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Genetic control of early events in protoplast division and regeneration pathways in sunflower

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Abstract Experiments were conducted to identify the genetic factors controlling protoplast division and to determine eventual relations between genetic factors involving organogenesis, somatic embryogenesis and protoplast division in sunflower. The present study involved protoplast culture and two traits: total division per 100 protoplasts (TOTD) and asymmetric division per 100 protoplasts (ASYD) were scored in 52 recombinant inbred lines (RILs) from a cross between PAC-2 and RHA-266. Asymmetric division is an early event in the formation of embryoids from protoplasts. Analysis of variance indicated the existence of highly significant differences among parental genotypes and their RILs. Heritability for the two protoplast division parameters (TOTD and ASYD) was high (0.87 and 0.89, respectively) and genetic gain expressed as percentage of the best parent for 10% of the selected RILs was significant. Twelve putative loci associated with total division per 100 protoplasts were identified. Eleven QTLs were also detected for asymmetric division per 100 protoplasts. The QTLs present high significant LOD scores and sum to a high percentage of phenotypic variance. The percentage of phenotypic variation explained by each QTL ranged from 2% to 24%. Some segments of the linkage groups I, XV and XVII are likely to contain genes important for organogenesis, somatic embryogenesis and protoplast division, as clustering of QTLs for these characters were described. The QTLs identified in these three linkage groups should be involved in cell division and in early events associated with cell differenciation.

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Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important sources of vegetable oil in the world. Plant improvement and biotechnology combined with tissue culture implies the ability to regenerate a great number of plants. In sunflower, fertile plants can be regenerated through organogenesis (Pugliesi et al. 1991; Chraîbi et al. 1992; Sarrafi et al. 1996 a, b; Flores Berrios et al. 1999a), embryogenesis (Pélissier et al. 1990; Thegane et al. 1994; Zezul et al. 1995; Fambrini et al. 1996; Bolandi et al. 2000) or protoplast culture (Chanabé et al. 1991; Ficher and Hahne 1992; Alibert et al. 1994; Krasnyansky and Menczel 1995, Bolandi et al. 1999).

Regeneration ability is influenced by culture medium components, developmental stage of the explant and the interaction of these parameters. However, regeneration also largely depends on genotype. It has been shown that organogenesis parameters are under additive genetic control (Sarrafi et al. 1996 a, b, Flores Berrios et al. 1999, 2000a). Evidence of genotype effect and nucleocytoplasmic interaction was also demonstrated (Deglene et al. 1997). Recently, Flores Berrios et al. (2000a) identified seven QTLs involved in the regeneration of buds from cotyledons in a set of recombinant inbred lines of sunflower.

Thin cell layers excised from hypocotyls have been shown to be totipotent in sunflower, the division occurs within the different layers and the embryos are produced directly at the surface of epidermic layers (Pélissier et al. 1990). These different responses are reactions to specific protocols, mainly differing in the hormonal combinations in the culture media (Jeanin et al. 1995). Many studies have demonstrated that somatic embryogenesis parameters are also genetically determined traits (Finer 1987; Freyssinet and Freyssinet 1988; Writzens et al. 1988; Espinasse and Lay 1989; Bolandi et al. 2000).

Moreover additive and dominant effects of genes controlling embryogenesis are reported by Bolandi et al. (2000). Recently Flores Berrios et al. (2000b) have shown that more than 54% of the variability in somatic embryogenesis induced on sunflower thin layer epidermic cells can be explained by four QTLs.

Protoplast culture is a refinement of single-cell regeneration in sunflower. Burrus et al. (1991) and Wingender et al. (1996) have described a protocol for plant regeneration from hypocotyl protoplasts. Henn et al. (1998) achieved the fusion of *Helianthus annuus* hypocotyl protoplasts with mesophyl protoplasts from *Sclerotinia*-resistant clones of *H. maximiliani*, *H. giganteus* and *H. nuttalii.* Genotype, explant source and environment are all critical factors affecting protoplast culture efficiency in sunflower. Petitprez et al. (1995) defined an original system in which very early events of somatic embryogenesis (i. e. the first asymmetric division) can be studied. Protoplast, produced from hypocotyls, divided symmetrically when cultured in liquid medium whereas, when included in agarose, they underwent an unequal division (asymmetric division) and gave rise to embryo-like structures. Barthou et al. (1999) showed that adhesion sites between the plasma membrane of protoplasts and the agarose matrix are involved in embryoid formation. The authors proposed a model in which the anchorage of the protoplast membrane to the agarose matrix is mediated by RGD-binding proteins connected with microtubules, consequently determining asymmetric division of the cell and polarized development.

The main purpose of the study reported here was to estimate the variability and genetic gain for protoplast division in 52 recombinant inbred lines (RILs) and their parents, PAC-2 and RHA-266. Moreover, we carried out a QTL mapping analysis to characterize genomic regions involved on symmetric and asymmetric division of protoplasts, using the protoplast culture system proposed by Petitprez et al. (1995). QTLs involved on organogenesis and somatic embryogenesis previously identified in the same RILs by Flores Berrios et al. (2000a, b), are compared with the results of protoplast division to detect chromosomic regions involved in common pathways of regeneration.

Materials and methods

Protoplasts culture

Fifty-two RILs developed through single-seed descent (SSD) and their two parents, PAC-2 and RHA-266, were used in this experiment.

Seeds were sterilized in a 5%(v/v) calcium hypochlorite solution for 25 min and rinsed three times in sterile distilled water. The sterilized seeds were germinated and grown for 8 days in Magenta boxes on a MS basal medium (Murashige and Skoog 1962) containing 2% sucrose and solidified with 0.7% agar; the pH of the medium was adjusted to 5.7 before autoclaving. Cultures were maintained in a light/dark cycle (16/8 h) at 25°C. Upon harvest, shredded hypocotyls were incubated for 1.5 h in S solution (Lenée and Chupeau 1986) macerated for 12 h in the dark, at 25°C, in the same medium but supplemented with cell degrading enzymes according to Burrus et al. (1991). Protoplasts were isolated and purified as described by Chanabé et al. (1989), and the culture density was adjusted before plating. Protoplasts were cultured in 55-mm-diameter plastic petri dishes according to the agarose bead method described by Shillito et al. (1983). The final density was 50,000 protoplasts/ml. The protoplast suspensions were gently mixed with a solution of 0.5% agarose (final concentration) and then pipetted in droplets of 0.25 ml into petri dishes (4–5 beads/dish) and incubated at 4°C for 1.5 h. The droplets were submerged by 12 ml of TLD liquid medium (Chanabé et al. 1991). Dishes were sealed with parafilm and maintained in the dark at 25°C for 10 days.

The experiment was designed as a randomized complete block with three replications and 54 genotypes (52 RILs and the two parents). Each replication consisted of ten petri dishes. In order to compare protoplast responses, 10 days after culture we examined ten random microscopic slides from each petri dish and calculated total division per 100 protoplasts (TOTD) and asymmetric division per 100 protoplast (ASYD).

QTL analysis

The linkage map used was described previously (Flores Berrios et al. 2000a). Briefly, a set of 99 RILs and their parents, PAC-2 and RHA-266 were used for DNA extraction and amplified fragment length polymorphism (AFLP) analysis. This set of RILs was screened with 20 AFLP primer combinations and a linkage map was constructed based on 254 linked loci out of 333 AFLP bands scored.

Variances and components of the variances were computed by equating mean squares to their expectations. Narrow-sense heritability was calculated according to Kearsey and Pooni (1996), using least square estimates of the genetic parameters.

The chromosomal location of QTLs for:total division per 100 protoplasts (TOTD) and asymmetric division per 100 protoplasts (ASYD) were resolved by composite interval mapping (CIM) using QTL CARTOGRAPHER version 1.13 model 6 (Basten et al. 1999). A window size of ten centimorgans and 15 markers were chosen to account for background. The data were transformed using arcsin of square roots for percentages. At each interval, the significance of the association is tested by a likelihood ratio statistic. (Haley and Knott 1992).

Results and discussion

In vitro regeneration responses

In our previous investigations (Flores Berrios et al. 2000 a, b) on organogenesis and somatic embryogenesis a high genetic variability was demonstrated in a set of RILs and their two parents, PAC-2 and RHA-266. Genetic gain, when the best parent was compared with 10% selected RILs was significant for the traits studied.

Analysis of variance of 52 RILs and their parental genotypes (PAC-2 and RHA-266) in the present experiment showed also a high significant genotype effect for protoplast division parameters (Table 1). Estimates of additive variance (V_A) and environmental variance (V_E) were determined for total protoplast division (V_A= 111.02, V_E = 15.88) and asymmetric division (V_A= 99.89, V_E = 11.81) from the mean squares of the ANOVA tables using least squares estimates. Parental inbred line RHA-266 showed significantly higher values than PAC-2 for total division per 100 protoplasts (TOTD) and asymmetric division per 100 protoplasts (ASYD). Genetic variability in protoplast culture responses in sunflower has been also observed by

Chanabé et al. (1991), Burrus et al. (1991), Krasnyanski and Menczel (1995), Wingender et al. (1996) and Bolandi et al. (2000). The difference between the sum of the recombinant inbred lines ($\bar{X}F_8$) and their parents ($\bar{X}P$) was not significant for ASYD, indicating that the RILs in this experiment are representative of the total possible recombinant lines from the cross PAC-2 × RHA-266. The opposite phenomenon was observed for TOTD, showing the lack of genotype number for this character (Table 2).

The best parent (RHA-266) compared with the best RIL (LR11) and with the mean of 10% of selected RILs, presented significant differences for the two traits TOTD and ASYD. This phenomenon, considered as genetic gain, might be due to the polygenic control of protoplasts division and the accumulation of favourable alleles for protoplast division ability in the RILs. Narrow-sense heritabilities were 0.87 for TOTD and 0.89 for ASYD, indicating that selection for these traits is possible in progenies of crosses.

Table 1 Mean squares for protoplast division in sunflower RILs and their two parents

Source of variation	*df*	TOTD[a]	ASYD[b]
Total	161	236.05	209.31
Genotype	53	682.04***	611.16***
Block	2	85.77 ns	27.73 ns
Residual	106	15.88	11.81

*** Significant at $P = 0.001$; ns, not significant
[a] Total division per 100 protoplasts
[b] Asymmetric division per 100 protoplasts

Table 2 Genetic gain (GG) and narrow-sense heritability (h^2) for total division per 100 protoplasts (TOTD) and asymmetric division per 100 protoplasts (ASYD) in RILs of sunflower

	TOTD	ASYD
RHA266 (P1)	47.17	42.50
PAC2 (P2)	7.58	3.84
P1-P2	39.59*	38.66*
$\bar{X}_P=(P1+P2)/2$	19.79	19.33
$\bar{X}_{RILs}$[a]	29.38	22.07
$\bar{X}_{RILs}-\bar{X}_P$	9.59*	2.74 ns
Best RIL (BRIL)	63.33	51.41
GG[c] = BRIL[a]-BP[b]	16.16*	8.91*
10% SF_8L[e]	57.45	48.99
GG[d]=10%SF_8L-BP	10.28*	6.49*
h^2	0.87	0.89

* Significant at $P = 0.05$; ns, not significant
[a] Mean of all recombinant inbreds lines
[b] Best parent (RHA-266)
[c,d] Genetic gain when the best RIL or 10% of the selected RILs are compared with the best parent ('RHA-266').
[e] 10% of the best recombinant F_8 lines

Table 3 Map positions and effect of QTLs detected in recombinant inbred lines (RILs) for protoplast division in sunflower

Trait	QTL	Linkage group	position[4]	Log-likelihood	Proportion of the phenotypic variance explained[a]	Additive effect (*a*)
TOTD[b]	*ptd1.1*	I	90.6	15.6		
	ptd7.1	VII	50.4	5.1	0.11	0.0042
	ptd7.2	VII	172.4	4.7	0.02	–0.0008
	ptd8.1	VIII	29.0	12.1	0.02	0.0006
	ptd8.2	VIII	88.1	12.5	0.06	0.0038
	ptd10.1	X	87.2	8.5	0.06	0.0034
	ptd13.1	XIII	25.4	10.7	0.07	–0.0056
	ptd14.1	XIV	74.7	14.3	0.05	0.0024
	ptd14.2	XIV	125.9	7.5	0.18	0.0244
	ptd15.1	XV	37.7	7.53	0.03	–0.0025
	ptd15.2	XVII	127.8	4.4	0.03	–0.0019
	ptd17.1	XVII	37.6	11.9	0.02	0.0007
					0.07	–0.0025
ASYD[c]	*pad1.1*	I	90.6	15.1	0.16	0.0092
	pad2.1	II	64.3	7.4	0.05	0.0038
	pad3.1	III	2.1	8.9	0.24	0.0163
	pad3.2	III	39.1	8.5	0.08	–0.0083
	pad3.3	III	112.7	4.38	0.03	–0.0032
	pad9.1	IX	9.4	10.6	0.11	–0.0070
	pad9.2	IX	89.6	3.8	0.02	0.0018
	pad10.1	X	22.1	4.1	0.03	–0.0024
	pad15.1	XV	53.2	16.5	0.24	–0.0134
	pad15.2	XV	100.2	7.2	0.07	0.0049
	pad17.1	XVII	139.1	9.1	0.09	–0.0045

[a] Value determined by QTL CARTOGRAPHER, version 1.13 (Basten et al. 1999)
[b] Total division per 100 protoplasts
[c] Asymetric division per 100 protoplasts
[d] Expressed, in Kosambi cM, from north of the linkage group (Flores-Berrios et al. 2000)

QTL analysis for protoplast division

Twelve putative loci associated with TOTD were identified. Eleven QTLs were also detected for asymmetric division per 100 protoplasts (ASYD) (Table 3). The QTLs were designed: *ptd* (protoplast total division) and *pad* (protoplast asymmetric division) followed by a number corresponding to the linkage group and corresponding number of the QTL on the linkage group. In the present study, the use of nearly fixed inbred lines (F_8) produced through many recombination events was considered to have increased the resolution of mapping putative QTLs. In fact, several linked putative QTLs were resolved on the same linkage groups. Examples are given by *ptd7.1* and *ptd7.2*, *ptd14.1* and *ptd14.2* or *pad3.1*, *pad3.2* and *pad3.3*. The protoplast division traits in our experiment were controlled by different QTLs with high LOD scores but with a relatively small percentage of phenotypic variance explained, particularly for total division (values of phenotypic variance explained ranged from 2 to 18%). All together, the *ptd* QTLs explained 72% of the phenotypic variation of total protoplast variation and *pad* QTLs explained almost all of the phenotypic variation of asymmetric protoplast division.

The transgressive phenotypes recorded and the significant observed genetic gain (Table 2) could be explained by the presence of QTLs of different sign in the same inbred. This dispersion is examplified with QTLs of linkage group XIV (*ptd14.1* and *ptd14.2*) or linkage group III (*pad3.1*, *pad3.2* and *pad3.3*). From a genetic point of view, it is logical to imagine that a large number of genes are involved in the cellular division. This process is essential on plant development, and many physiological mechanism involved in plant development should affect total division of protoplasts. The large number of phenotypic and physiological changes in protoplast growth demonstrates the complexity of plant response to protoplasts culture, and it should be expected that the number of QTL involved in the expression of division traits can be large. Very significant QTLs were detected on linkage group I and XV, involved in both total and asymmetric division of protoplats (*ptd1.1*, *pad1.1* and *ptd15.1*, *pad15.1* respectively). One can speculate that these QTLs may represent common mechanisms involved in cell division. However, QTLs for ASYD and TOTD were not always detected in the same regions, considering the intrinsic or extrinsic fate determining asymmetric cell division (Scheres and Benfey 1999). The chromosomic regions identified can be associated with specific processes of this trait. As an example *pad17.1* not associated with a QTL for TOTD, could represent such a situation.

Analysis of *in vitro* regeneration pathways in sunflower

We reported in this experiment the identification of QTLs for protoplast division together with QTLs for organogenesis and embryogenesis in contigous intervals of linkage group I, XV and XVII (Fig 1). On linkage group I, two QTLs associated with protoplast division (*pad1.1* and *ptd1.1*) are *contiguous* to somatic embryogenesis QTLs (*tee1.1* and *ete1.1*) (Fig 1 A). Linkage group XV exhibit a region where QTLs for organogenesis (*osr15.1*), somatic embryogenesis (*tee15.1*) and protoplast division parameters (*pad15.2, ptd15.2*) are clustered (Fig 1B). Linkage group XVII harbor a region involved in organogenesis and somatic embryogenesis parameters:*ose17.1, osr17.1, ete17.2* (Fig 1 C). These results clearly define three regions of the genome that are of upmost importance for the traits under study.

The induction of cells toward organogenesis and somatic embryogenesis involve a reprogramming of gene expression and the differentiation of the induced cells into embryos or tissue. Luo and Koop (1997) described that highly similar process to those in the somatic embryos seem occur in early division pattern of protoplast culture. Petitprez et al. (1995) showed that the protoplasts with asymmetric division display a polar organisation at the onset of their development giving rise to compact embryo-like structures. Studies in plant cell reveal that transmembrane proteins could play structural and signalling roles in controlling cell polarity and morphogenesis (Quatrano et al. 1991, Wyatt and Carpita 1993). One can thus speculate that some QTLs associated with the control of protoplast division could be important in the initiation of cell division occuring during the first steps of organogenesis and somatic embryogenesis. In particular, associations between QTLs for asymmetric protoplast division and somatic embryogenesis could be expected. This is in fact the case on linkage group I, XV and XVII. It is difficult to speculate about the biochemical background of the genetic differences in regeneration ability. Even a physiological description can be done in different ways. The common regions found for all organogenesis traits in linkage groups I, XV and XVII could thus be considered to control the early division patterns. If morphogenesis occurs through a common pattern of gene expression for different shapes or organs, thus clustering of QTLS involved in both somatic embryogenesis and organogenesis may represent genetic systems involved in developmental genetics. Targets such as the cluster *ose17.1, osr17.1, ete17.2* could be of interest with respect to this hypothesis.

The correspondence observed between the chromosomal regions identified as potentially important in the control of *in vitro* regeneration is of interest for testing hypothesis regarding linkage vs. pleiotropy and the identification of regions as candidates for fine structure mapping of *in vitro* regeneration. From a plant improvement perspective, this implies that additional efforts such as the use of large populations will be required to recover recombinants in the event of repulsion linkages. The results of our experiments should be considered as the first step in molecular studies of regeneration in sunflower, allowing the possibility to identify:(1) candidates genes determining *in vitro* regeneration expression, (2) loci where regeneration traits will have to be

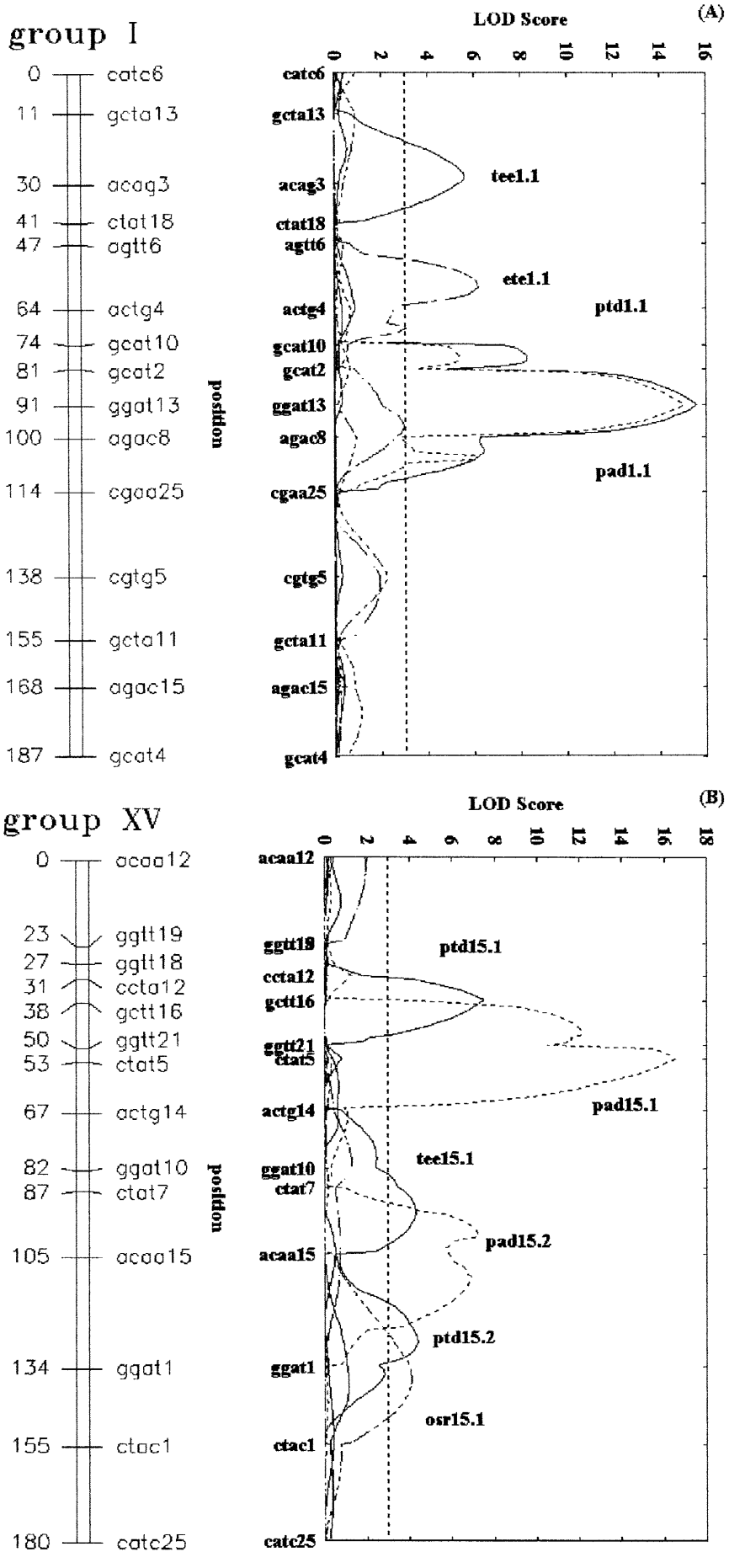
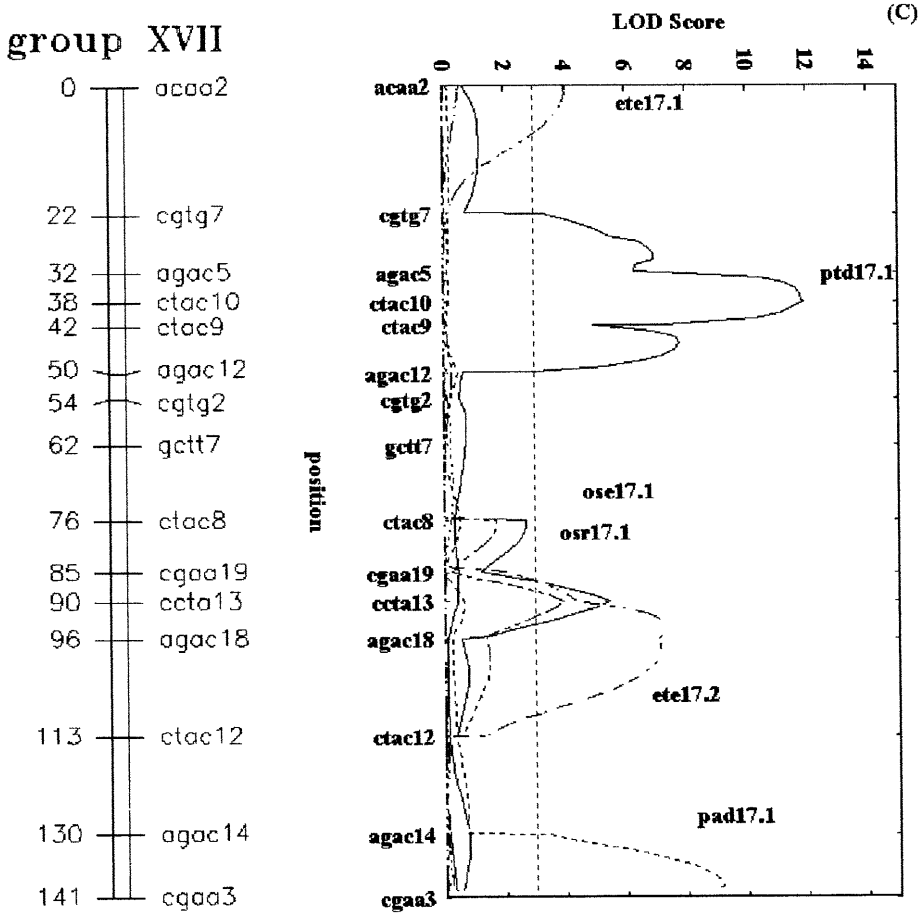


Fig 1A–C Genetic maps and LOD plots showing some location of putative QTLs associated with organogenesis, somatic embryogenesis and protoplast division detected by composite interval mapping (CIM). *ose*: organogenic shoots per explant, *osr*: organogenic shoots per regenerant explant, *tee*: total embryogenic explants, *ete*: embryos per total explant, *ptd*: protoplast total division and *pad*: protoplast asymmetric division. (A) linkage map of linkage group I, (B) linkage map of linkage group XV, (C) linkage map of linkage group XVII



accessed in other germplasm sources, (3) pleiotropic effects on regeneration traits, and (4) regions of the genome important for a range of regeneration traits where no candidate loci have been identified. Additional research works are needed to more accurately locate and identify the specific genes controlling regenerability in sunflower. Although the control of somatic embryogenesis and organogenesis is polygenic, the most important aspect of QTL analysis with respect to breeding procedure is to detect major, previously unknown loci. It should thus be possible to use molecular markers to facilitate the transfer of regenerability into elite, recalcitrant genotypes.

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