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Genotypic variation and chromosomal location of QTLs for somatic embryogenesis revealed by epidermal layers culture of recombinant inbred lines in the sunflower (*Helianthus annuus* L.)

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Abstract The present study was conducted to identify the genetic factors controlling somatic embryogenesis in the sunflower. Two traits, the number of embryogenic explants per 40 explants plated (EE/40 E) and the number of embryos per 40 explants (E/40 E), were scored in 74 recombinant inbred lines (RILs) from a cross between 'PAC-2' and 'RHA-266'. The experiment was designed as a randomized complete block with 76 genotypes (74 recombinant inbred lines and two parents) and three replications. Each replication consisted of three Erlenmeyer flasks with 40 epidermal layers (explants). Analyses of variance indicated the existence of highly significant differences among parental genotypes and their RILs. Heritabilities for the somatic embryogenesis traits studied, EE/40 E and E/40 E, were high (0.64 and 0.77 respectively) and the genetic gain, in percentage of the best parent for 10% of selected RILs, was significant. Four QTLs for EE/40 E (*tee*) and seven for E/40 E (*ete*) were detected using composite interval mapping and AFLP mapping. The QTLs for EE/40 E explained 48% of the phenotypic variation while the QTLs for E/40 E explained about 89% of the variation.

Keywords AFLP · Recombinant inbred lines · Somatic embryogenesis · Sunflower · QTL

Introduction

An interest in oil crops has led to the sunflower being developed into one of world's most important oil seed crops. The ability to regenerate large numbers of plants from tissue culture is important for the successful application of most of biotechnological techniques, such as genetic engineering. Over the last few years, regeneration methods have been developed for sunflower. There are two main types of regeneration method:organogenesis (Pugliesi et al. 1991, Chraîbi et al. 1992, Sarrafi et al. 1996a, b) and somatic embryogenesis (Pélissier et al. 1990, Bronner et al. 1993, Thegane et al. 1994, Jeannin et al. 1995, Zezul et al. 1995). Embryogenesis capacity is influenced by cultural conditions, genotype, and their interaction. In sunflower the number of embryogenic events increase with increasing sucrose concentration, while organogenesis shows the opposite trend (Jeannin et al. 1995). In order to obtain a high rate of embryo formation, it is important to maintain the explants in complete darkness (Carola et al. 1997).

Direct somatic embryogenesis responses from immature embryos of sunflower genotypes was variable, which confirm the dependence of embryogenesis frequency on genotype (Pélissier et al. 1990). Bolandi et al. (2000) reported that the production of somatic embryos from epidermal layers in sunflower is highly variable depending mainly upon the genotype. At present, the number of reports about the genetic control of regeneration in sunflower remains limited. Organogenesis parameters have been shown to be under quantitative genetic control in sunflower (Sarrafi et al. 1996a, b). Additive and dominant effects of genes controlling embryogenesis traits have been reported by Bolandi et al. (2000) in this species.

The construction of genetic maps has provided a tool for identification of the number, significance and location of quantitative trait loci (QTLs) associated with a variety of phenotypic characteristics. In sunflower, maps have been developed (Berry et al. 1995, Gentzbittel et al. 1995, Jan et al. 1998, Gentzbittel et al. 1999) and linkages of molecular markers with resistance genes to rust (Lawson et al. 1998), QTLs for *Sclerotinia sclerotiorum* resistance (Mestries et al. 1998) and candidate-genes for downy mildew (Gentzbittel et al. 1998) have been identified. The utilization of molecular markers linked to dif-

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ferent traits would simplify and accelerate the identification process (Paterson et al. 1985, Yung et al. 1988; Martin et al. 1991). Estimates of genetic variation and determination of chromosomal regions (QTLs) that control somatic embryogenesis can be used to determine the value of genotypes in a breeding programme.

DNA markers have been used to locate genes contributing to somatic embryogenesis in:tomato (Koornef et al. 1993), alfalfa (Yu and Pauls 1993) and barley (Komatsuda et al. 1995; Mano et al. 1996). Somatic embryogenesis in alfalfa is a quantitative trait under the control of two dominant genes with complementary effects (Yu and Pauls 1993). A cross between two highly embryogenic genotypes of *Medicago sativa* produced only slightly more embryos than progeny of these genotypes crossed with a non-embryogenic genotype; this is evidence that there is more than one pathway for somatic embryogenesis (Kris and Bigham 1988).

Single-seed-descent (SSD) plant production provides a rapid way to obtain recombinant inbred lines (RILs), which are homozygous progenies from a selected cross. The RILs undergo multiple rounds of meiosis before homozygosis is reached; in consequence, linked genes have a greater probability of recombination (Burr and Burr 1991). This effect increases the power of testing differences between genotypic classes.

The objective of the investigation presented here was to carry out a QTL mapping analysis to characterize genomic regions involved in somatic embryogenesis. We also evaluate the variability and genetic gain for somatic embryogenesis in 74 recombinant inbred lines (RILs) of a cross between 'PAC-2' and 'RHA-266', including their parents.

Materials and methods

Scoring and measurement of regeneration by somatic embryogenesis

A population of 74 recombinant inbred lines (RILs) developed by the SSD method from the cross between 'PAC-2 and RHA-266' were used in this experiment. These recombinant inbred lines (F_8) and their parents were produced by INRA-France. Before culturing, pericarps were surface sterilized in a 5% (w/v) sodium hypochlorite solution with 0.01% (v/v) tween-20 for 20 min and rinsed three-times in sterilized water. Sterile seeds were germinated in culture tubes on agar-solidified MS basal medium (Murashige and Skoog 1962) and the pH was adjusted to 5.7. Cultures were maintained at 24°C±1 under a light flux of 50 $\mu E^{-2}s^{-1}$ (16-h light, 8-h dark cycle). Culture media were prepared according to Pélissier et al. (1990). The epidermal layers from 7-day-old hypocotyls were excised in 2-cm sections and transfered to 250-ml Erlenmeyer flasks containing 100 ml of the MS basal medium (MSb) for 5 days, then on B5–90 medium for 8 days. Cultures were maintained at 24±1°C in the dark with shaking at 120 rpm. After this period of 13 days, explants were transfered to MS-120 embryo-development medium for 15–20 days at 26±1°C in the dark. Then embryos were separated from thin layers and transfered to B-60 medium in order to develop secondary embryos for 10 days. The embryos were finally transferred to the same medium for 15 more days.

The experiment was designed as a randomized complete block with 76 genotypes (74 recombinant inbred lines and two parents) and three replications. Each replication consisted of three Erlenmeyer flasks with 40 epidermal layers (explants) each. The following traits were determined for each genotype per replication:the number of embryogenic explants per 40 explants plated (EE/40 E) and the number of embryos per 40 explants (E/40 E). Analysis of variance was performed for embryogenic traits and the means separated using a Newman-Keuls-test (P=0.05). Additive, environmental variances and narrow-sense heritabilities were calculated according to Kearsey and Pooni (1996), using least-square estimates of the genetic parameters.

Data analysis and QTL mapping

A set of 99 RILs and their parents 'PAC-2' and 'RHA-266' were used for DNA extraction and AFLP analysis. Then the same set was screened with 333 AFLP markers and a linkage map was constructed based on 254 linked loci, as previously described (Flores Berrios et al. 2000b).

The chromosomal location of QTLs for embryogenic traits were resolved by composite interval mapping (CIM) using QTL Cartographer v1.13 model 6 (Basten et al. 1999). The QTL-carte model 6 integrates two parameters:the number of markers which control the genetic background (n_p) and a window size (w_s) that will block out a region of the genome on either side of the markers flanking the test site (Basten et al. 1999). Inclusion of the background makes the analysis more sensitive to the presence of a QTL in the target interval. A window size of 10 cM and 15 markers were chosen to account for background. At each marker locus, the significance of the association was tested by the likelihood-ratio statistic (LRS) (Haley and Knott 1992). The LRS test is statistically convenient because asymptotically it is a χ^2 distribution (Manly and Olson 1999).

Results and discussion

Results of the analysis of variance indicated the existence of highly significant differences among parental genotypes and their RILs for the embryogenic traits studied. Mean performance concerning the genetic variability of parental inbred lines for two embryogenic traits are presented in Table 1. The parental genotype 'PAC-2' showed higher values when compared with 'RHA-266' for EE/40 E and E/40 E. Pelissier et al. (1990), Fambrini et al. (1996) and

Table 1 Genetic gains and heritabilites for somatic embryogenesis traits in recombinant inbred lines (RILs) of sunflower

Item	EE/40E[a]	E/40E[b]
PAC2 (P1)	18.67	141.00
RHA266 (P2)	4.67	8.00
P1-P2	14.00*	133.00*
$\bar{X}_P$=(P1+P2)/2	11.67	74.50
$\bar{X}_{RILs}$[c]	5.66	28.38
$\bar{X}_{RILs}$-$\bar{X}_P$	–6.01 ns	–46.12 ns
Best RIL (BRIL)	26.67	180.33
GG=BRIL-BP[d]	8.00*	39.33*
10% SF_8L	25.25	170.82
GG=10%BSF_8L-BP[e]	6.58*	29.82*
h^2	0.64	0.77

* P<0.05; ns, not significant at P<0.05

[a] EE/40 E, number of embryogenic explants per 40 explants plated

[b] E/40 E, number of embryos per 40 explants plated

BP, best parent ('PAC-2')

[c] $\bar{X}$RIL, mean of all recombinant inbreds lines

[d, e] GG, genetic gain when the best RIL or 10% of the selected RILs are compared with the best parent ('PAC-2')

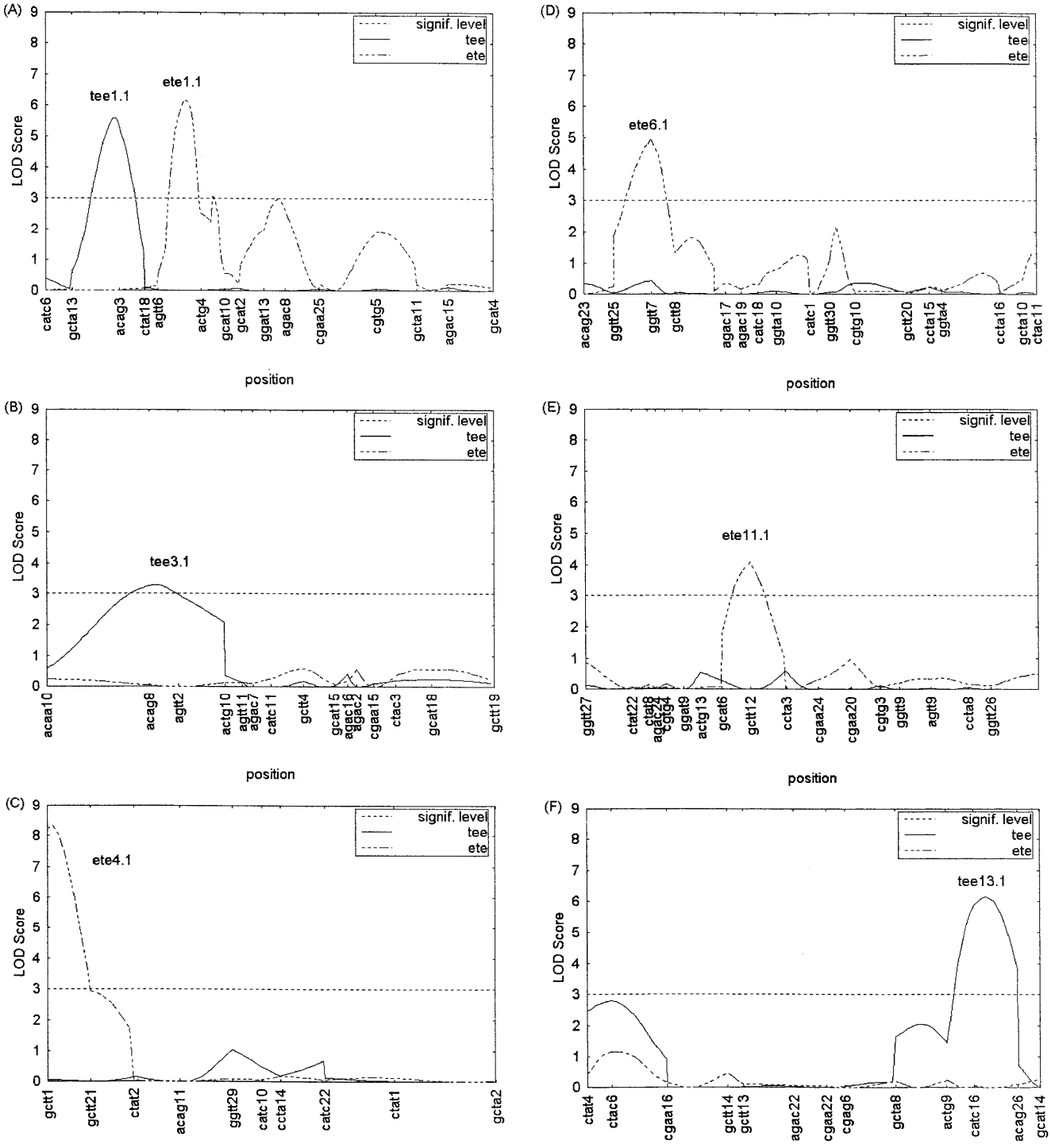

Fig 1A–I Genetic maps and LOD plots showing the locations of putative QTLs associated with somatic embryogenesis detected by composite interval mapping (CIM). *tee:* total embryogenic explants, *ete:* embryos per total explant. (**A**) Linkage map of linkage group I, (**B**) linkage map of linkage group III, (**C**) linkage map of linkage group IV, (**D**) linkage map of linkage group VI, (**E**) linkage map of linkage group XI, (**F**) linkage map of linkage group XIII, (**G**) linkage map of linkage group XV, (**H**) linkage map of linkage group XVI, (**I**) linkage map of linkage group XVII

Bolandi et al. (2000) have also demonstrated that embryogenic parameters are genotype-dependent. The difference between all recombinant inbred lines ($\bar{X}_{F8}$) and their parents ($\bar{X}_P$) was not significant, indicating that RILs in this experiment are representative of the total possible recombinant lines from the cross 'PAC-2×RHA-266' (Table 1). The best parent (PAC-2) compared with the best RIL (BRIL) and with the mean of 10% of RILs with the highest values for embryogenic parameters, presented significant differences for the two traits studied (Table 1). This phenomenon, considered as genetic gain, might be due to the

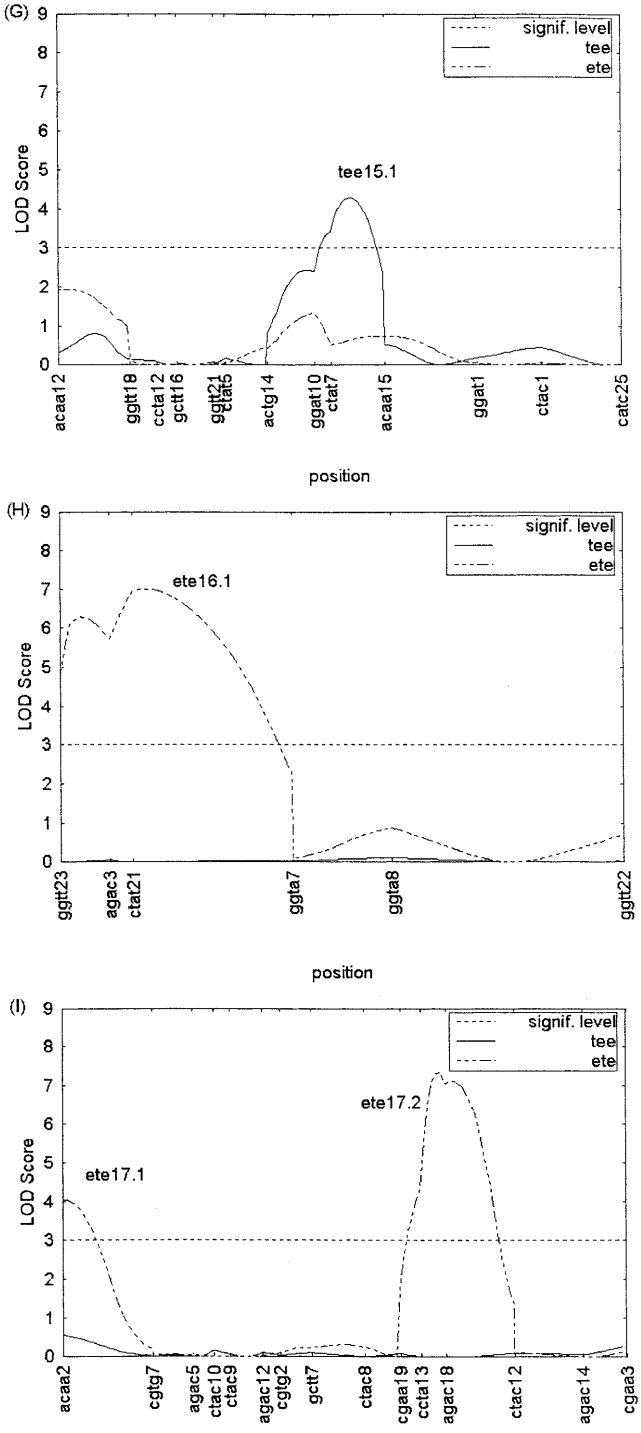

Fig 1A–I continued

polygenic nature of embryogenesis and the accumulation of favourable alleles for embryogenesis ability in the RILs. Polygenic control of embryogenesis parameters has also been reported in sunflower (Bolandi et al. 2000), as in:alfalfa (Reisch and Binghman 1980), tomato (Koornef et al. 1987) and cotton (Gawel and Robacker 1990). Narrow-sense heritabilities were 0.64 and 0.77 for EE/40 E and E/40 E, respectively, indicating that selection for these embryogenic traits will be possible in progenies of this cross.

Significant peak values of LOD scores, the position of these peaks, the percentage of phenotypic variance explained and the estimate of QTL effects based on a composite interval mapping analysis for embryogenic traits studied, are shown in Table 2. Four QTLs were identified for embryogenic explants per 40-explants plated and seven QTLs were detected for the number of embryos per 40-explants plated. These QTLs were designed according to the traits as follow:*tee* (total embryogenic explants) for embryogenic explants per 40 explants plated and *ete* (embryos per total explant) for the number of embryos per 40 explants plated (Fig. 1). The effects of each QTL are moderate (ranging from 7 to 20%). The transgressive phenotypes observed could be explained by the presence of QTLs of opposite sign in each parents. For the two components of somatic embryogenesis capacity, EE/40 E and E/40 E, the detected QTLs together explain 48 and 89% of the phenotypic variation respectively. As described for organogenesis parameters (Flores Berrios et al. 2000b), linked and opposite QTLs, such as *ete17.1* and *ete17.2*, are present on linkage group 17. This demonstrates the suitability of RILs to allow for the detection of linked and opposite QTLs, even with moderate effects.

Wan et al. 1992 performed RFLP analysis on regenerable calli formed from embryo-like structures in maize. They hypothesized that some of regions found might be related both to the induction of embryos and the ability to produce regenerable calli. Histological studies showed that division occurred within the different layers and that embryos were produced directly at the surface of the epidermial layers (Nonohay et al. 1999). Koorneff et al. (1993) demonstrated that shoot regeneration from various tissue culture systems, including established callus and protoplast cultures, are controlled by a major gene in tomato. The ability of cultured plant cells to produce embryos without a sexual process suggest that signals for embryogenesis are supplied in vitro to a wide range of somatic or gametic cells that have the potential to express an egg-cell gene-expression programme (Henry et al. 1994). The QTLs we have detected for embryogenic traits could be correlated with the perception of these signals.

A model where somatic embryogenesis is divided into two different steps can be considered. The first step is cell re-programming, giving the explant the ability to produce embryos. The second step is the expression of the trait, leading to the production of embryos by an induced explant. The first step, called the induction step, could be mesured by the total number of embryogenic explants, irrespective of the number of embryos produced per explant. The second step could be evaluated by the number of embryos per embryogenic explant. Thus, the two parameters studied could be related either to the induction or the expression of embryogenic abilities. The large number of detected QTLs, together with the fact that only one region (on linkage group I) is associated with the induction and expression parameters, suggest that the genetic control of this trait is probably complex. It also sug-

Table 2 Mapping positions and effect of the QTLs detected in recombinant inbred lines (RILs) and their parents for in vitro embryogenic traits in sunflower

Trait	QTL	Linkage group	Position[a]	Log-likelihood	Proportion of the phenotypic variance explained[b]	Additive effect (*a*)
[2]EE/40 E	*tee1.1*	I	29	5.6	0.15	–3.56
	tee3.1	III	33	3.3	0.07	2.24
	tee13.1	XIII	103	6.2	0.15	3.74
	tee15.1	XV	93	4.3	0.11	2.89
[3] E/40 E	*ete1.1*	I	58	6.2	0.16	30.37
	ete4.1	IV	1	8.3	0.20	–27.17
	ete6.1	VI	27	4.9	0.09	–23.23
	ete11.1	XI	62	4.1	0.07	–16.50
	ete16.1	XVI	8	7.0	0.15	21.97
	ete17.1	XVII	1	4.0	0.07	–16.33
	ete17.2	XVII	95	7.1	0.15	23.84

[a] Expressed, in Kosambi cM, from north of the linkage group (Flores-Berrios et al. 2000b)
[b] Values determined by QTL Cartographer, Version 1.13 (Basten et al. 1999)
[c] EE/40E: embryogenic explants per 40 explants plated
[d] E/40E: number of embryo per 40 explants plated

gests that the two components of the model (induction and expression) would be supported by different genetic systems. Although the interesting region on linkage group I needs to be more precisely mapped, the available information should help the transfer of embryogenic ability to genotypes that respond poorly.

The description of a few mutants in *Arabidopsis thaliana* (Mayer et al. 1991, Busch et al. 1996) and pea (Liu et al. 1999 as examples) led to the identification of some limiting steps in embryo patterning. However, little is known about the overall gene regulations and genetic pathways leading to somatic and zygotic embryogenesis in dicots. The results presented here revealed several regions related to in vitro somatic embryogenesis in recombinant inbred lines of sunflower. In a companion paper (Flores-Berrios et al. 2000a), we described the presence of three major chromosomal regions associated with organogenesis, embryogenesis and cell division. These results should provide a starting point for the deciphering of the molecular mechanism leading to somatic embryogenesis.

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