*. One approach has been to use promoterless constructs to eliminate somaclonal variants induced in culture and focus on transformants in which T-DNA insertion into active genes has occurred (Koncz et al., 1989, 1990). A second approach has involved screening plants for mutations produced after seed transformation with *A. tumefaciens* (Feldmann and Marks, 1987). This method has generated a wide range of putatively tagged mutants (Feldmann et al., 1989, 1990) and resulted in the isolation of genes required for trichome formation (Herman and Marks, 1989; Marks and Feldmann, 1989) and floral development (Yanofsky et al., 1990).

Embryonic mutants are the most common type of genetic defect identified after x-irradiation and ethyl methanesulfonate (EMS) seed mutagenesis in *Arabidopsis* (Müller, 1963; Meinke, 1986). Arrested embryos from these *emb* mutants differ with respect to lethal phase (Meinke, 1985), pattern of abnormal development (Marsden and Meinke, 1985), ultrastructure (Patton and Meinke, 1990), accumulation of seed storage proteins (Heath et al., 1986), response in culture (Baus et al., 1986; Franzmann et al., 1989), and gametophytic gene expression

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(Meinke, 1982). Sixteen genes have been mapped relative to visible markers in preparation for gene isolation through chromosome walking (Patton et al., 1991). Some mutants are defective in essential housekeeping functions (Schneider et al., 1989; Shellhammer and Meinke, 1990). Others appear to be altered in genes with a more direct role in the regulation of embryogenesis (Meinke, 1991).

Embryonic mutants should also be common after seed transformation if T-DNA insertion occurs at random chromosomal sites. The objective of this study was, therefore, to screen for mutants defective in embryo development after seed transformation, compare the diversity of mutants found with those obtained following EMS mutagenesis, and identify mutants that appeared to result from T-DNA insertion. Embryonic mutants could result from (1) insertion of a functional T-DNA that conferred kanamycin resistance and nopaline synthesis, (2) insertion of a silent T-DNA that failed to confer kanamycin resistance or nopaline synthesis, (3) mutagenesis associated with the infection process, or (4) mutations present before transformation. All of these mechanisms appear to be responsible for mutations identified after seed transformation. We describe here the isolation and characterization of 18 transgenic families segregating for embryonic mutations, nine of which appear to be tagged, and outline a strategy to facilitate the molecular isolation of genes with essential functions during plant embryo development.

RESULTS

Mutant Isolation

Embryonic mutants were identified after seed transformation (Feldmann and Marks, 1987) by screening transgenic families for the presence of abnormal seeds. The C58C1rif strain of *A. tumefaciens* used to produce transformants (Velten and Schell, 1985) contained a cointegrate Ti plasmid with neomycin phosphotransferase (*npt*II) and nopaline synthase (*nos*) gene markers, as shown in Figure 1. Forty-one transgenic families that appeared to produce a high frequency of aborted seeds were identified from preliminary screens. Sixteen of these were false positives that failed to produce aborted seeds in subsequent generations. Seven other families were not examined further because they produced a low ratio of aborted seeds with variable phenotypes. The remaining 18 families segregated for recessive mutations affecting embryogenesis, as shown in Table 1. Mutant phenotypes were similar to those observed after x-irradiation (Müller, 1963) and EMS seed mutagenesis (Meinke and Sussex, 1979; Meinke, 1985). Segregation ratios were consistent with the presence of a single recessive mutation affecting embryo development.

Classification of Mutants

Nopaline assays and responses on kanamycin were used to classify mutants as putatively tagged or not tagged with a functional insert. Nine families were identified that appeared to contain a tagged mutant allele (Table 1). Seven of these were embryonic lethals arrested early in development. Allelism tests demonstrated that five mutants examined in most detail (*emb76*, *emb83*, *emb84*, *emb86*, *emb87*) were defective in different genes. Abnormal suspensors observed in several families were similar to those described previously (Meinke, 1985). Two mutants produced embryos that completed morphogenesis but were either pale (*emb82*) and produced albino seedlings, or accumulated anthocyanin (*emb78*) and resembled the fusca type described previously (Weiland and Müller, 1972). Anthocyanin in *emb78* was limited to mature cotyledons and failed to accumulate in a genetic background (*ttg*/*ttg*) known to disrupt anthocyanin formation in vegetative tissues (Koornneef, 1981). Mature seeds germinated in culture but failed to develop leaves or extensive roots. This mutant, therefore, appears to be defective in some aspect of embryo maturation that results in accumulation of anthocyanin. One family (*emb93*) was classified as possibly tagged because it contained multiple inserts that made interpretation of kanamycin responses and nopaline assays difficult. The remaining eight families were clearly not tagged with a functional insert because they contained nop$^-$ heterozygotes that produced 100% kan^s progeny. Two of these mutants (*emb77* and *emb79*) had identical

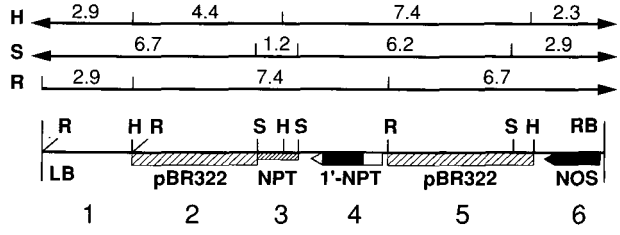

Figure 1. T-DNA Structure in Cointegrate Ti Plasmid 3850:1003.

Sequences expressed in plants are highlighted in black. Right (RB) and left (LB) T-DNA borders are noted. Expected sizes (in kilobases) of fragments produced following cleavage of genomic plant DNA with HindIII (H), SalI (S), and EcoRI (R) are shown above. Important regions of T-DNA structure include: (1) part of Ti fragment 10 from HindIII digest, (2) partial pBR322 used in plasmid construction, (3) Tn903 *npt*II gene with promoter for expression in bacteria, (4) Tn5 *npt*II gene (1.3 kb) with 1′ promoter from T_r-DNA and 3′ OCS sequences for kanamycin resistance in plants, (5) complete pBR322 used in plasmid construction, and (6) *nos* gene (1.7 kb) from HindIII fragment 23 with regulatory sequences for nopaline production in plants (Velten and Schell, 1985).

Table 1. Overview of Embryonic Mutants Identified in Transgenic Families

Mutant Family	Gene Symbol	Mutant Phenotype[a]	Seed Color[b]	Seeds Screened	Percent Aborted	Percent Top Half[c]	Heterozygotes[d]
Putatively tagged with T-DNA							
T-76	*emb76*	Globular (S)	1–2	2885	24.4	50.5	All nop$^+$
T-578	*emb78*	Dark red cotyledons	4–5	3030	23.0	50.2	All nop$^+$
T-739	*emb82*	Albino embryos	1	1482	23.1	50.9	All nop$^+$
T-869[e]	*emb83*	Globular-heart	1–2	776	23.6	49.7	All nop$^-$
T-508	*emb84*	Globular (S)	2	1014	25.6	42.1	All nop$^+$
T-811	*emb87*	Globular	1	1030	25.1	52.5	All nop$^+$
T-731	*emb86*	Globular-heart	1	883	25.8	50.4	All nop$^+$
T-1002[e]	*emb88*	Globular (S)	1	880	27.7	48.4	Variable
T-1793[e]	*emb95*	Early globular	1	1708	23.0	56.9	Variable
Possibly tagged with T-DNA							
T-562[f]	*emb93*	Globular-mature	1	1971	23.4	49.1	All nop$^+$
Not tagged with functional insert							
T-77	*emb77*	Late preglobular	1	2031	24.1	52.1	Some nop$^-$
T-79	*emb79*	Late preglobular	1	2151	18.6	53.2	Some nop$^-$
T-98	*emb80*	Late preglobular	2	3926	23.4	50.4	Some nop$^-$
T-668	*emb81*	Early heart	1	1853	25.3	43.8	Some nop$^-$
T-543	*emb89*	Globular-mature	1	1051	25.7	47.4	Some nop$^-$
T-749	*emb90*	Globular-heart	1	415	26.5	49.1	Some nop$^-$
T-685	*emb91*	Early globular	1	769	25.4	50.3	Some nop$^-$
T-1790	*emb94*	Globular-heart	1	1037	24.6	47.0	Some nop$^-$

[a] Some arrested embryos contained an abnormally large suspensor (S).
[b] Seeds were either white (1), pale yellow-green (2), or a mixture of green (4) and dark red (5).
[c] Percentage of total aborted seeds located in the top half of heterozygous siliques. This should equal 50% if the mutant gene does not disrupt pollen germination or pollen-tube growth (Meinke, 1982).
[d] The presence of nop$^-$ heterozygotes indicates that the mutant gene is not tagged with a functional insert.
[e] The T-DNA insert in these families has altered NOS activity.
[f] Analysis of this family has been complicated by the presence of additional inserts.

phenotypes but were shown through complementation tests to define different genes. Other mutants exhibited a wide range of lethal phases consistent with defects in different target genes.

Expected Response of Tagged Mutants

Families segregating for a tagged embryonic lethal should produce a 2:1 ratio of kan^r:kan^s progeny in the absence of additional inserts. Plants homozygous for the tagged allele should not be recovered because they fail to complete embryogenesis. Every kan^r nop$^+$ plant in these families should be heterozygous for the lethal and every kan^s nop$^-$ plant should be wild type. Tagged mutants with additional inserts should produce a higher percentage of kan^r progeny and a significant number of kan^r nop$^+$ wild-type plants. Families with a single insert unlinked to an embryonic lethal not caused by T-DNA insertion should produce a 3:1 ratio of kan^r:kan^s progeny. One-third of the kan^r nop$^+$ plants in these families should be wild type. The ratio of kan^r:kan^s progeny and the frequency of kan^r wild-type plants in families segregating for an embryonic lethal can, therefore, provide an estimate of the number of functional inserts and the relationship between these inserts and the recessive mutation. The presence of nop$^-$ heterozygotes with 100% kan^s progeny demonstrates that the mutation is not caused by a functional T-DNA insert. DNA gel blot analysis is then required to demonstrate that lethality is not caused by a silent insert.

Table 2. Ratio of Kan^r:Kan^s Progeny Produced after Self-Pollination of Heterozygous (*EMB/emb*) Plants[a]

Mutant	Parent	Phenotype of Progeny Seedlings		
		Kan^r	Kan^s	Kan^r/Kan^s
emb76	Nop$^+$	1008	455	2.2
	Nop$^+$	504	41	12.3[b]
emb78	Nop$^+$	1282	747	1.7
emb82	Nop$^+$	553	330	1.7
emb83	Nop$^-$	650	420	1.5
emb84	Nop$^+$	4074	1893	2.2
	Nop$^+$	97	0	All Kan^r
	Nop$^+$	72	272	0.3[c]
emb87	Nop$^+$	1029	571	1.8
	Nop$^+$	99	248	0.4[c]
emb86	Nop$^+$	1033	82	12.6[b]
	Nop$^+$	1395	617	2.3
	Nop$^+$	130	346	0.4[c]
emb88	Nop$^-$	253	122	2.0
emb95	Nop$^+$	481	46	10.5[b]
	Nop$^+$	420	254	1.7
emb93	Nop$^+$	395	52	7.6[b]

[a] Subfamilies with different responses are listed separately.
[b] Contain a second insert not closely linked to the lethal.
[c] Low ratios in these subfamilies remain to be explained.

Response of Seedlings on Kanamycin

Kanamycin responses of seedlings produced from heterozygotes in putatively tagged families are summarized in Table 2. Many of these heterozygotes produced a 2:1 ratio of kan^r:kan^s progeny consistent with a single insert responsible for the mutation. Others produced higher ratios consistent with the presence of additional inserts. Kanamycin ratios obtained from a number of heterozygotes in three putatively tagged families (*emb76*, *emb86*, *emb95*) were consistent with the 11:1 ratio expected for plants containing a second insert unlinked to a tagged lethal. Two families (*emb83* and *emb87*) produced mottled plants on kanamycin that appeared normal when transplanted to soil. This suggests that NPTII activity in these plants is reduced. One of these families (*emb83*) also failed to produce nop$^+$ plants and, therefore, appeared to be defective in expression of both *nos* and *npt*II genes. Kanamycin ratios in several families were slightly lower than the 2:1 ratio expected for a single insert. This may reflect variable expression of the *npt*II gene, particularly in families with mottled plants on kanamycin, or reduced viability of gametes carrying the tagged allele. Extremely low ratios observed in rare subfamilies of *emb84*, *emb87*, and *emb86* remain to be explained.

Kanamycin ratios in *emb93* were consistent with the presence of a second insert distantly linked to a tagged lethal. Other heterozygotes in this family are being tested for the presence of a single insert to provide more definitive evidence for tagging. The remaining families produced at least one nop$^-$ heterozygote that gave rise to all kan^s progeny. Embryonic mutations in these families are, therefore, not caused by a functional insert, and resistance noted in previous generations must have resulted from a T-DNA not associated with the mutation. One family (*emb88*) was not initially thought to be tagged because several nop$^-$ heterozygotes were identified, but these plants were subsequently shown to produce a 2:1 ratio of progeny seedlings on kanamycin. This mutant, therefore, appears to be tagged with a defective insert with altered *nos* function.

Analysis of Kan^r Transplants

Kanamycin-resistant plants started in culture were transplanted to soil and screened for the presence of the lethal to provide more definitive evidence for insertional mutagenesis. Every kan^r transplant in families putatively tagged with a single insert was expected to be heterozygous for the mutation. Results obtained with several families were consistent with this prediction, as shown in Table 3. Other families produced a low frequency of kan^r wild-type plants. Two models were examined to explain the origin and significance of these wild-type plants. The first assumed that lethality was caused by T-DNA insertion and that wild-type plants resulted from (1) recombination between the tagged locus and a second closely linked insert, (2) contamination by pollen or seeds from adjacent plants, (3) transient activation of a silent insert unlinked to the mutation, or (4) modifications at the insertion site that restored normal function to the mutant allele. The second model assumed that lethality was not caused by T-DNA insertion and that wild-type transplants were products of recombi-

Table 3. Identification of Rare Kan^r Wild-Type Plants in Putatively Tagged Families

Mutant Family	Kan^r Plants Screened	Wild-Type Plants Found	Map Distance (cM) if T-DNA Is Linked to Embryonic Lethal[a]
emb76	320	11	3.4[b]
emb78	749	4	0.5
emb82	362	0	<0.3
emb83	379	10	2.6[b]
emb84	1433	94	6.6[b]
emb87	1152	1	<0.1
emb86	867	21	2.4[b]
emb88	158	0	<0.6
emb95	144	0	<0.7

[a] Assumes that rare wild-type plants are recombinants.
[b] These families appear to contain a second insert closely linked to the embryonic lethal.

nation between the mutant gene and a closely linked insert. Results obtained to date are consistent with the first model. One family (*emb84*) appears to contain a second insert located approximately 7 centimorgans (cM) from the tagged gene. Several *emb84* heterozygotes have recently been identified that appear to lack this second insert. Three families (*emb76*, *emb83*, *emb86*) appear to contain a second insert even more closely linked to the tagged locus. The origin of rare wild-type transplants in the remaining families (*emb78* and *emb87*) remains to be determined.

Results of Nopaline Assays

Kanamycin-resistant wild-type plants were originally thought to represent recombinants between a T-DNA insert and a linked *emb* locus not tagged with T-DNA. If this model were correct, kan^r nop^+ wild-type plants and kan^s nop^- heterozygotes should be present in equal frequencies because they represent reciprocal products of recombination. Therefore, we performed nopaline assays with a large number of plants in putatively tagged families in an attempt to identify the rare nop^- heterozygotes predicted by this model. The absence of nop^- plants among 1045 heterozygotes (329 *emb76*, 210 *emb87*, 206 *emb78*, 128 *emb84*, 172 *emb86*) grown under nonselective conditions was inconsistent with the model, and thus provided further evidence in support of tagging. The most definitive evidence was obtained with *emb76* because 11 of 329 heterozygotes tested should have been nop^- if kan^r wild-type plants (Table 3) were indeed recombinants between a T-DNA insert and a closely linked *emb* locus. Nopaline assays also identified a number of nop^+ wild-type plants in putatively tagged families. In several cases, these plants were found at the same frequency as kan^r wild-type plants and were probably generated through the same mechanism. Nopaline assays with *emb95* revealed 118 nop^+ heterozygotes, 10 nop^- heterozygotes, and 42 nop^- wild types. The absence of nop^+ wild types indicates that nop^- heterozygotes in this family probably result from transient inactivation of *nos* rather than recombination between the mutant allele and a linked insert. The combined results of nopaline assays and kanamycin responses are, therefore, consistent with the general conclusion that five families (*emb78*, *emb82*, *emb87*, *emb88*, *emb95*) contain a single insert responsible for the mutation, three contain an insert with altered NOS activity (*emb83*, *emb88*, *emb95*), two contain an insert with altered NPTII activity (*emb83* and *emb87*), and four have a second insert closely linked to the tagged allele (*emb76*, *emb83*, *emb84*, *emb86*). None of the families appears to contain an insert closely linked to a mutant allele not tagged with T-DNA.

DNA Gel Blot Hybridization with T-DNA Probes

DNA gel blot hybridizations between genomic DNA isolated from transgenic families and probes representing the T-DNA right border and internal pBR322 regions were performed to determine whether T-DNA insertion patterns were consistent with the models described above. Four mutants that did not appear from nopaline assays and kanamycin responses to be tagged with a functional insert (*emb77*, *emb79*, *emb90*, *emb91*) also lacked sequences homologous to right border and pBR322 probes (data not shown). This is consistent with the model that some mutations recovered following seed transformation are not tagged and result instead from mutagenesis associated with the infection process.

Five putatively tagged families (*emb76*, *emb78*, *emb83*, *emb84*, *emb87*) appeared to contain rearranged or duplicated inserts. The simplest pattern was observed with *emb87* heterozygotes where the presence of a single band in blots probed with pBR322 was consistent with a truncated insert missing the left half of the T-DNA, as shown in Figure 2A. The mottled appearance of heterozygotes in this family appears to result from reduced expression of the *npt*II gene adjacent to the break point. Another family (*emb83*) that failed to produce any nop^+ plants contained a truncated insert missing right border sequences (data not shown). Blots prepared from *emb76* kan^r wild-type plants appeared to contain a subset of the bands present in heterozygotes (Figure 2B). This is consistent with the model that heterozygotes are tagged with T-DNA but contain a second linked insert that becomes separated through recombination. The insert putatively responsible for the mutation in this family appears to contain a mixture of tandem and inverted repeats. Similar rearrangements have been noted in other mutants isolated from this population of transgenic plants (Feldmann et al., 1989; Herman and Marks, 1989). Results obtained with *emb78* were inconsistent with the presence of a second insert linked to the mutation because bands produced by kan^r wild-type plants were not a subset of those present in heterozygotes (Figure 2B). Wild-type plants in this family may, therefore, have resulted from pollen or seed contamination or rearrangement of unstable inserts. The presence of multiple bands in blots of kan^r wild-type plants from *emb84* (Figure 2C), when combined with genetic data noted above, suggests that several inserts may be clustered within 5 cM to 10 cM of this mutant allele. Efforts to identify *emb84* subfamilies without kan^r wild-type plants may lead to the elimination of these additional inserts through recombination and the recovery of plants with a less complex pattern of hybridization.

DISCUSSION

Seed transformation with *Agrobacterium tumefaciens* has become a valuable method for insertional mutagenesis and gene isolation in *Arabidopsis*. More than 8000 transgenic families produced after seed transformation await further characterization. Preliminary screening of these transgenic

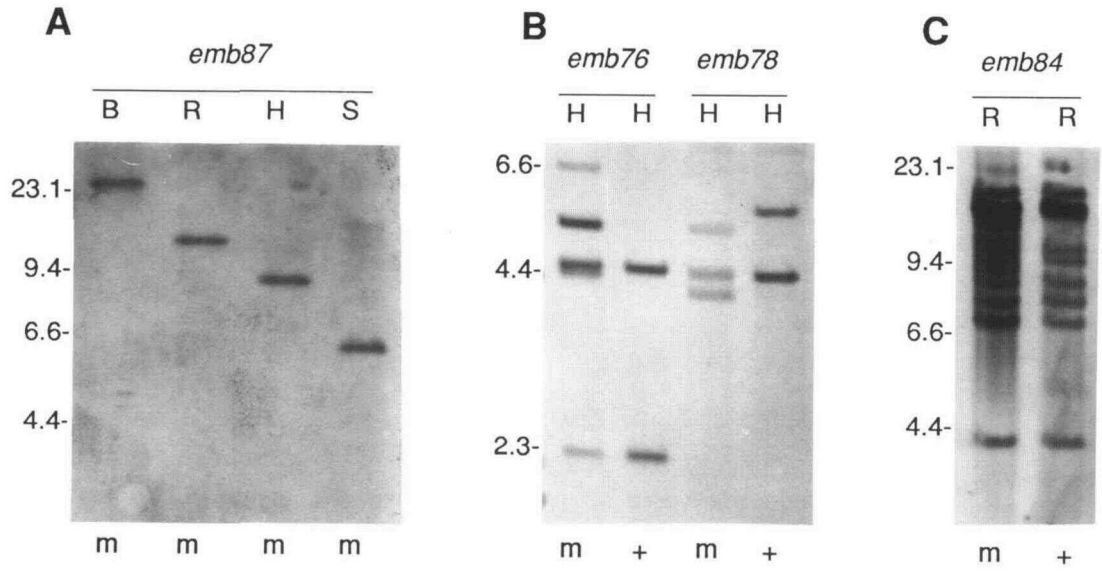

Figure 2. DNA Gel Blot Hybridization with Right Border and pBR322 Probes.

Genomic DNA from heterozygous (m) and kan^r wild-type (+) plants was digested with either BglII (B), EcoRI (R), HindIII (H), or SalI (S).
(A) DNA from *emb87* probed with pBR322. Results are consistent with the presence of a truncated insert missing regions 1 through 3 in Figure 1.
(B) DNA from *emb76* and *emb78* probed with right border sequences. Three *emb76* bands are present in heterozygotes but not in wild-type transplants. Tandem and inverted duplications appear to be associated with the mutation. A duplicated insert may also be responsible for lethality in *emb78*.
(C) DNA from *emb84* probed with right border sequences. The complexity of bands in wild-type transplants is consistent with the presence of multiple inserts. Several bands unique to heterozygotes appear to be associated with the mutation.

families has revealed the presence of mutations affecting many different aspects of plant growth and development (Feldmann et al., 1990). We demonstrate in this report that (1) embryonic mutants are among the most common defect identified following seed transformation, (2) a wide range of T-DNA insertion patterns can be identified among these transformants, and (3) further analysis of embryonic mutants tagged with T-DNA may greatly facilitate the molecular isolation of genes with essential functions during plant embryo development.

Repeated and rearranged T-DNA inserts have been noted previously in *Arabidopsis* (Feldmann et al., 1989; Herman and Marks, 1989), petunia (Jones et al., 1987), and tomato (Jorgensen et al., 1987) plants transformed with *A. tumefaciens* C58 strains carrying derivatives of the cointegrate pGV3850 Ti plasmid. The present study documents the extent and complexity of these rearrangements in transgenic *Arabidopsis* families with embryonic mutations produced after seed transformation. Nopaline assays and responses on kanamycin revealed the presence of inserts with partial NPTII activity (*emb83* and *emb87*), negligible NPTII activity (additional insert in *emb76* subfamily), variable NOS activity (*emb88* and *emb95*), and negligible NOS activity (*emb83*). Both deletions and altered expression appear to be responsible for these patterns. Hypermethylation has been correlated with altered NOS activity in transgenic tobacco (Matzke and Matzke, 1990) and may be responsible for some of the variations observed after seed transformation in *Arabidopsis*. The relatively high frequency of T-DNA inserts with altered NOS or NPTII activity indicates that when screening seed transformants for insertional mutants, care must be taken not to eliminate families that exhibit features potentially inconsistent with tagging.

The identification of five families with a second insert closely linked to a putatively tagged mutant allele is difficult to explain. If insertion occurs at random chromosomal sites and families with two inserts are produced through independent events, it seems unlikely that five families would contain both a tagged allele and a second insert within 3 cM to 7 cM of the mutation. One model to explain this apparent nonrandom pattern of insertion might be that linked insertion sites represent chromosomal regions located in close proximity when integration takes place. This model is difficult to evaluate in part because the exact timing of infection and integration after seed transformation remains to be established. Genetic localization of inserts through mapping with visible and molecular markers may

help to resolve this question of nonrandom insertion sites after transformation.

With this perspective on mutant phenotypes and insertion patterns observed among transgenic families, we propose the following approach to identifying additional embryonic mutants tagged with T-DNA after seed transformation in *Arabidopsis*: (1) screen immature siliques from several T_3 or T_4 plants within a given family for the presence of 25% abnormal seeds, (2) verify the presence of putative mutations in the next generation, (3) assay tissue from a limited number of heterozygotes for nopaline to determine whether nop^- heterozygotes are present, (4) estimate the number of functional inserts present by plating progeny seeds from heterozygotes on kanamycin and determining the ratio of resistant to sensitive progeny, (5) screen kan^r transplants for the presence of wild-type plants indicative of inserts not responsible for the mutation, (6) perform DNA gel blots with genomic DNA isolated from heterozygotes to determine the complexity of T-DNA insertion patterns, (7) identify putatively tagged families with simple patterns of insertion, and (8) eliminate through recombination and random assortment any additional inserts identified in other families. A similar approach could be pursued to identify embryonic mutants produced through other methods of *Agrobacterium*-mediated transformation. We estimate that several hundred embryonic mutants tagged with T-DNA could be isolated in this manner over the next 3 years. These mutants could greatly facilitate gene isolation before the completion of a physical map of the genome.

METHODS

Isolation and Characterization of Mutants

Embryonic mutants were isolated from transgenic families produced following seed transformation of *Arabidopsis thaliana* wild-type strain Wassilewskija with *Agrobacterium tumefaciens* strain C58C1rif containing the cointegrate 3850:1003 Ti plasmid (Velten and Schell, 1985) with *nos* and *npt*II gene markers. Details of this transformation method have been described previously (Feldmann and Marks, 1987; Feldmann et al., 1990). Mutants described in this study were isolated from a random sample of transgenic families. Mutation frequencies could not be determined because the entire population was not sampled. Embryonic mutants were identified by screening mature T_3 seeds produced by T_2 plants, mature T_4 seeds produced by several T_3 plants from a single T_2 family, and immature siliques produced by T_3 or T_4 plants. The final approach allowed the most definitive identification of mutants defective in embryo development. Most of the preliminary screening of transgenic families was performed at Du Pont (Wilmington, DE). Putative mutants were subsequently examined in more detail at Oklahoma State University. Plants were grown in pots at 23°C ± 3 °C beneath fluorescent lights maintained on daily 16-hr light/8-hr dark cycles (Meinke, 1985; Heath et al., 1986). Heterozygous plants were identified by the presence of siliques containing 25% abnormal seeds (Meinke and Sussex, 1979; Meinke, 1985). Complementation tests and mutant analysis were performed as described previously (Meinke, 1985).

Nopaline Assays

Extracts for nopaline assays were prepared by grinding single leaves from mature plants with a glass rod in a microcentrifuge tube containing 10 μL of distilled water. Exposure of plant tissues to arginine was not required for nopaline detection. Samples were centrifuged at 15,000*g* for 3 min to 5 min, and 10 μL of supernatant was spotted on a piece of Whatmann 3MM chromatography paper (20 × 32 cm) with air drying. Arginine and nopaline standards were included in the initial chromatograms to facilitate positive identification of spots. Subsequent chromatograms contained 10 experimental samples without standards. Paper electrophoresis was performed with a formic acid:acetic acid buffer in an electrophoresis apparatus (IBI Model HRH), as described by Rogers et al. (1986). Nopaline was detected by staining dried chromatograms with 0.005% (w/v) phenanthrenequinone in 80% ethanol and 2% NaOH and visualizing spots with UV light. Sensitivity was increased by preparing the phenanthrenequinone solution within 12 hr of staining and adding NaOH immediately before use.

Response of Seedlings on Kanamycin

Resistance to kanamycin was determined by germinating dry seeds on 100 × 15 mm Petri plates containing the inorganic salts of Murashige and Skoog (1962), 3% glucose, 0.8% (w/v) purified agar, and 50 mg/L kanamycin sulfate (Sigma). Seeds were surface sterilized by a 30-sec exposure to 95% ethanol and a 6-min treatment with 50% Clorox and 0.02% Tween 20, followed by extensive washing with sterile water. Plates containing 50 seeds were first incubated at 4°C for 2 days to increase germination frequencies and then maintained at room temperature beneath fluorescent lights on 16-hr light/8-hr dark cycles. The resulting seedlings were screened after 10 days to 14 days for the presence of kan^r plants with green leaves and kan^s plants with white cotyledons. Resistant plants were transplanted to pots without kanamycin after 2 weeks to 3 weeks in culture and screened for the presence of aborted seeds after self-pollination.

Molecular Analysis of Transgenic Families

Arabidopsis genomic DNA was prepared from whole plants as described by Richards (1990). The resulting DNA (2 μg to 3 μg) was digested with endonucleases (Bethesda Research Laboratories), electrophoresed in 0.7% agarose in 1 × TAE buffer (Maniatis et al., 1982), and vacuum blotted onto Nytran (Schleicher & Schuell) membranes. Blots were probed with T-DNA right border and pBR322 DNA sequences labeled with nonradioactive digoxigenin using the Genius kit (Boehringer Mannheim). The right border probe was a 1.1-kb HindIII-BamHI fragment from pTiC58 HindIII fragment 23 containing part of the *nos* gene. Hybridization (42°C in 50% formamide, 10% dextran sulfate, 5 × SSC, 1.5%

blocking reagent, 0.1% sarkosyl, 0.02% SDS) and subsequent washes and color detection were performed according to Genius protocols. Fragment sizes were calculated from HindIII-digested λ fragments visualized with digoxigenin-labeled λ probe.

ACKNOWLEDGMENTS

We thank Ralph Quatrano and Pablo Scolnik for valuable discussions and Florence Garlick for assistance in generating and maintaining transgenic families at Du Pont. Research at Oklahoma State University was supported by grants from the National Science Foundation (DCB-8905137) and U.S. Department of Agriculture (88-37261-3708). Jennifer Schnall was supported by U.S. Department of Agriculture Grant 89-372624456 to Ralph Quatrano.

Received November 8, 1990; accepted November 29, 1990.

REFERENCES

Baus, A.D., Franzmann, L., and Meinke, D.W. (1986). Growth in vitro of arrested embryos from lethal mutants of *Arabidopsis thaliana*. Theor. Appl. Genet. **72,** 577–586.

Chang, C., Bowman, J.L., DeJohn, A.W., Lander, E.S., and Meyerowitz, E.M. (1988). Restriction fragment length polymorphism linkage map for *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **85,** 6856–6860.

Feldmann, K.A., and Marks, M.D. (1987). *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: A non-tissue culture approach. Mol. Gen. Genet. **208,** 1–9.

Feldmann, K.A., Marks, M.D., Christianson, M.L., and Quatrano, R.S. (1989). A dwarf mutant of *Arabidopsis* generated by T-DNA insertional mutagenesis. Science **243,** 1351–1354.

Feldmann, K.A., Carlson, T.J., Coomber, S.A., Farrance, C.E., Mandel, M.A., and Wierzbicki, A.M. (1990). T-DNA insertional mutagenesis in *Arabidopsis thaliana*. In Horticultural Biotechnology, A.B. Bennett and S.D. O'Neill, eds (New York: Wiley-Liss), pp. 109–120.

Franzmann, L., Patton, D.A., and Meinke, D.W. (1989). In vitro morphogenesis of arrested embryos from lethal mutants of *Arabidopsis thaliana*. Theor. Appl. Genet. **77,** 609–616.

Heath, J.D., Weldon, R., Monnot, C., and Meinke, D.W. (1986). Analysis of storage proteins in normal and aborted seeds from embryo-lethal mutants of *Arabidopsis thaliana*. Planta **169,** 304–312.

Hehl, R., and Baker, B. (1990). Properties of the maize transposable element *Activator* in transgenic tobacco plants: A versatile inter-species genetic tool. Plant Cell **2,** 709–721.

Herman, P.L., and Marks, M.D. (1989). Trichome development in *Arabidopsis thaliana*. II. Isolation and complementation of the *GLABROUS1* gene. Plant Cell **1,** 1051–1055.

Jones, J.D.G., Gilbert, D.E., Grady, K.L., and Jorgensen, R.A. (1987). T-DNA structure and gene expression in petunia plants transformed by *Agrobacterium* C58 derivatives. Mol. Gen. Genet. **207,** 478–485.

Jones, J.D.G., Carland, F., Lim, E., Ralston, E., and Dooner, H.K. (1990). Preferential transposition of the maize element *Activator* to linked chromosomal locations in tobacco. Plant Cell **2,** 701–707.

Jorgensen, R., Snyder, C., and Jones, J.D.G. (1987). T-DNA is organized predominantly in inverted repeat structures in plants transformed with *Agrobacterium tumefaciens* C58 derivatives. Mol. Gen. Genet. **207,** 471–477.

Koncz, C., Martini, N., Mayerhofer, R., Koncz-Kalman, Z., Korber, H., Redei, G.P., and Schell, J. (1989). High-frequency T-DNA mediated gene tagging in plants. Proc. Natl. Acad. Sci. USA **86,** 8467–8471.

Koncz, C., Mayerhofer, R., Koncz-Kalman, Z., Nawrath, C., Reiss, B., Redei, G.P., and Schell, J. (1990). Isolation of a gene encoding a novel chloroplast protein by T-DNA tagging in *Arabidopsis thaliana*. EMBO J. **9,** 1337–1346.

Koornneef, M. (1981). The complex syndrome of *ttg* mutants. *Arabidopsis* Inf. Serv. **18,** 45–51.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Marks, M.D., and Feldmann, K.A. (1989). Trichome development in *Arabidopsis thaliana*. I. T-DNA tagging of the *GLABROUS1* gene. Plant Cell **1,** 1043–1050.

Marsden, M.P.F., and Meinke, D.W. (1985). Abnormal development of the suspensor in an embryo-lethal mutant of *Arabidopsis thaliana*. Am. J. Bot. **72,** 1801–1812.

Matzke, M.A., and Matzke, A.J.M. (1990). Gene interactions and epigenetic variation in transgenic plants. Dev. Genet. **11,** 214–223.

Meinke, D.W. (1982). Embryo-lethal mutants of *Arabidopsis thaliana*: Evidence for gametophytic expression of the mutant genes. Theor. Appl. Genet. **63,** 381–386.

Meinke, D.W. (1985). Embryo-lethal mutants of *Arabidopsis thaliana*: Analysis of mutants with a wide range of lethal phases. Theor. Appl. Genet. **69,** 543–552.

Meinke, D.W. (1986). Embryo-lethal mutants and the study of plant embryo development. Oxford Surv. Plant Mol. Cell Biol. **3,** 122–165.

Meinke, D.W. (1991). Genetic analysis of plant development. In Plant Physiology: A Treatise, Vol. 10: Plant Growth and Development, F.C. Steward and R.G.S. Bidwell, eds (New York: Academic Press), in press.

Meinke, D.W., and Sussex, I.M. (1979). Isolation and characterization of six embryo-lethal mutants of *Arabidopsis thaliana*. Dev. Biol. **72,** 62–72.

Meyerowitz, E.M. (1987). *Arabidopsis thaliana*. Annu. Rev. Genet. **21,** 93–111.

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

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 493–497.

Nam, H.-G., Giraudat, J., den Boer, B., Moonan, F., Loos, W.D.B., Hauge, B.M., and Goodman, H.M. (1989). Restriction fragment length polymorphism linkage map of *Arabidopsis thaliana*. Plant Cell **1,** 699–705.

Patton, D.A., and Meinke, D.W. (1990). Ultrastructure of arrested embryos from lethal mutants of *Arabidopsis thaliana*. Am. J. Bot. **77,** 653–661.

Patton, D.A., Franzmann, L.H., and Meinke, D.W. (1991). Mapping genes essential for embryo development in *Arabidopsis thaliana*. Mol. Gen. Genet., in press.

Richards, E. (1990). Preparation of genomic DNA from plant tissue. In Current Protocols in Molecular Biology, F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds (New York: Greene Publishing and Wiley-Interscience), pp. 2.3.1–2.3.3.

Rogers, S.G., Horsch, R.B., and Fraley, R.T. (1986). Gene transfer in plants: Production of transformed plants using Ti plasmid vectors. Methods Enzymol. **118,** 627–640.

Schmidt, R., and Willmitzer, L. (1989). The maize autonomous element *Activator* (*Ac*) shows a minimal germinal excision frequency of 0.2%–0.5% in transgenic *Arabidopsis thaliana* plants. Mol. Gen. Genet. **220,** 17–24.

Schneider, T., Dinkins, R., Robinson, K., Shellhammer, J., and Meinke, D.W. (1989). An embryo-lethal mutant of *Arabidopsis thaliana* is a biotin auxotroph. Dev. Biol. **131,** 161–167.

Shellhammer, J., and Meinke, D. (1990). Arrested embryos from the *bio1* auxotroph of *Arabidopsis thaliana* contain reduced levels of biotin. Plant Physiol. **93,** 1162–1167.

Velten, J., and Schell, J. (1985). Selection-expression plasmid vectors for use in genetic transformation of higher plants. Nucl. Acids Res. **13,** 6981–6998.

Voytas, D.F., and Ausubel, F.M. (1988). A copia-like transposable element family in *Arabidopsis thaliana*. Nature **336,** 242–244.

Ward, E.R., and Jen, G.C. (1990). Isolation of single-copy-sequence clones from a yeast artificial chromosome library of randomly sheared *Arabidopsis thaliana* DNA. Plant Mol. Biol. **14,** 561–568.

Weiland, U., and Müller, A.J. (1972). In-vitro-Kultur der Wurzeln von letalen *fusca*-Mutanten von *Arabidopsis thaliana*. Kulturpflanze **20,** 151–164.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A., and Meyerowitz, E.M. (1990). The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. Nature **346,** 35–39.

Yoder, J.I. (1990). Rapid proliferation of the maize transposable element *Activator* in transgenic tomato. Plant Cell **2,** 723–730.