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Somatic embryogenesis by liquid culture of epidermal layers in sunflower: from genetic control to cell development

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Abstract

Embryos were obtained using liquid medium culture of sunflower hypocotyl epidermis layers according to the Pélissier et al. (1990) method. In the present work we identified genetic factors controlling somatic embryogenesis and we evidenced the role of ionic channels in embryogenic tissues. Two traits, the number of embryogenic explants (EE) and the number of embryos (EM) were scored in 74 recombinant inbred lines (RILs) from a cross between lines PAC-2 and RHA-266. Analysis of variance indicated the existence of highly significant differences among the parental genotypes and their RILs. Heritability for the somatic embryogenesis traits studied were high (0.64 for EE and 0.77 for EM). Four quantitative trait loci (QTLs) for EE and seven for EM were detected using composite interval mapping. The QTLs for EE explained 48% of the phenotypic variation while the QTLs for EM explained about 89% of the variation, thus revealing several genomic regions related to somatic embryogenesis control in sunflower. In order to study the distribution of ion channels in somatic embryos as compared to zygotic ones, we used a fluorescent-labelled phenylalkylamine, DM-Bodipy PAA, as a probe. Fluorescence labelling was determined by confocal microscopy. The probe intensively labelled the protoderm and epidermis cells in both zygotic and somatic embryos. Callus exhibited labelling on sites where somatic embryos developed. Considering that the location of phenylalkylamine (PAA) binding sites is related to the distribution of ion channels, the high intensity in the protoderm and epidermis of embryos, point to similar properties and functions and their key role in embryo development.

Abbreviations: EE – embryogenic explants; EM – number of embryos; PAA – phenylalkylamine; QTLs – quantitative trait loci; RILs – recombinant inbred lines

Introduction

The ability to regenerate large numbers of plants from tissue culture is important for the successful application of most biotechnological techniques, such as genetic engineering. During the last few years, regeneration methods have been developed for sunflowers (*Helianthus annuus*). There are two main types of regeneration methods: Organogenesis and somatic embryogenesis (Pelissier et al., 1990; Jeannin et al., 1995). Embryogenesis capacity is influenced by cultural conditions, genotype and their interaction. In sunflower, embryogenic events increase with increasing sucrose concentration (Jeannin et al., 1995) and darkness (Carola et al., 1997). Direct somatic embryogenesis could be obtained either from immature embryos or from epidermal layers;

both responses are highly variable depending upon the genotype (Pelissier et al., 1990; Bolandi et al., 2000). At present, the number of reports about the genetic control of regeneration in sunflower remains limited. 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 and linkage of molecular markers with resistance genes have been identified (Gentzbittel et al., 1998, 1999). The utilization of molecular markers linked to different traits would help to identify the genes involved. Moreover, estimates of genetic variation and determination of chromosomal regions that control somatic embryogenesis can be used to determine the value of genotypes in a breeding program.

Beside genotype, culture conditions of explant are known to assume particular importance in somatic embryo development. Tissues, which are at the interface between the organ and the external medium are essential sites for communication between plant and environment. This is particularly crucial for the protoderm of the young embryo. Recent investigations have revealed that specific genes are expressed during the development of embryonic protoderm and epidermis (Vroemen et al., 1996) and embryonic protoderm shows a calcium-binding pattern, which differs from that in the inner embryonic cells (Timmers et al., 1996). These findings call attention upon the functions of the plant epidermis system, especially in signal transduction pathways. Although it is well known that ion channels play an important role in signalling and control of morphogenesis in plants, their distribution in higher plant tissues has not been described yet. Phenylalkylamines (PAAs) are pharmacological drugs able to block specifically the L-type Ca^{2+}-channel activity in animal cells (Norris and Bradford, 1985) and has been shown to block the entry of calcium into plant cells (Thuleau et al., 1990). A fluorescently labelled PAA, DM-Bodipy PAA, has been used as a probe for labelling Ca^{2+}-channels in animal cell membranes (Knaus et al., 1992) and in sunflower protoplasts (Vallée et al., 1997). It appears to be a good tool to study the distribution of Ca^{2+}-channels antagonist binding sites in higher-plant tissues. In the present work we have studied the distribution of PAA binding sites in epidermal layer systems giving rise to somatic embryos as compared to non-embryonic explants.

The objective of the investigation presented here was to carry out a QTL mapping analysis to characterize the genomic regions involved in somatic embryogenesis in order to localize more precisely the genes involved and to clone them. The coupled approaches of genetic mapping and cell biology could allow us to decipher both genetic control and cellular mechanisms involved in somatic embryo determination.

Material and methods

Scoring of somatic embryogenesis

A population of 74 recombinant inbred lines (RILs) developed by the SSD method from the cross between lines PAC-2 and RHA-266 were used in this experiment. Surface-sterile seeds were germinated on agar-gelled MS basal medium (Murashige and Skoog, 1962) pH 5.7. Cultures were maintained at 24 °C under a light flux of 50 μmol $m^{-2}s^{-1}$ (16-h light, 8-h dark). Epidermal strips from 7-day-old hypocotyls were peeled, cut in 2 cm sections and transferred to MS basal medium for 5 days, then to B5-90 medium for 8 days, according to Pelissier et al. (1990). The strips, including the epidermis and about 4 subepidermal layers, were cultured at 24 °C in the dark with shaking at 120 rpm. After this period, explants were transferred to MS-120 embryo-developing medium for 15–20 days at 26 °C in the dark. The embryos were separated from the layers and transferred to B-60 medium in order to develop secondary embryos for 10 days. The experiment was designed as a randomized complete block with 76 genotypes (74 RILs and 2 parents) and three replicates. Each replicate consisted of three Erlenmeyer flasks each with 40 epidermal strips. The following traits were determined for each genotype per replicate: the number of embryogenic layers per 40 plated strips, and the number of embryos per 40 strips. Variance analysis was performed and the means separated using a Newman-Keuls-test ($p = 0.05$).

Additive, environmental variances and heritability were calculated according to Kearsey and Pooni (1996), using least-square estimates of the genetic parameters.

QTL mapping

This set of 74 RILs and 2 parents was used for DNA extraction, AFLP products screened with 333 markers and a linkage map was constructed based on 254 linked loci, as previously described (Flores-Berrios et al., 2000). The chromosomal location of QTLs for embryogenic traits were resolved by composite interval mapping (CIM) using QTL cartographer v1.13 model 6 software (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 the background. At each marker locus, the significance of the association was tested by the likelihood ratio statistic (LRS) (Haley and Knott, 1992).

Probe loading

Fluorescently labelled PAA (Molecular Probes, Eugene, Oregon, USA) was prepared as a 2.5 mmol stock solution in dimethyl sulfoxide. Before use, the DM-Bodipy PAA stock solution was diluted to 1 mmol in MS medium supplemented with 120 g sucrose per litre (MS-120). Embryos and epidermal layers were hand-cut in 0.5–1 mm thick sections, incubated in 1 mmol DM-Bodipy PAA solution at 25 °C for 30 min, rinsed in MS-120 medium and mounted in an observation chamber. A confocal laser-scanning microscope equipped with an argon-ion laser (488/510 nm) was used to analyse the labelling. For image acquisition a × 25/0.8 Plan objective was used with a 32% attenuation filter. Each image corresponds to the average of four frames.

Results and discussion

We have previously shown that the fluorescent probe DM-Bodipy PAA labels sunflower cell plasma membranes (Vallée et al., 1997), and is selectively removed by Ca^{2+}-antagonist treatment. Thus we may speculate that the location of labelling is highly related to the distribution of Ca^{2+}-channels, and to a lesser extent, K^{+}-channels (Xu XuHan et al., 1999).

In epidermal strips cultured in liquid medium, callus developed on the outermost cells – subepidermal cells. On this callus, mainly at the side opposite from the epidermis, embryogenic masses appeared (Figures 1A, 2A) developing next in a rounded "globular" structure (Figure 2B). Such globular somatic embryos develop further a protoderm layer (Figure 2B) and take a classical heart shape. In cultured epidermal strips, the original epidermis, which could still be attached to the callogenic subepidermal tissue, showed a strong labelling by DM-Bodipy PAA (Figure 1B). Most callus cells exhibited no, or a very weak, fluorescence, except in the areas where somatic embryos were to be formed (Figure 1B). Such

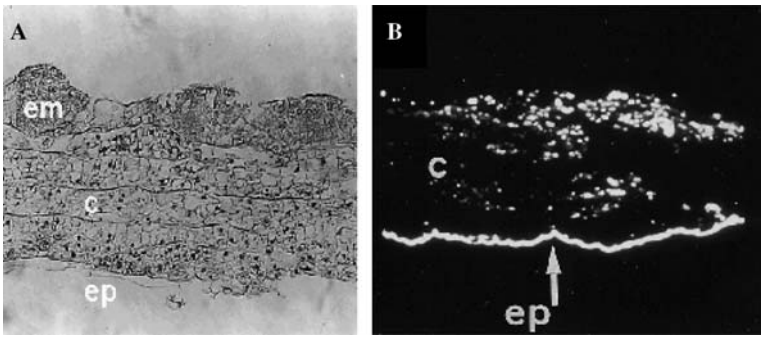

Figure 1. (*A*) Thin section of an epidermal strip showing the initiation sites of somatic embryos (em) in the outermost part of the strip in relation to the epidermis (ep). (*B*) Confocal optical section of an epidermal strip in culture, labelled with DM-Bodipy PAA. The original epidermis (ep) is strongly fluorescent, whereas the inner cells of the callus (*c*) are weakly labelled. The outermost cell layers of the callus are intensively labelled, corresponding to the embryo-forming cells.

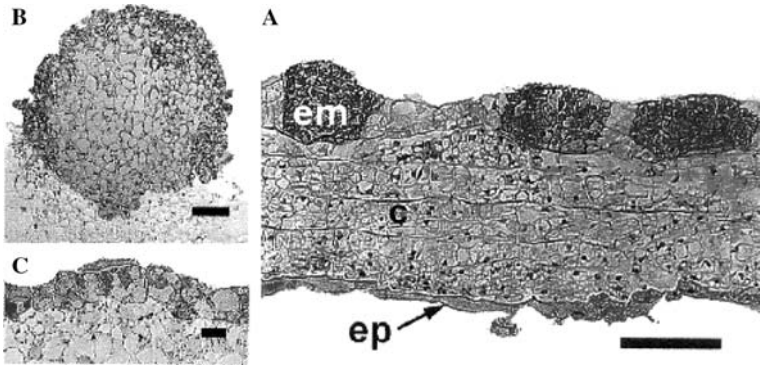

Figure 2. (*A*) On this section of an epidermal strip presented in false colour, the original hypocotyls epidermis (red) was still attached to the subepidermal cell layers that became callogenic (green). In the outermost cell layers of the callus there appeared initiation sites of somatic embryos (blue) (Bar = 100 μm). (*B*) Thin section of a somatic embryo at the globular stage (blue) surrounded by callus cells at its base (Bar = 100 μm). (*C*) Part on a somatic embryo at the heart-shaped stage, showing aligned protodermal cells (red) covering the embryonic ground tissue (green) (Bar = 20 μm).

somatic embryos developed in the upper cell layers of the explant (Figure 2A). Calli, which did not develop protoderm and epidermis did not show DM-Bodipy PAA labelling. When somatic embryos developed further, they showed a fluorescence pattern, which was similar to that of zygotic embryos: DM-Bodipy PAA labelled only the outermost cell layers (Figure 3). Such a similar labelling strongly argues in favour of common developmental pattern in somatic and zygotic embryos. Moreover the major role of protoderm is brought out: These cells are characterized by a high density of K^+ and Ca^{2+}-channels. Such a specificity has been reported concerning the ML1 gene, an homeobox gene, which expressed very early in the 8-celled embryo

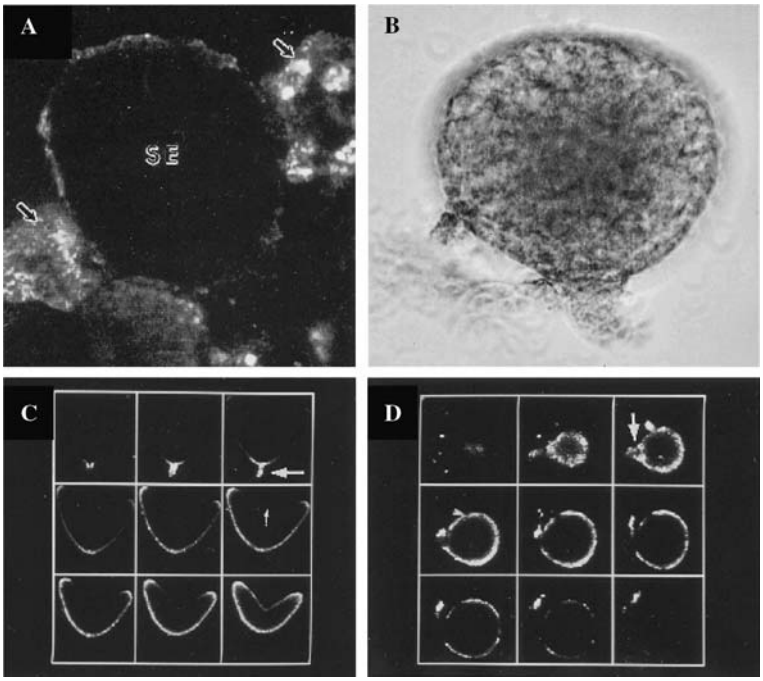

Figure 3. (*A, B*) Somatic embryo as observed under confocal scanning microscope, with (*A*) or without (*B*) labelling with DM-Bodipy PAA; note the intensive labelling of embryonic protoderm. (*C, D*) Series of optical sections of a zygotic embryo of sunflower at the globular (*D*) or heart-shaped (*C*) stage. Note in both cases the strong labelling of the outer cell layers corresponding to protoderm.

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

	EE	EM
PAC2 (P1)	46.67	352.5
RHA266 (P2)	11.67	20
P1-P2	35*	332.5*
Xp = (P1-P2)/2	29.17	186.25
XRILs	14.15	70.95
XRILs-Xp	−15.02 ns	−115.3 ns
BRIL (Best RIL)	66.67	450.82
GG = BRIL-BP	20*	98.32*
10% SRIL	63.12	427.05
GG = 10% SRIL-BP	16.45*	74.55*
Heritability	0.64	0.77

*$p < 0.05$; ns, not significant at $p < 0.05$.
EE, number of embryogenic explants per 100 explants plated.
EM, number of embryos per 100 explants plated.
BP, best parent (PAC2).
XRILs, mean of all recombinant inbred lines.
GG, genetic gain when either the best RIL (BRIL) or the selected 10% (10% SRIL) are compared with the best parent (BP).

and, further, only in protoderm cells up to heart-shape embryo (Lu et al., 1996).

Analysis of variance for the number of embryogenic explants and the number of embryos per explant are presented in Table 1. The parental genotype "PAC-2" showed higher values as compared with RHA-266 for the two tested traits. Bolandi et al. (2000) have also demonstrated that embryogenic parameters are highly genotype-dependent. The difference between all recombinant inbred lines and their parents was not significant, indicating that the RILs obtained are representative of the total possible recombinant lines from the cross PAC-2 X RHA-266 (Table 1). The best parent (PAC-2) compared with the best RIL or with the mean of the best 10% of the RILs, presented significant differences for the 2 traits studied (Table 1). This genetic gain might be due to the accumulation of favourable alleles for embryogenic ability. Narrow-sense heritability was 0.64 and 0.77 for EE and EM, respectively, indicating that selection for these embryogenic traits will be possible in progeny of this cross.

Significant peak values of LOD score, the position of the peaks, the percentage of phenotypic variation 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 detected for embryogenic explants and seven for embryo development. The effect of each QTL is moderate (ranging from 7 to 20%). The transgressive phenotypes observed could be explained by the presence of QTLs of opposite sign in each parent. For the two components of somatic embryogenesis capacity, the detected QTLs explain together 48 and 89% of the phenotypic variation respectively. Wan et al. (1992) performed RFLP analysis on regenerable calli formed from embryo-like

Table 2. Mapping position and effects of the QTLs detected in recombinant inbred lines for somatic embryogenesis traits in sunflower

Trait	QTL	Linkage group	Position [(a)]	Log-likelihood	Variance explained [(b)]	Additive effect [(b)]
EE	ee1.1	I	29	5.6	0.15	−3.56
	ee3.1	III	33	3.3	0.07	2.24
	ee13.1	XIII	103	6.2	0.15	3.74
	ee15.1	XV	93	4.3	0.11	2.89
EM	em1.1	I	58	6.2	0.16	30.37
	em4.1	IV	1	8.3	0.20	−27.23
	em6.1	VI	27	4.9	0.09	−23.23
	em11.1	XI	62	4.1	0.07	−16.50
	em16.1	XVI	8	7.0	0.15	21.97
	em17.1	XVII	1	4.0	0.07	−16.33
	em17.2	XVII	95	7.1	0.15	23.84

[a]Expressed in Kosambi cM, from north of the linkage group (Flores-Berrios et al., 2000).
[b]Values determined by QTL Cartographer, version 1.13 (Basten et al., 1999).

structures in maize. They hypothesized that some regions found might be related to the induction of embryos and the ability to produce embryogenic calli. Histological studies showed that division occurred within the different layers and that embryos were produced directly at the surface of the epidermal layers (Nonohay et al., 1999).

A model where somatic embryogenesis is divided into two different steps can be considered. The first step, an induction phase of the explant, can be approached by the number of embryogenic explants, the second step, expression of embryogenic potential, can be evaluated by the number of embryos formed. The large number of detected QTLs together with the fact that only one region (on linkage group I) is associated with both induction and expression parameters, suggest that the genetic control of this trait is probably complex. It also suggests that the two components of the model would be supported by different genetic controls. Although the interesting region on linkage group needs to be more precisely mapped, the available information should help the transfer of embryogenic ability to genotypes that respond poorly.

The first step corresponds to cell re-programming, giving the subepidermal layers the ability to differentiate, divide and initiate an embryo. During this induction phase, some cells strongly expressed Ca^{2+}-channel proteins on their plasma membrane. Such an ionic channel activity seems to be determinant in their cell fate. The second step consists of the expression of the embryogenic potential, leading to the development of embryos on the induced explant. In this expression phase, setting up of the protoderm is a critical event. In the protodermal cells, ionic channel activity appears to characterize their specificity early in controlling the relationships between the embryo proper and the surrounding medium. This focuses on the determinant role of calcium, not only as a compound of the culture medium, but mainly as a second messenger in signal transduction pathways.

These results should provide a starting point for the deciphering of molecular mechanisms leading to somatic embryogenesis. The coupled approaches of genetic mapping and cell biology could allow us to unravel both genetic control and cellular events involved in somatic embryo determination.

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