Somatic Embryogenesis in *Arabidopsis thaliana* Is Facilitated by Mutations in Genes Repressing Meristematic Cell Divisions

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Manuscript received January 14, 1998
Accepted for publication March 20, 1998

ABSTRACT

Embryogenesis in plants can commence from cells other than the fertilized egg cell. Embryogenesis initiated from somatic cells *in vitro* is an attractive system for studying early embryonic stages when they are accessible to experimental manipulation. Somatic embryogenesis in Arabidopsis offers the additional advantage that many zygotic embryo mutants can be studied under *in vitro* conditions. Two systems are available. The first employs immature zygotic embryos as starting material, yielding continuously growing embryogenic cultures in liquid medium. This is possible in at least 11 ecotypes. A second, more efficient and reproducible system, employing the *primordia timing* mutant (*pt* allelic to *hpt*, *cop2*, and *amp1*), was established. A significant advantage of the *pt* mutant is that intact seeds, germinated in 2,4-dichlorophenoxyacetic acid (2,4-D) containing liquid medium, give rise to stable embryonic cell cultures, circumventing tedious hand dissection of immature zygotic embryos. *pt* zygotic embryos are first distinguishable from wild type at early heart stage by a broader embryonic shoot apical meristem (SAM). In culture, embryogenic clusters originate from the enlarged SAMs. *pt* somatic embryos had all characteristic embryo pattern elements seen in zygotic embryos, but with higher and more variable numbers of cells. Embryogenic cell cultures were also established from seedling, of other mutants with enlarged SAMs, such as *clavata* (*clv*). *pt clv* double mutants showed additive effects on SAM size and an even higher frequency of seedlings producing embryogenic cell lines. *pt clv* double mutant plants had very short fasciated inflorescence stems and additive effects on the number of rosette leaves. This suggests that the *PT* and *CLV* genes act in independent pathways that control SAM size. An increased population of noncommitted SAM cells may be responsible for facilitated establishment of somatic embryogenesis in Arabidopsis.

As in other higher eukaryotic organisms, plant embryogenesis commences with the zygote, the fusion product of the haploid egg cell and the sperm cell. In dicots, embryos pass through a stereotyped sequence of characteristic stages. These are, first, the globular stage, in which the embryo is spherical and attached via the suspensor to the surrounding maternal tissue. Upon formation of the cotyledons, the spherical shape changes into a heart shape when viewed from the side. Longitudinal expansion of the cotyledons and the hypocotyl then gives the embryo its torpedo shape. During embryogenesis all body pattern elements are established, including the shoot apical meristem (SAM) and the root meristem (RM). Almost the entire plant is continuously produced postembryonically from these apical meristems, ensuring that the pattern established in the embryo is propagated and elaborated further during plant development (Jürgens *et al.* 1994; Jürgens 1995; Scheres *et al.* 1996). In plants, the entire process of embryogenesis can be recapitulated from cells other than the zygote. The origin of these asexual embryos is diverse: apomictic embryos derive from unfertilized eggs or even from surrounding maternal tissue, androgenetic embryos develop from *in vitro* cultured immature pollen grains (microspores), and somatic embryos originate from somatic cells grown *in vitro* (reviewed by Mordhorst *et al.* 1997). Somatic embryos pass through the same sequence of stages of embryogenesis as their zygotic counterparts. Morphological, cytological, and molecular similarities between zygotic and somatic embryos are abundant (reviewed by Zimmerman 1993; Merkle *et al.* 1995; Yeung 1995; Mordhorst *et al.* 1997), suggesting that the same molecular mechanisms are employed in both forms of embryogenesis. Somatic embryogenesis in Arabidopsis is an important tool for the analysis of embryos developing outside the maternal environment, especially because of the availability of many zygotic embryo mutants in this species. The *in vitro* system offers, for instance, the possibility to manipulate the environment of the developing embryo by

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adding specific signaling molecules and to analyze the effect in wild-type and mutant somatic embryos.

Somatic embryos or embryo-like structures have been obtained in Arabidopsis from callus cultures originated from seeds or roots (Huang and Yeoman 1984; Marton and Browse 1991; Huang and Yeoman 1995). Ford (1990) and Mathur *et al.* (1995) described the development of embryo-like structures from cell colonies obtained from cell culture-derived protoplasts. The direct development of somatic embryos from leaf protoplasts was reported by O'Neill and Mathias (1993). Initial cell divisions in leaf protoplast-derived cells that were very similar to early cell division patterns of zygotic embryos were reported (Luo and Koop 1997). Unfortunately, callus formation occurred next, followed by root and shoot regeneration, rather than continued embryogenesis (Luo and Koop 1997). Immature zygotic embryos, known to have a high regeneration capability (Patton and Meinke 1988; Sangwan *et al.* 1992), were used to induced somatic embryogenesis in Arabidopsis (Wu *et al.* 1992; Pillon *et al.* 1996; Luo and Koop 1997). Wu *et al.* (1992) and Luo and Koop (1997) obtained somatic embryos in a single step procedure after culturing dissected immature zygotic embryos in liquid 2,4-dichlorophenoxyacetic acid (2,4-D) containing medium. From such primary somatic embryos, continuous initiation of new somatic embryos after monthly subculture on solidified 2,4-D containing medium was reported by Pillon *et al.* (1996). Ecotypes Landsberg *erecta*, Nd-0, C24, and Nossen (Wu *et al.* 1992; Luo and Koop 1997) and Columbia (Pillon *et al.* 1996; Luo and Koop 1997) could be used successfully. Here, we report that immature zygotic embryos of all these plus six additional ecotypes can be used for continuous initiation of somatic embryogenesis in liquid medium. In addition we have used available mutants with high regeneration capability. One of these is *altered meristem program1* (*amp1*), for which a 10-fold higher frequency of shoot regeneration on root explants was described (Chaudhury *et al.* 1993). *amp1* belongs to a complementation group of which other alleles have been named *häuptling* (*hpt*) (Jürgens *et al.* 1991; Ploense 1995), *constitutive photomorphogenic2* (*cop2*) (Hou *et al.* 1993), and *primordia timing* (*pt*) (Vizir *et al.* 1995). Allelism tests for these mutants have been reported by Telfer *et al.* (1997) and Conway and Poethig (1997) and in our laboratory for *hpt* and *pt* (A. Mordhorst and S. de Vries, unpublished results). Using the *pt* allele we were able to establish a system for continuous induction of somatic embryogenesis directly from seedlings germinated in liquid 2,4-D containing medium. This system turned out to be more efficient compared with immature zygotic embryo-derived cultures. The initial embryogenic clusters all derived from the SAM of the seedlings. Based on one of the characteristics of the *pt*, *clv*, and *pt clv* seedling phenotypes, a much larger SAM than wild type (WT), we propose that a larger than normal number of dividing and noncommitted meristematic cells in the SAM facilitates the establishment of somatic embryogenesis in Arabidopsis.

MATERIALS AND METHODS

Plant material: Seeds of the *primordia timing* (*pt*), the *häuptling* (*hpt*), and the *constitutive photomorphogenic2* (*cop2*) of the *hpt/cop2/amp1/pt* complementation group were kindly provided by I. Vizir (University of Nottingham, UK) (Vizir *et al.* 1995), by U. Mayer (University of Tübingen, Germany) (Jürgens *et al.* 1991), and by V. Raz (University of Wageningen, The Netherlands) (Hou *et al.* 1993), respectively. Seeds of *clavata1* (*clvl-4*) and *clavata3* (*clv3-2*) mutants were kindly provided by S. Clark (University of Michigan, Ann Arbor, MI). The *clv* alleles used represent the strongest mutant allele of the respective locus (Clark *et al.* 1993, 1995). All mutations behaved in crosses as single monogenic recessive mutations. All mutants were in the Landsberg *erecta* background, except *cop2*, which was in the Columbia background. Seeds of the ecotypes Estland (Est), Enkheim (En-T), Dijon G (Di-G), Weinigen (Wei-0), and Cvi (Cvi-0) were kindly provided from the Nottingham Arabidopsis stock center. Seeds of the ecotypes Nossen (No-0) and Coïmbra (Coï) were kindly provided from the Arabidopsis Biological Research Center, Ohio.

For the analysis of *pt clv1-4* and *pt clv3-2* double mutants, plants were grown in a growth chamber with Philips TLD50W/84HF illumination and 22° in a 16/8 dark/light period.

***In vitro* culture:** For the initiation of cell lines from immature embryos, siliques containing embryos at the early bent-cotyledon stage (before desiccation started) were surface sterilized for 10 sec in 70% ethanol followed by a 10-min incubation in commercial bleach (final concentration 4% sodium hypochlorite, containing 0.3% Tween 20) and washed three times with sterile water. Immature zygotic embryos were isolated under the dissecting microscope. Twenty embryos were incubated in 20 ml autoclaved B5-4 medium (Gamborg *et al.* 1968) containing 2% w/v sucrose, 4.5 μm 2,4-D at pH 5.8. Cultures were kept on a rotary shaker (100 rpm) at 25° in the light (~3000 lux for 16 hr, 8 hr dark). Embryos unfolded, and each embryo developed into an individual callus aggregate.

For the initiation of cell lines from seedlings, seeds were surface sterilized for 10 sec in 70% ethanol followed by a 10-min incubation in commercial bleach (final concentration 2% sodium hypochlorite, containing 0.3% Tween 20), washed four times with sterile water, dried on filter paper, and stored at room temperature before use. Approximately 30 seeds were incubated in 20 ml liquid medium in 190-ml Greiner plastic containers (Alphen a/d Rijn, The Netherlands). For initiation of cultures, autoclaved MS-medium (Murashige and Skoog 1962) (Duchefa, Haarlem, The Netherlands) containing 2% w/v sucrose, 4.5 μm 2,4-D, and 10 mm 2-(*N*-morpholino)-ethanesulonic acid (MES) at pH 5.8 was used (MS-4). Zeatin used in one series of experiments was filter-sterilized and added to autoclaved medium. After a cold pretreatment of 2 days at 6–8°, cultures were kept in the same conditions as mentioned above. Seeds germinated, and each seedling developed a callus aggregate.

After 2 wk the medium was replaced for both types of culture by 20 ml fresh B5-4 or MS-4 medium, respectively. After 3 wk the number of immature zygotic embryos or seedlings that developed embryogenic green clusters with a smooth surface in addition to yellowish nonembryogenic callus was determined. Embryogenic cell lines of both culture types were established by subculturing only the green, embryogenic clusters originally initiated from one to three immature zygotic em-

bryos or seedlings in 20 ml medium. Nonembryogenic lines were obtained by subculturing the yellowish callus in a similar way. If the embryogenic clusters increased in size above 0.5 cm, they were gently broken in smaller pieces with the tip of the medium pipette. Embryogenic lines were maintained by a weekly subculture of ~30–50 mg (*pt* cultures) or ~50–100 mg (immature zygotic embryo cultures) embryogenic green clusters in 20 ml B5-4 medium. Upon weekly transfer of the cultures the ratio of green embryogenic to yellowish nonembryogenic aggregates was determined.

Culture in light with the resulting green aggregates was a condition not required to induce somatic embryogenesis, because initiation, maintenance, and development of somatic embryos were all equally efficient in the dark. However, the dark-grown cultures were completely yellowish, making selective subculture of embryogenic clusters more difficult. After transfer to light, dark-grown cultures turned green and were indistinguishable from light-grown control cultures (data not shown). Considerable effort was made to produce fine cell suspensions without losing the embryogenic capacity. Neither changing the subculture procedure (time of subculture interval, cell density, chopping, or sieving of aggregates) nor using different growth regulator combinations and concentrations improved the appearance of the culture (data not shown).

For the development of somatic embryos, parts of aggregates from liquid cultures (~1.5 × 1.5 mm in size) were transferred to agar (0.8% w/v)-solidified grown regulator free 1/2 MS medium containing 1% w/v sucrose. After 7–10 days, somatic embryos had developed. The number of embryos that developed were counted only on clusters of 5 mm diameter to allow comparison between different cultures. Single embryos were dissected and transferred to new, vertically oriented 1/2 MS plates. Germinated embryos were transferred to the greenhouse if the roots were longer than 4 cm.

Histology: For preparation of semithin sections, tissue of *in vitro* cultures was fixed in 0.1 m phosphate buffer pH 7.2 with 4% glutaraldehyde and dehydrated in serial steps with ethanol. Siliques at various developmental stages or dissected developing seeds were fixed in 0.1 m phosphate buffer pH 7.2, 4% glutaraldehyde and 4% dimethylsulfoxide (DMSO) and dehydrated in serial steps with acetone. All samples were embedded in technovit 7100 (Kulzer, Wehrheim/TS, Germany) as recommended by the manufacturer. Sections of 5–7 μm were stained with toluidine blue.

The SAM of mature embryos was analyzed in whole mount preparations. Seeds were imbibed overnight in 15% ethanol, and the seed coat was removed under the dissecting microscope. If mature embryos had three cotyledons, one of them was gently removed with a small scalpel to allow a better view of the SAM. Embryos were dehydrated in serial steps of ethanol and then transferred via serial steps to xylene where tissue clearing took place overnight in 100% xylene. Embryos were mounted in glycerol and squeezed gently between slide and cover slip to spread the cotyledons, and the SAM was viewed under Normarski optics.

RESULTS

Establishment of embryogenic cell lines from immature zygotic embryos: Early bent cotyledon stage embryos were incubated as intact embryos in B5-4 medium. Zygotic embryos first unfolded their cotyledons (Figure 1A). On a variable percentage of the zygotic embryos, bright green callus with a smooth surface developed after 9 days from the cotyledons or the SAM region (outlined arrows in Figure 1B). This type of callus appeared organized (with distinct compact subunits on the surface) and is similar to the preglobular embryos described by Luo and Koop (1997). It will be referred to here as green embryogenic cluster. After 3 wk callus clumps of 0.5 to 0.7 cm diameter were developed from each individual zygotic embryo (Figure 1C). All ecotypes tested (L*er*, RLD, Col, En-T, Est, No-0, Wei-0, Di-G, Cvi-0, Coï, and C24) responded similarly, but with variable efficiency. In L*er*, 100% of immature zygotic embryos developed embryogenic clusters, and in others up to 75% of the immature embryos produced unorganized yellowish callus with a rough surface or did not respond at all. Selective subculture of only the embryogenic clusters gave rise immediately to embryogenic cell lines. Immature embryos producing embryogenic clusters also produced nonembryogenic callus; therefore, corresponding nonembryogenic lines could be established simultaneously from the same explanted embryo. Embryogenic lines of all ecotypes consisted of a mixture of green clusters with a diameter between 5 and 15 mm (outlined arrow in Figure 1D) and of nonembryogenic yellowish aggregates with a diameter between 2 and 10 mm (white arrow in Figure 1D). Cultured under the same conditions, the ratio of green embryogenic clusters to yellowish nonembryogenic aggregates varied between 1:1 and 1:9. The variation depended in part on the ecotype but was also present between different lines of the same ecotype. In general, the embryogenic capacity dropped with prolonged time of subculture and became zero after 6 to 12 mo.

Somatic embryos developed upon transfer to agar-solidified media without growth regulators (outlined arrows in Figure 1E). The morphology of these somatic embryos was highly variable. After 7–10 days, 2.3 ± 2.8 ($n = 13$) somatic embryos had developed on a L*er* aggregate of 0.5 cm diameter. Single embryos could be separated and germinated, demonstrating that both apical meristems are present and functional. Fertile plants developed after transfer to the greenhouse (Figure 1F). Regenerated (R_1) plants were more bushy than seed-grown plants (Figure 1F), but this was not seen any more in the next (R_2) generation (data not shown).

Characterization of *pt*, *clv* and *pt clv* mutants: The *pt* allele in the L*er* background displays many pleiotropic features similar to the *amp1* allele in the Columbia background. This includes the presence of three cotyledons (data not shown) instead of two in ~30% of the seedlings (polycotyly), more but smaller rosette leaves (Table 1), and an increased number of side shoots, which leads to a bushy appearance due to the reduced apical dominance of the main shoot (Figure 2A). To help interpret the "enhanced somatic embryogenesis" properties of the *pt* and *clv* mutants and their double mutants, it is useful to describe them at embryo and plant level. In the F_2 of crosses between *pt* and *clv1-4* and *clv3-2*, both double mutants segregated with a similar phenotype (see below) that was clearly distinguishable from

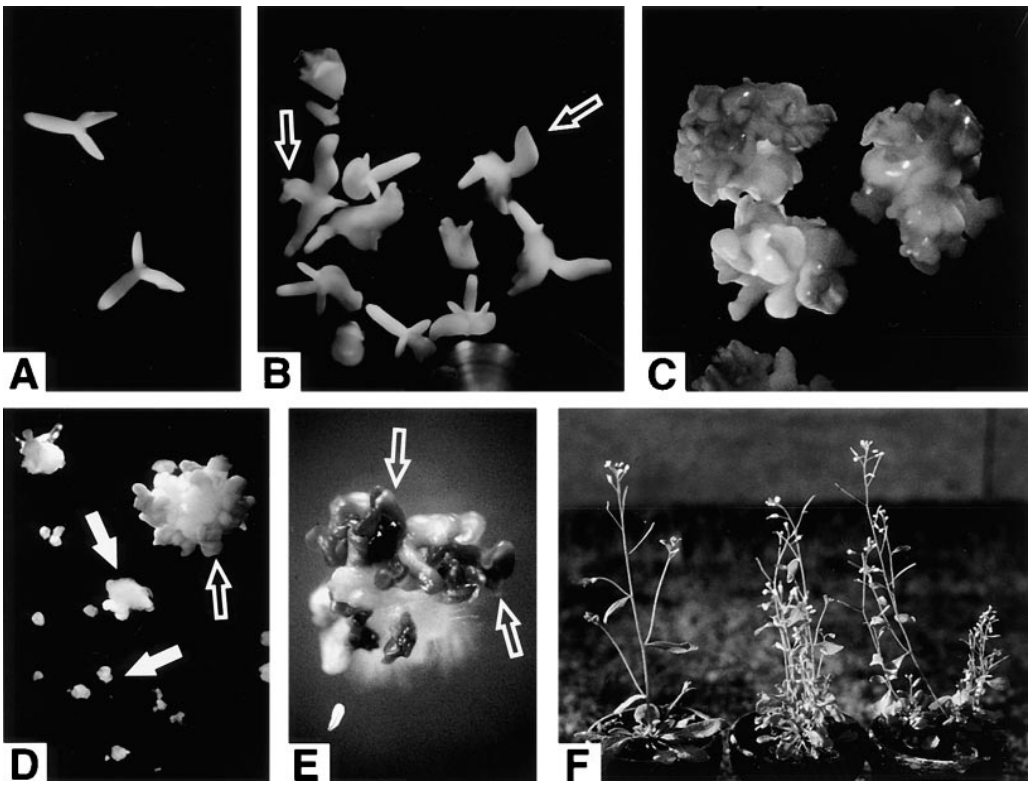

Figure 1.—Culture initiation of embryogenic cultures from immature zygotic embryos and plant regeneration. (A) 4 days; (B) 9 days; (C) 3 wk after culture initiation; (D) embryogenic suspension; (E) development of somatic embryos on growth regulator free medium; (F) left, seed-grown Col plant; middle, No-0; and right, En-T plant, both somatic embryo derived.

each parental phenotype (Figure 2, B and C). Like the *pt* single mutant, ~30% of the seedlings have three cotyledons. Individuals with four or partly fused cotyledons were occasionally observed. Both *clv* single mutants had a slightly higher number of rosette levels as compared to wild type (Table 1). The number of rosette leaves in *pt* single and *pt clv* double mutants was very much increased over the wild-type level (Table 1). The very high number of rosette leaves in both double mutants therefore seemed an additive effect of the respective single mutants. The rosette leaf blades of *pt* and *clv* single mutants were about three quarters, and those of *pt clv* double mutants about half, the size of L*er* (Table 1). Remarkable was the reduced inflorescence stem elongation in both double mutants, reaching only 3.1 cm (*pt clv1-4*) or 2.5 cm (*pt clv3-2*) (Table 1). For both *clv* single alleles, fasciation (abnormal development of the meristem as a band-like structure) of inflorescence stems was described (Clark *et al.* 1993, 1995). Under our conditions 63% of *clv3-2* inflorescence stems fasciated only in the upper region, whereas 89 and 100% of the respective double mutants showed fasciation over the whole inflorescence stems (Table 1). Extreme forms of fasciation such as twisted, hooked, split, and even completely burst stems were seen in the double mutants (data not shown). In contrast to the regular numbers in *pt* and L*er*, the number of organs per flower in all four whorls was higher and more variable in the *pt clv*

TABLE 1

Leaf sizes, inflorescence stem elongation, and percentage of fasciated inflorescence stems of wild type (L*er*), *pt*, *clv1-4*, *clv3-2*, and the respective double mutants

Genotype	Number of rosette leaves	Length (mm)	Width (mm)	*n*	Inflorescence stem elongation (cm)	*n*	Fasciated inflorescence stems (%)	*n*
WT (L*er*)	6.2 ± 0.6	20 ± 5	12 ± 3	29	20.8 ± 3.9	28	0	40
pt	15.7 ± 3.5	14 ± 3	10 ± 3	21	15.9 ± 1.4	27	0	40
clv1-4	9.3 ± 1.4	16 ± 4	9 ± 2	33	21.6 ± 4.8	31	0	40
clv3-2	8.2 ± 1.4	16 ± 6	9 ± 3	31	22.4 ± 4.4	30	63	30
pt clv1-4	21.8 ± 6.6	10 ± 3	7 ± 2	20	3.1 ± 2.2	27	89	44
pt clv3-2	23.0 ± 7.0	10 ± 3	7 ± 2	16	2.5 ± 2.5	25	100	38

The leaf blades of the last or biggest rosette leaves were measured.
Values are ±SE.

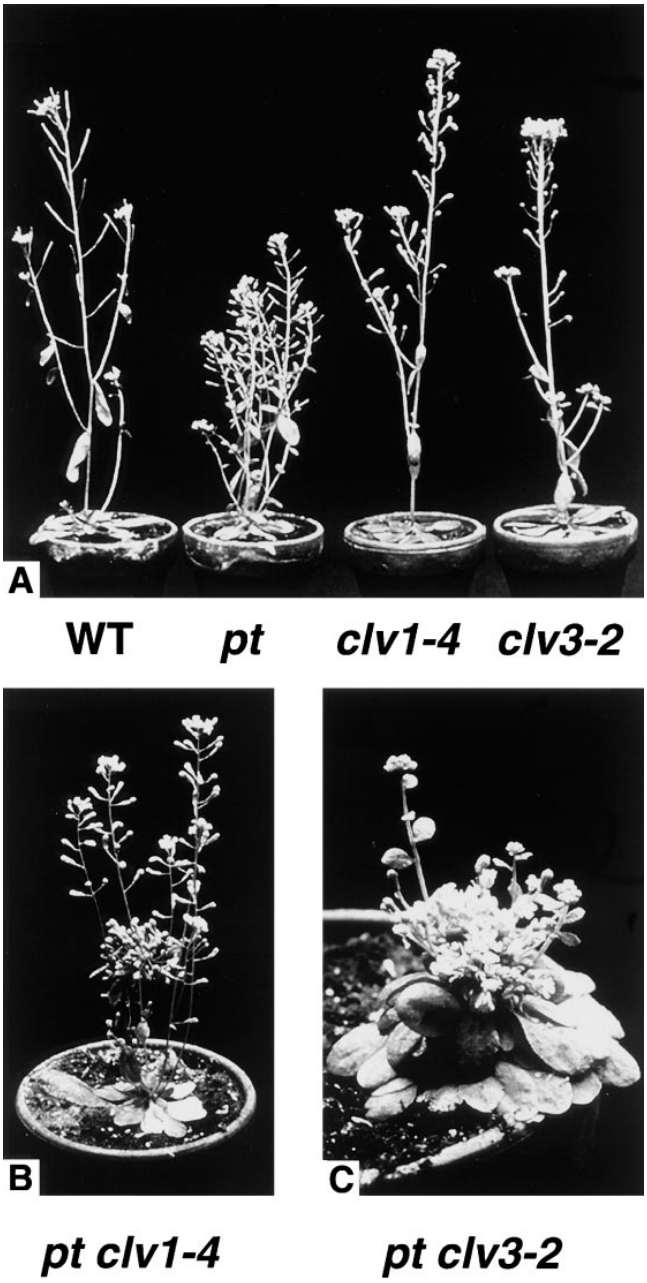

Figure 2.—Phenotypes of (A) wild type (WT) (L*er*), *pt*, *clv1-4*, and *clv3-2* plants; (B) *pt clv1-4* plant; (C) *pt clv3-2* plant.

double mutants similar to the *clavata* single mutants (Table 2). The siliques of both double mutants were shorter than the single mutants, which themselves were shorter than wild type (L*er*) (Table 2). The double mutant siliques had a typical cone shape (data not shown). characteristic of the *clavata* mutants caused by additional internal whorls of carpels (Clark *et al.* 1993, 1995). Siliques of double mutants only contained a few seeds, but those seeds were completely viable.

The first visible effect of the *pt* mutation in embryogenesis is an enlarged late globular stage due to extra cell divisions (compare Figure 3, B and H). In the heart stage, these extra cell divisions are confined to the future SAM region and result in an increased number of cells between the cotyledon primordia (compare Figure 3, C and D and Figure 3, I and J). Later stages show an enlarged embryonic SAM (compare Figure 3, E and F and Figure 3, K and L). The SAM of the mature *pt* embryo (Figure 4D) is dome-shaped when compared to the flattened apices of wild-type (Figure 4A) and *clavata* mutants (Figure 4, B and C). The SAM of both double mutants was almost half spherical (Figure 4, E and F). Counting the number of cells in the L1 layer of the SAMs confirmed the substantial increase of the presumed SAM volume especially in the *pt* and even more in *pt clv* mutants (Table 3). These data suggest that the effects on increased SAM size in each single mutant are additive in the *pt clv1-4* and *pt clv3-2* double mutants.

Taken together, the morphological phenotypes described for the single *pt* and *clv* mutants appear to be either additive or clearly present in the double mutants, suggesting that both genes are not likely to be genetically interacting.

Establishment of embryogenic cell lines from *pt*, *clv*, and *pt clv* seedlings: Seed germination in liquid medium in the presence of 2,4-D led to stunted seedlings without root growth and hypocotyl elongation for both *pt* (Figure 5A) and L*er* (Figure 5E). Under these culture conditions, a yellowish, rough, and unorganized callus aggregate developed from the leaf veins of the entire cotyledons of a L*er* seedling (Figure 5, F–H). In addition to this type of callus, using *pt* (Figure 5A to D), *pt clv1-4* (Figure 5K), and *pt clv3-2* (Figure 5L) seedlings developed another type of SAM-derived callus, characterized by a smooth surface and a bright-green color. These embryogenic clusters resembled the ones found when using immature zygotic embryos, but they contained more and smaller compact subunits on the surface. In the cases of *clv1-4* (Figure 5I) and *clv3-2* (Figure 5J), the amount of embryogenic clusters produced is rather low. The percentage of seedlings producing embryogenic clusters was correlated with the increased size of the SAM of the original seedling (Table 3). Subculture of only the embryogenic clusters immediately gave rise to embryogenic cell lines containing green aggregates with a diameter between 3 mm and 1.5 cm. In the *pt*, *pt clv1-4*, and *pt clv3-2* lines, the ratio of green embryogenic aggregates to yellowish nonembryogenic aggregates varied from 4:1 to 19:1 between individual cell lines. In *clv1-4* and *clv3-2* lines that had a phenotype similar to *pt* but with a lower embryogenic capacity, the ratio of green embryogenic aggregates to yellowish nonembryogenic aggregates varied from 1:1 to 1:9 between individual cell lines. With the *pt* alleles *hpt* and *cop2*, similar embryogenic cultures could be established (data not shown).

The embryogenic capacity of *pt* lines remained stable for over 2 years. New embryogenic clusters were continuously formed on the surface of existing embryogenic

TABLE 2

Number of organs per flower and length of mature siliques of wild type (L*er*), *pt*, *clv1-4*, *clv3-2* and the respective double mutants

Genotype	Number of organs per flower				Silique length (cm)	*n*
	Sepals	Petals	Stamen	Carpels		
WT (L*er*)	4.0 ± 0.0	4.0 ± 0.0	6.0 ± 0.1	2.0 ± 0.0	1.1 ± 0.2	100
pt	4.0 ± 0.0	4.0 ± 0.0	6.0 ± 0.4	2.0 ± 0.0	0.4 ± 0.1	100
clv1-4	4.9 ± 0.6	5.0 ± 0.6	7.5 ± 1.2	5.1 ± 0.7	0.5 ± 0.2	100
clv3-2	4.8 ± 0.5	4.6 ± 0.6	7.7 ± 1.0	5.9 ± 1.1	0.4 ± 0.1	100
pt clv1-4	4.2 ± 0.4	4.2 ± 0.2	6.5 ± 1.0	4.7 ± 0.7	0.2 ± 0.1	91
pt clv3-2	4.4 ± 0.4	4.5 ± 0.5	6.6 ± 1.0	5.1 ± 0.7	0.3 ± 0.1	92

Only the first 10 flowers on any given plant were analyzed according to Clark *et al.* (1993).
Values are ± SE.

clusters leading to large aggregates (Figure 6A) that occasionally break apart. The center of such aggregates became yellowish and was no longer embryogenic. After transfer to agar-solidified media lacking growth regulators, mature somatic embryos developed. The morphology of somatic embryos was highly variable. After 10 days, 17.5 ± 10.1 (n = 13) somatic embryos had developed on an aggregate of 0.5 cm diameter, which is approximately eight times higher than using immature zygotic embryos of L*er* (see above) and in an order similar to that of cultures of immature *pt* zygotic embryos (data not shown). Single somatic embryos developed with fused or separated cotyledon(s) (Figure 6, B and C). In most cases two to six somatic embryos were fused to each other. Examples with a single common root (Figure 6H), fused roots and hypocotyls (white arrows in Figure 6E), or common cotyledons (outlined arrows in Figure 6E) were found. In general, embryos were oriented with the basal (root) pole attached to the callus tissue (Figure 6, B, C, and E). Up to 25% of the embryos were attached with the apical (shoot) pole to the callus (Figure 6D). Single embryos of different developmental

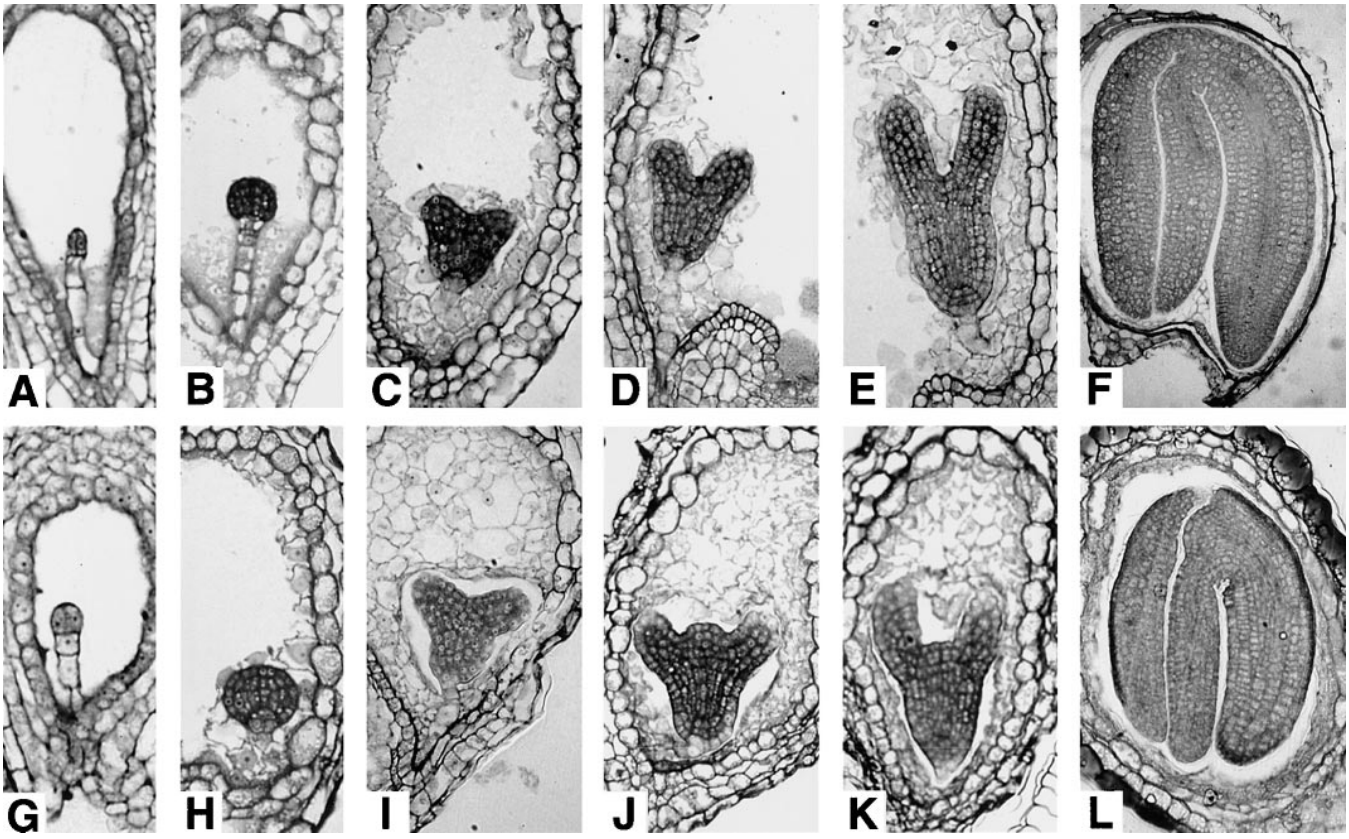

Figure 3.—Semithin tissue sections of different stages of zygotic embryo development. (A)–(F) wild type (L*er*) and (G)–(L) *pt*.

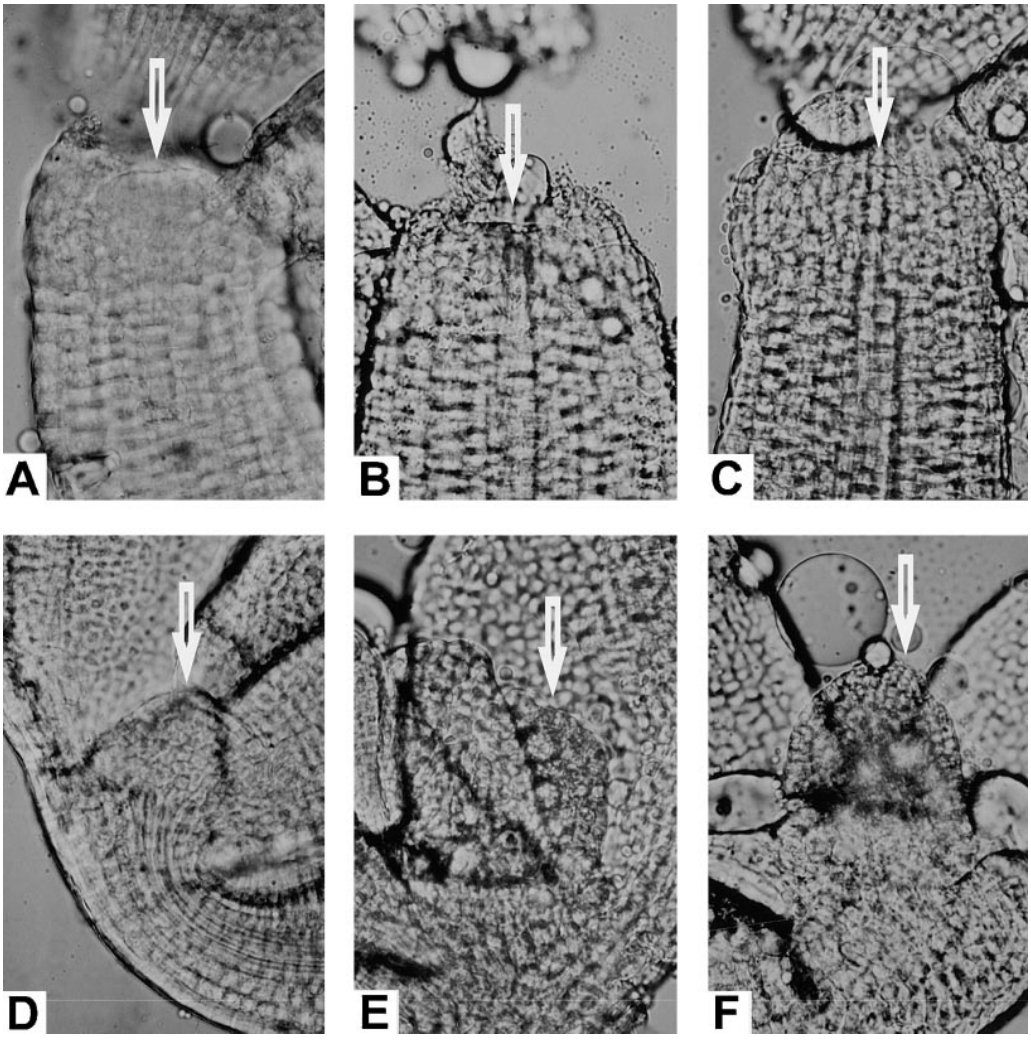

Figure 4.—Whole mount preparations of shoot apices of mature embryos of (A) wild type (L*er*), (B) *clv1-4*, (C) *clv3-2*, (D) *pt*, (E) *pt clv1-4*, (F) *pt clv3-2* plants. White arrows point to the SAM.

TABLE 3

Number of cells in the L1 layer of SAMs of mature embryos in the central optical section and percentage of seedlings developing embryogenic clusters out of the SAM upon germination in liquid 2,4-D containing media of wild type, *pt, clv1-4, clv3-2*, and the respective double mutants

Genotype	Number of cells in L1	*n*	Percentage of seedlings developing EC[a,b]
WT (L*er*)	8.2 ± 0.7	6	0
pt	17.8 ± 2.3	8	30
clv1-4	11.9 ± 1.8	7	16
clv3-2	10.3 ± 1.2	6	4
pt clv1-4	21.0 ± 3.2	8	41
pt clv3-2	25.5 ± 4.0	11	42

Values are ±SE.
[a] EC, embryogenic clusters.
[b] Mean of two independent experiments each with two replicates and ~30 seedlings per replicate.

stages were detached from the callus tissue and compared to the corresponding stages of zygotic *pt* embryos. As seen in other species, Arabidopsis somatic embryos were larger than their zygotic counterparts (Figure 7). It is of interest to see that polycotyly, part of the *pt* phenotype, is reproduced in somatic embryos (Figures 6C and 7). To promote germination, somatic embryos were separated from each other and cultured as a single embryo or in groups of two to four. The hypocotyl/root junction became clearly visible after development of the primary root (Figure 6, D and F). Similar to seed-derived seedlings, primary leaves of somatic embryos can develop in whorls of three (Figure 6G). However, in most cases a single first leaf with a high number of trichomes is produced (Figure 6H), which is never observed in seed-grown seedlings. No somatic embryos develop after transfer of nonembryogenic aggregates to growth regulator free medium (Figure 6J). Somatic embryo-derived seedlings were transferred into the greenhouse and developed into fertile plants (Figure 6I, left plant) even shorter and more bushy than *pt* seed-grown plants (Figure 6I, right plant). The *pt* R_2 generation was indistinguishable from the *pt* plants only propagated through

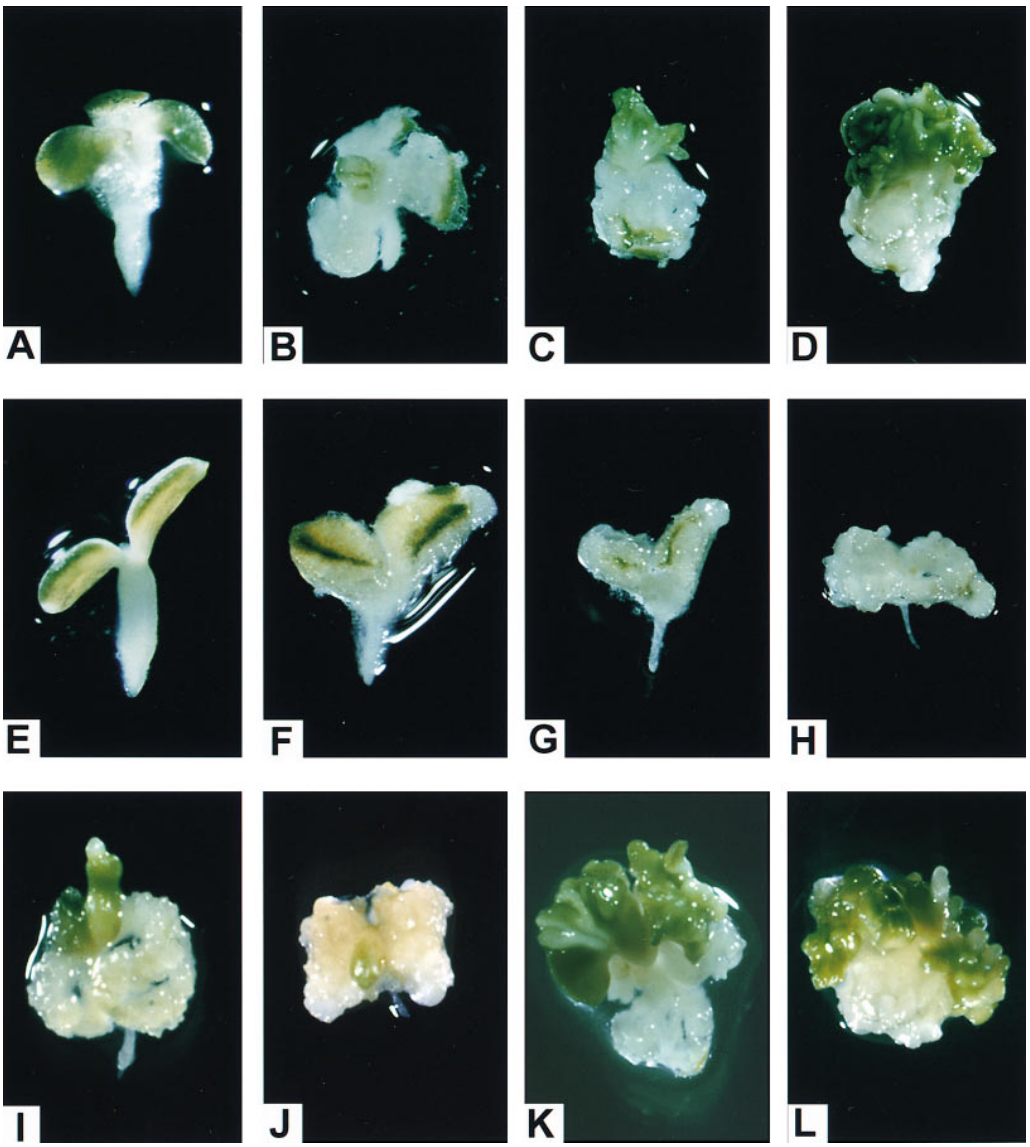

Figure 5.—Culture initiation from seedlings germinated in 2,4-D containing liquid media. (A)–(D) *pt*; (E)–(H) wild type (L*er*); (I) *clv1-4*; (J) *clv3-2*; (K) *pt clv1-4*; (L) *pt clv3-2*. (A), (E) 4 days; (B), (F) 8 days; (C), (G), 12 days; (D), (H), (I)–(L) 21 days after germination.

seeds. Cultures of *clv1-4*, *clv3-2*, *pt clv1-4*, and *pt clv3-2* developed mature somatic embryos exactly as described for the *pt* cultures and were embryogenic for over 6 months (data not shown).

Cytological analysis of *pt* somatic embryos: Because the somatic embryos display differences in morphology from zygotic embryos, it was essential to confirm their internal organization. To qualify as a true somatic embryo, all apical-basal and radial pattern elements must be demonstrated in the same structure. Longitudinal sections through *pt* somatic embryos showed that the provascular strands connected root and shoot apical meristems and branched into the cotyledons (Figure 8A). The root morphology was clearly visible in roots attached to tissue (Figure 8B) and in roots protruding outward (Figure 8C). SAM cells in embryos attached with their basal end (Figure 8A) or their apical end (Figure 8D) showed the characteristic intense staining. The internal structure as seen in sections confirmed the presence of both apical meristems, as predicted by the fact that the somatic embryos germinated. Sections through fused structures showed that they indeed consist of fused somatic embryos (Figure 8E). In rare cases longitudinal sections showed that whereas the apical part resembles a true somatic embryo, no root meristem was present and the provascular strands continued into the callus tissue (Figure 8F), reflecting organogenesis rather than somatic embryogenesis.

Hypocotyls of both wild-type (L*er*) and *pt* zygotic embryos are characterized by an invariant number of three continuous cell layers of the ground tissue (two cortex and one endodermis layer) and eight cell files in the provascular tissue (Figure 9, A and B). In somatic *pt* embryos, 4.4 ± 1.0 ($n = 13$) layers of ground tissue and 12.0 ± 5.6 ($n = 13$) cell files in the provascular tissue were found (see, *e.g.*, Figure 9C). The morphological difference between cortex and endodermis cell files was also less pronounced in somatic than in zygotic embryos

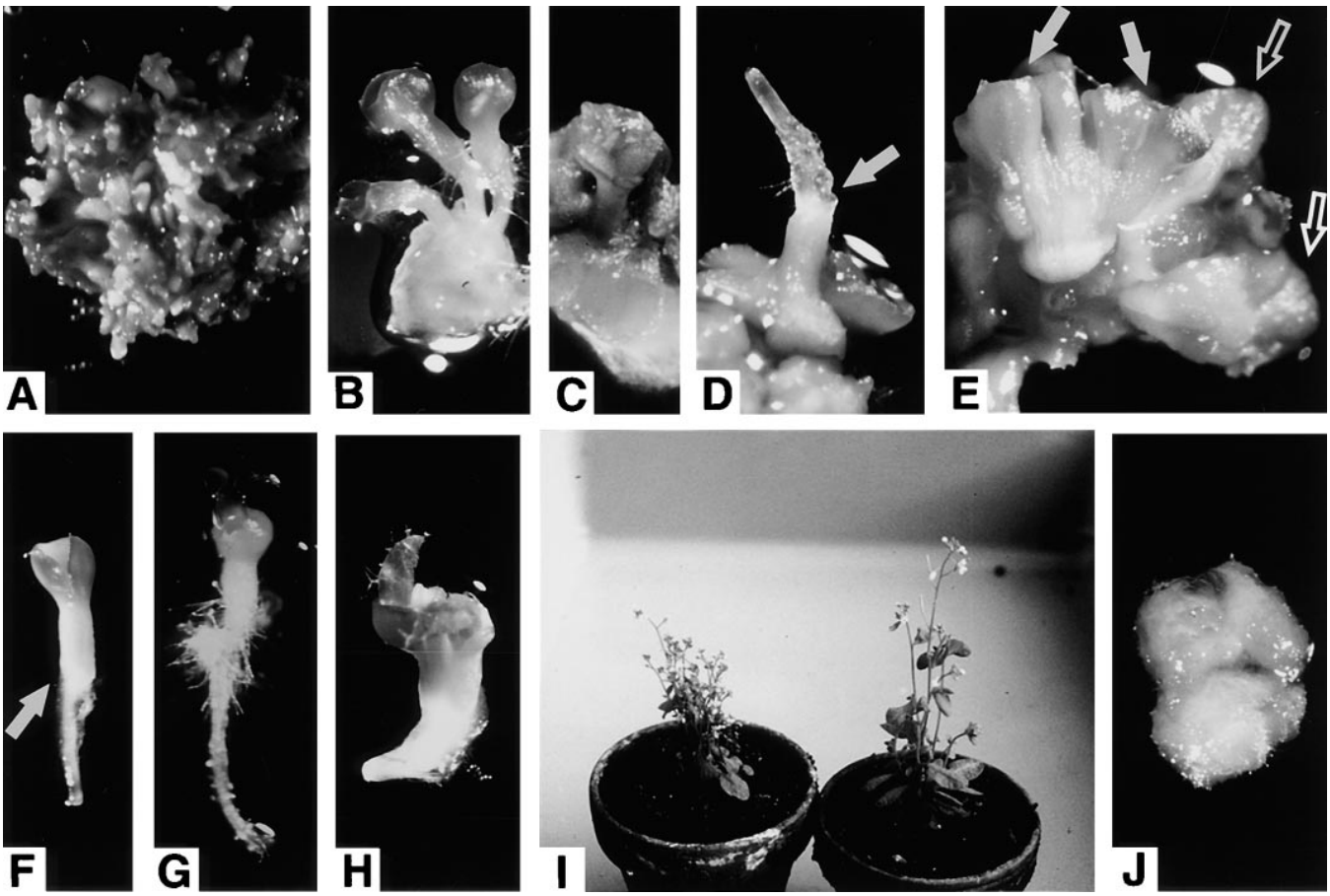

Figure 6.—Development of *pt* somatic embryos. (A) unbroken embryogenic aggregate from 2,4-D containing medium, (B)–(H) and (J) development on growth regulator free medium, (B) somatic embryos with fused cotyledon(s), (C) somatic embryo with three separate cotyledons, (D) somatic embryo with inverse orientation (the arrow points to the root/hypocotyl boundary), (E) fused and fasciated somatic embryos (the white arrows point to embryos with fused root/hypocotyls, the outlined arrows to embryos fused with the cotyledons), (F), (G) germinating separated single somatic embryos (the arrow in (F) points to the root/hypocotyl boundary), (H) germinating fused embryo (one common root and hypocotyl, two separated SAMs), (I) somatic embryo-derived (left) and seed-derived (right) *pt* plants in the greenhouse, (J) on nonembryogenic aggregate, no somatic embryos develop.

(compare white arrows in Figure 9, C and D). In zygotic *pt* embryos developing on plants derived from somatic embryos, the numbers of cortex/endodermis cell layers and vascular cell files were identical to seed-derived plants again (Figure 9D). The primary root of L*er* contains one layer of cortex and one layer of endodermis, each consisting of eight cell files per layer (Dolan *et al.* 1993). Primary roots of *pt* seed-grown seedlings had 10.4 ± 1.5 (n = 8) cortex and 9.4 ± 1.3 (n = 8) endodermis cell files per layer. Primary roots of somatic *pt* embryos contained additional layers, and the number of cells per layer was increased up to 26 and was highly variable. Primary roots of seedlings germinated from seeds of somatic embryo-derived plants displayed the original organization of *pt* primary roots again (10.2 ± 1.4 cortex and 9.9 ± 1.3 endodermis cell files). These findings demonstrate that the observed increased variability in hypocotyls and roots of *pt* somatic embryos is due to the tissue culture conditions and is not heritable.

Exogenous cytokinin cannot phenocopy the enhanced somatic embryogenesis trait of *pt*: The pleiotropic phenotype of the *amp1* allele was attributed to an elevated endogenous cytokinin content (Chaudhury *et al.* 1993) and the de-etiolated phenotype of dark-grown *amp1* seedlings (short hypocotyl, open cotyledons) could be phenocopied in wild type with additional zeatin (Chin-Atkins *et al.* 1996). Therefore, we tested whether exogenous zeatin could phenocopy the enhanced somatic embryogenesis trait of *pt* in wild-type (L*er*) seedlings. None of the zeatin concentrations tested led to formation of embryogenic clusters in L*er*, and the percentage of *pt* seedlings developing embryogenic clusters dropped dramatically with increased zeatin concentration (Figure 10). Both wild type and *pt* reacted to increasing zeatin concentrations with an increased amount of light-green nonembryogenic callus produced from the cotyledons (data not shown). Cytokinin-treated, established nonembryogenic *pt* and wild-type lines remained non-

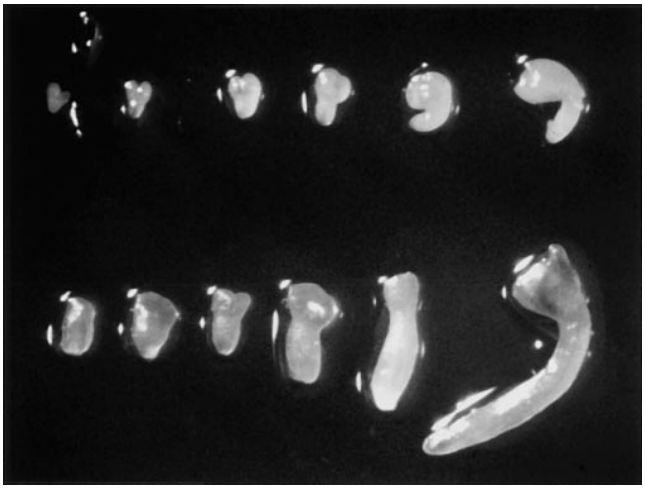

Figure 7.—Comparison of *pt* zygotic (top row) and *pt* somatic (bottom row) embryo development.

embryogenic, and the embryogenic capacity of embryogenic *pt* lines declined (data not shown). We conclude that the enhanced somatic embryogenesis trait of *pt* cannot be phenocopied by application of exogenous zeatin to wild type (L*er*).

DISCUSSION

Two systems for continuous somatic embryogenesis in liquid medium of Arabidopsis are now available. One system employs dissected immature zygotic embryos as starting material and yields embryogenic cell lines maintained through selective subculturing of green embryogenic aggregates. After transfer to growth regulator free medium, mature somatic embryos develop, which are able to germinate and to grow into fertile plants. This system is a continuation of the work of Wu *et al.* (1992), Pillon *et al.* (1996), and Luo and Koop (1997) and is applicable now to at least 11 different ecotypes. The second system, described here, employs seedlings developed from seeds directly germinated in liquid media. Based on the origin of the embryogenic clusters in *pt* seedlings, the enlarged SAM, embryogenic capacity was also found in *clv* mutants, a property not reported previously for these mutants.

The embryogenic seedling-derived *pt* cultures closely resembled immature zygotic embryo-derived embryogenic wild-type cultures but were much more efficient. This is evident from a higher ratio of embryogenic to nonembryogenic aggregates and from the high amounts of somatic embryos produced for a long period of time. One of the main advantages of the *pt* culture system is that it employs dry seeds as starting material, making laborious dissection of immature zygotic embryos and continuous plant growth unnecessary (Sangwan *et al.* 1992; Wu *et al.* 1992; Pillon *et al.* 1996; Luo and Koop 1997). It is therefore a valuable improvement when high amounts of Arabidopsis embryogenic cells and somatic embryos are required. A potential drawback of the *pt* approach is that it is restricted to mutants that themselves have an embryo or seedling phenotype. This necessitates extensive genetic analysis when the *pt* lines are used to combine high embryogenic capacity with other embryo mutants with the aim of analyzing their behavior *in vitro.* In those cases, the immature zygotic embryo system is preferable.

Recurrent somatic embryogenesis: The morphological appearance of all Arabidopsis embryogenic cultures described by us and others (Wu *et al.* 1992; Pillon *et al.* 1996) suggests that propagation occurs via recurrent embryogenesis. This differs from the mode of embryogenesis extensively described in carrot (De Vries *et al.* 1988; Krikorian and Smith 1992) and other species (Kreuger *et al.* 1995, 1996) where proembryogenic masses are propagated as a cell type in finely dispersed suspension cultures. Recurrent, secondary, repetitive, or cycling embryogenesis is also observed in many other plant species such as soybean (Finer and Nagasawa 1988), apple (Paul *et al.* 1994), olive (Rugini and Caricato 1995), peanut (Durham and Parrott 1992), Camellia (Pedroso and Pais 1995), cacao tree (Alemanno *et al.* 1996), and oak tree (Puigderrajols *et al.* 1996). In these species recurrent embryogenesis leads to rather large aggregates with a characteristic variability in morphology and arrangement of somatic embryos, including complete or partly fused embryo axes and fused cotyledons. Fused embryos can emerge when they are initiated simultaneously, close enough to prevent complete separation. Besides a fusion of entire somatic embryos, cotyledons of single embryos were also generally fused or at least partly fused. The failure of cotyledon separation has also been described in the context of a reduced polar auxin transport, caused either by an auxin transport inhibitor (Liu *et al.* 1993) or by the *pin-1* mutation (Okada *et al.* 1991). It is possible that the auxin present in the culture medium is interfering with the internal auxin gradient, and as a result, partitioning of cotyledon primordia is distorted in somatic embryos.

Separation of body pattern formation and cell division pattern: Early wild-type Arabidopsis zygotic embryo development is characterized by a highly regular pattern of divisions leading to an almost invariant number of cortex cell layers and provascular cell files in hypocotyls and primary roots (Dolan *et al.* 1993). In somatic *pt* embryos the number of cell layers in hypocotyl and root is higher and more variable. This is likely to be due to a less strict division pattern in the earlier stages of somatic embryos, resulting from the *in vitro* conditions because zygotic embryos from plants regenerated via somatic embryos had the original invariant number of cell layers and cell files. This observation suggests that a regular pattern of embryogenic cell divisions is not required for patterning in somatic embryos. This observation is in line with the less regular patterns of cell division in

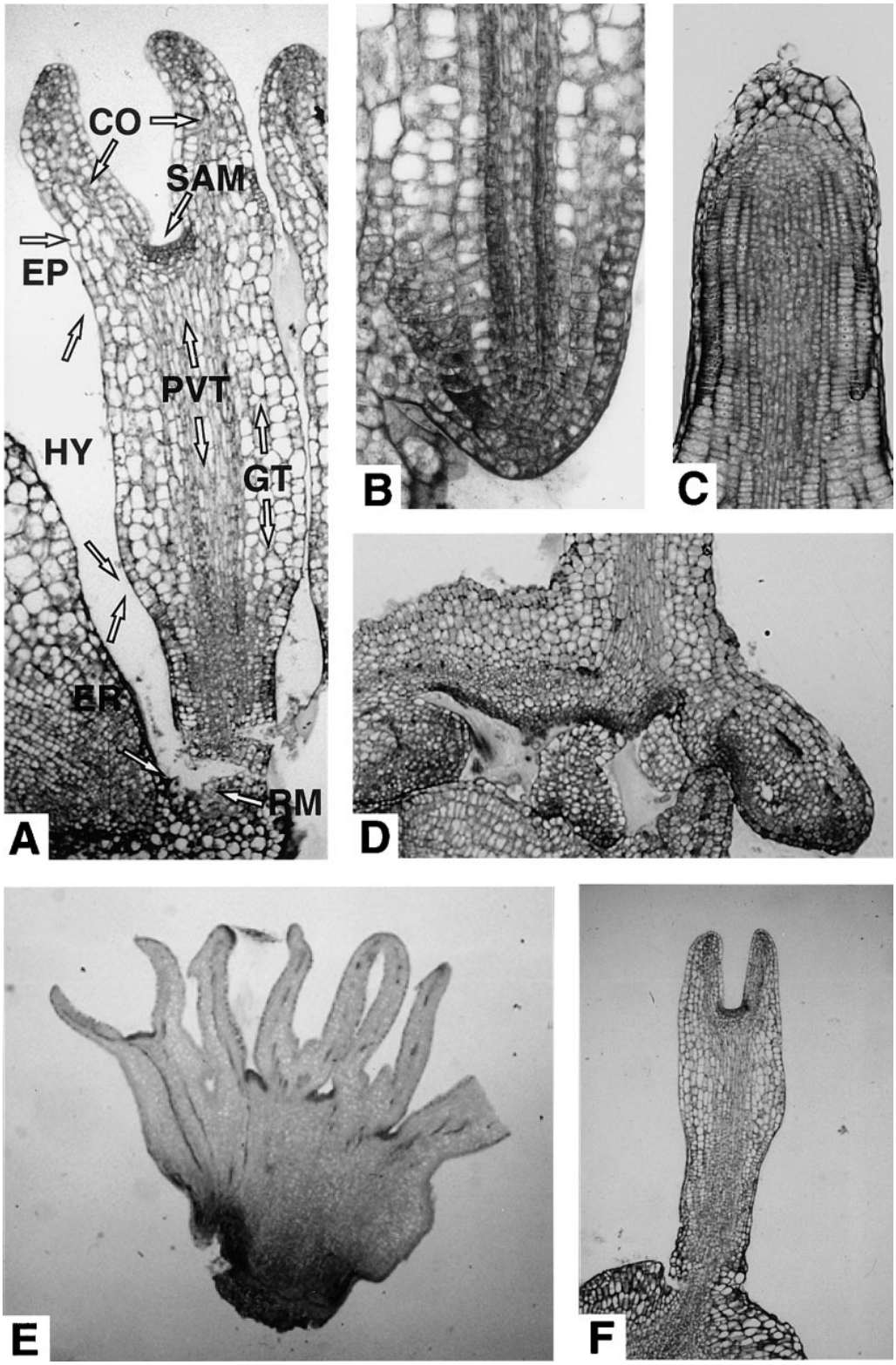

Figure 8.—(A)–(E) Semithin longitudinal tissue section through *pt* somatic embryos. (A) complete somatic embryo; (B), (C) sections through roots of somatic embryos with "normal" (B) and inverse (C) orientation; (D) section through apex region of somatic embryo with inverse orientation; (E) section through fused somatic embryos; (F) section through a structure resembling a somatic embryos, but provascular strands connect SAM and adjacent callus. CO, cotyledons; SAM, shoot apical meristem; EP, epidermis; PVT, provascular strands; HY, hypocotyl; GT, ground tissue; ER, embryonic root; RM, root meristem.

zygotic embryo mutants such as *fass/emb40* (Torres Ruiz and Jürgens 1994) and with the less regular cell division patterns in zygotic embryos of other plant species (reviewed by Mordhorst *et al.* 1997); in both cases, a complete embryo pattern is formed.

Additive phenotypes in *pt clv* double mutants: The additive effects observed in both *pt clv1-4* and *pt clv3-2* double mutants, particularly on SAM size and on the number of rosette leaves, show that the *PT* and *CLV1/CLV3* genes all act on the same target tissue. However, they appear to control either different developmental stages, distinct pathways, and/or act on a different sub-

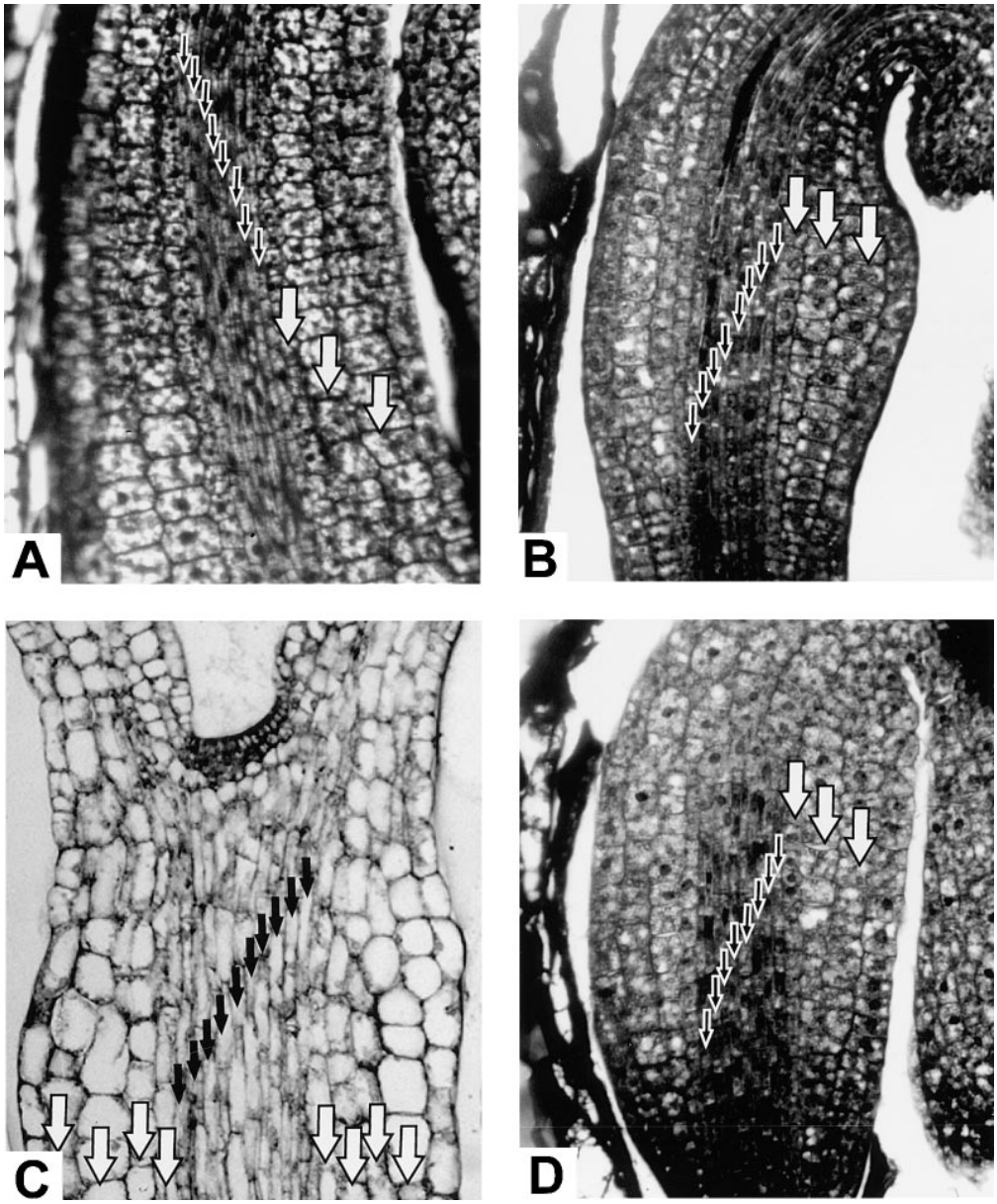

Figure 9.—Semithin completely median longitudinal tissue sections through hypocotyl regions of (A) L*er* zygotic embryo, (B) *pt* zygotic embryo, (C) *pt* somatic embryo, and (D) *pt* zygotic embryo from an R1 plant regenerated through somatic embryogenesis (all in same magnification). Small outlined arrows indicate individual provascular cell files; large white arrows indicate individual cortex cell files.

set of SAM cells. The consequences of the *pt* mutation are detected earlier than those of *clavata*, because the effect of the *pt* mutation on the size of the SAM is most prominent during embryogenesis and early seedling development (Ploense 1995; Conway and Poethig 1997). The size of the inflorescence meristem of *pt* mutant plants is not increased, and the number of organs per flower remains unchanged. In contrast, *clv1* and *clv3* mutant zygotic embryos have only a slightly enlarged SAM (Clark *et al.* 1995; Running *et al.* 1995), and the size of the meristem increased continuously during plant development, leading to fasciated inflorescence stems and flowers with extra organs in all whorls (Clark *et al.* 1993, 1995). In the last aspect, the double mutant flowers are similar to *clv* single mutant flowers, suggesting that *PT* does not have a role in flower development, a conclusion already made by Conway and Poethig (1997). Although the cloning of the *PT* and the *CLV3* genes has not yet been reported, *CLV1* encodes a leucine-rich repeat transmembrane receptor kinase, suggesting a role in signal transduction. *CLV1* is expressed in shoot and flower meristems in the L3 layer (Clark *et al.* 1997). Two models for the action of the *CLV* genes have been discussed: (1) regulation of organ formation and (2) regulation of proliferation of undifferentiated cells in the central zone of the meristem (Clark *et al.* 1995, 1997; Laux and Schoof 1997; Meyerowitz 1997). In both models, a mutation in the *CLV* genes disrupts the balance between cell proliferation and cell differentiation in the SAM. This causes an accumulation of undifferentiated or uncommitted cells in the meristem. For the *pt* mutant it was shown that the increased size of the embryonic SAM could be the result of precocious development of the shoot apex in

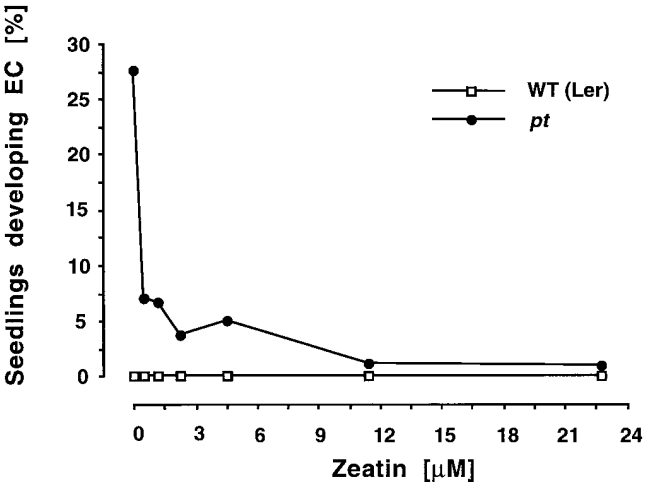

Figure 10.—Effect of additional zeatin in 2,4-D containing culture media on the percentage of WT (L*er*) and *pt* seedlings developing embryogenic clusters (ECs).

combination with a delayed development of the embryo proper (Conway and Poethig 1997). The enlarged SAM of *pt clv* double mutants can be a much larger platform than in wild type on which genes controlling the formation of leaf primordia act, thus resulting in an increased number of much smaller rosette leaves, due to spacing and competition constraints. A continuous increase of the meristem size during plant development as observed in the *clv* mutants, but starting from an already enlarged meristem due to the *pt* mutation, might also be responsible for the extreme forms of fasciation observed in the *pt clv1-4* and *pt clv3-2* double mutants.

A larger embryonic SAM facilitates initiation of somatic embryogenesis: *pt* zygotic embryos are characterized by extra cell divisions and by an enlarged SAM. Because embryogenic clusters developed from the SAM, we propose that it is this property of the *pt* mutant that is responsible for the enhanced somatic embryogenesis phenotype. This is supported by a similar response in the other mutants used, such as *clv1-4* and *clv3-2* (Clark *et al.* 1995; Running *et al.* 1995). The fact that both *clv* single mutants had less extra SAM cells and both *pt clv* double mutants had more than *pt* single mutants was reflected in their respective embryogenic cluster formation, suggesting even a quantitative relation between both traits. The pleiotropic phenotype of the Columbia allele *amp1* was attributed to elevated endogenous cytokinin levels (Chaudhury *et al.* 1993; Chin-Atkins *et al.* 1996). Although cytokinins promote shoot meristem development (Grayburn *et al.* 1982), we were not able to phenocopy the enhanced somatic embryogenesis phenotype of *pt* with exogenous zeatin in wild type. Instead, increasing exogenous cytokinin inhibited induction of somatic embryogenesis in wild type (Luo and Koop 1997) as well as in *pt*. In contrast to the deetiolated *amp1* phenotype (Chin-Atkins *et al.* 1996), it is therefore unlikely that the enhanced somatic embryogenesis can be directly correlated with an elevated endogenous cytokinin level in *pt* seedlings. Our interpretation is that, with an increased SAM size, more undifferentiated or uncommitted cells are present that are able to respond to 2,4-D by initiating a new embryonic program. In cereals, somatic embryogenesis can be initiated only by dividing undifferentiated cells (Lazzeri and Lörz 1990; Jähne and Lörz 1995), suggesting that the Arabidopsis system is more like that of cereals than that of carrot, where somatic embryogenesis can be initiated from vascular cells of the hypocotyl (Guzzo *et al.* 1994, 1995; Schmidt *et al.* 1996). Recently, roots of the *pickle* (*pkl*) mutant were shown to retain an embryonic characteristic such as the synthesis of particular seed-type fatty acids. After removal from the mutant plant and transfer to growth regulator free medium, *pkl* roots spontaneously produced embryo-like structures (Ogas *et al.* 1997). For *amp1*, it was discussed that extra cotyledons could be produced before germination through precocious development of the SAM during embryogenesis but also after germination (Conway and Poethig 1997). This could be interpreted as a delayed switch from an embryonic to an adult SAM program.

Together with our observations, this suggests that the initiation of somatic embryogenesis in Arabidopsis seems to be facilitated by the presence of cells that have retained certain "embryonic" or "undifferentiated" characteristics. Such cells are present in all immature zygotic embryos and are normally reduced in number after germination in wild type. Due to mutations in certain genes affecting the SAM such as *pt*, *clv1*, and *clv3* and affecting the root such as *pkl*, such embryonic cells persist until after embryogenesis is completed. In this aspect the propagation of embryogenic cells *in vitro* under appropriate conditions can be interpreted as a default mechanism occurring when normal controlling elements of cell division and/or cell differentiation are inhibited. *CLV1*, as putative member of a signalling pathway (Clark *et al.* 1997), of course suggests that signalling pathways are crucial in exerting such control. If the hypothesis that embryogenic cell formation is a default mechanism is true, this implies that mutations in many different genes can lead to an enhanced somatic embryogenesis phenotype. Nevertheless, working with Arabidopsis will make screens for such mutants feasible and can be expected to contribute to a better understanding of the concept of totipotency in plant cells.

We thank I. Vizir (University of Nottingham, United Kingdom), S. Clark (University of Michigan, Ann Arbor), U. Mayer (University of Tübingen, Germany), V. Raz (University of Wageningen, The Netherlands), the Nottingham Arabidopsis Stock Centre and the Arabidopsis Biological Research Center, Ohio, for the kind gift of seeds. We also thank W. van Veenendaal, M. Thijssen, and B. Dubreucq for advice during the preparation of tissue sections and A. van Kammen for fruitful discussions. A.P.M. was supported by grants from the Research School of Experimental Plant Sciences, and Agricultural University Wageningen, the European Community (EC) programs Human Capital and Mobility, Project of Technological Priority (PTP)-

Biotech and the EC, Life Sciences and Technologies, Biotechnology Program (1994–1998) funding the European Plant Embryogenesis Network. E.A.M. was supported by PTP-Biotech.

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Communicating editor: D. Preuss