Somatic embryogenesis from thin epidermal layers in sunflower and chromosomal regions controlling the response

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Abstract

Somatic embryogenesis of sunflower recombinant inbred lines from the cross 'PAC-2' × 'RHA-266' was investigated and three QTLs were resolved for the number of embryogenic explants per 100 explants cultured. In order to increase the resolution in our mapping of the QTL regions, two consistently highly embryogenic lines 'LR35' and 'C40' that have alleles with positive effects at all three loci were crossed to a poorly embryogenic line 'C149' having alleles with negative effects at these three QTLs. The genotypes of F2 plants of both crosses were determined using a large number of AFLP and some SSR markers and two linkage maps were constructed. Based on the marker profile at QTL regions, 16 F2 plants, 9 presenting QTLs expected to produce F3 families with high and 7 with low expected embryogenic responses, were selected and somatic embryogenesis of F3 families was studied. In 14 out of 16 cases the phenotype of F3 families agreed with the genotype of their respective F2 plants. Therefore, these three QTLs accounted for most of the embryogenic variations. This study enabled the localization of the QTL regions to much shorter intervals and the identification of four AFLP markers on linkage groups 5 and 13 closely linked to somatic embryogenesis potential.
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1. Introduction

The ability to regenerate large numbers of plants from cultured tissues is important for the successful application of genetic engineering to crop plants. A variety of techniques for regenerating sunflower by organogenesis [1,2] or somatic embryogenesis [3–6] have been developed. Organogenesis deals with the production of a unipolar structure, namely the shoot, while somatic embryogenesis leads to the production of a bipolar structure that develops into the root/shoot axis [7]. Culture systems successful for plant regeneration in different species are usually difficult or impossible to adapt to sunflower, although regeneration efficiency appears to be rather high in direct regeneration systems. Among these, direct somatic embryogenesis is distinguished by its rapidity and the vigorous sunflower plantlets that can be easily transferred to the greenhouse [8]. Hypocotyl-derived epidermal thin layers [6]

and immature embryos [4,5,9,10] have been used successfully for the direct induction of somatic embryos.

Regeneration capacity is influenced by cultural conditions, genotype and their interactions [11–13]. While Yu and Pauls [14] and Crea et al. [15] have suggested that somatic embryogenesis in tetraploid alfalfa is controlled by two complementary genes, other studies with other species have resulted in identification of several relatively broad genomic regions (quantitative trait loci, QTLs) each controlling different percentages of phenotypic variations [16]. Previous studies have suggested that the genetic control of morphogenesis in sunflower is rather complex and is controlled by several QTLs [11,12,17–21]. QTLs are usually mapped into large genomic regions that are far too large for positional cloning. Several mapping approaches have been developed to increase the mapping precision, but even with these methods it is not feasible to map a QTL of moderate effect to a sufficiently narrow confidence interval [22]. Therefore, methods have been proposed to further confirm the confidence interval during or after localizing the QTLs of interest. Construction and use of near isogenic lines (NILs) [23], so-called stepped aligned

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inbred recombinant strains (STAIRs) [24], interval-specific congenic strains (ISCS) [22], and QTL isogenic recombinants (QIR plants) [25] are some important options for increased resolution in QTL mapping. In all of these approaches, the main idea is to homogenize the genetic background and focus only on the QTL region(s). Another strategy to enhance the resolution of the QTL region is to start with two parents that differ at the QTL of interest but are homogenous for most of the rest of their genome. In theory, any two inbred lines derived from the same single-cross hybrid should share 50% of their genome because they are inherited from the same parents. In F2 populations of such crosses, two cycles of meiosis have occurred with concomitant cross-overs in the QTL region which should increase the number of markers and map resolution in those regions. The transfer of genes controlling regeneration to elite germplasm, via crossing may be one of the most effective means of realizing the potential advantages of tissue culture. An interesting aspect of tissue culture response QTLs/genes is that they can be used at the very same time, both as transgenes and as safe marker genes in transforming recalcitrant genotypes through genetic engineering [26].

The aim of this study was to fine map sunflower QTLs related to somatic embryogenesis through increasing the number of markers and the rate of cross-over in QTL regions in order to find markers closely linked to somatic embryogenesis.

2. Materials and methods

Somatic embryogenesis data concerning recombinant inbred lines (RILs) from a previous study [19] were re-analyzed using an improved version of the genetic linkage map which was constructed with 123 RILs and 333 AFLP markers [27]. QTLs were resolved with QTL Cartographer 1.16 [28] using the composite interval method, model 6, with steps of 2 cM, 15 background markers and a window size of 10 cM. Based on the results of the assays of phenotypic embryogenesis of RILs and their genotypes at QTL regions, two RILs ('LR35' and 'C40') with high, and one ('C149') with low embryogenesis response were selected (Table 1). In order to increase the resolution of genomic regions harboring the QTLs for embryogenesis, two crosses were made ('LR35' × 'C149' and 'C149' × 'C40') which were followed to the F2 generation. The F2 population of the first cross included 376 plants, while in the second population there were 94 plants. Genotypes of the F2 individuals of the first population were determined using 112 AFLP and 8 SSR markers and for the second population with 93 AFLP and 6 SSR markers. Linkage groups were constructed using MAPMAKER/EXP 3.0 [29] with a LOD of 3.0 for the F2 population of the first cross and 4.2 for the second one.

Based on the marker/genotype at the QTL region, 16 F2 plants were selected; 7 from 'LR35' × 'C149' and 9 from 'C149' × 'C40'. Among these 16 selected plants 9 presented alleles with positive effects for high embryogenic response and 7 had alleles with negative effects for it, in both combinations. Upon selfing, these F2 plants were expected to produce F3 families, 9 with high and 7 with low embryogenic responses.

Table 1
Comparison of percentage of embryogenic explants in two F1 hybrids, 16 F3 families and their three parents

	LR35 × C149	C149 × C40
LR35 (high)	68.40	–
C149 (low)	6.57	–
LR35–C149	61.83[*]	–
C40 (high)	–	75.30
C149 (low)	–	6.57
C40–C149	–	68.73[*]
F3-624	–	3.84
F3-726	–	4.95
F3-670	–	5.79
F3-703	–	10.65
F3-605	–	12.62
F3-638	–	17.17
F3-743	–	50.38
F3-687	–	68.25
F3-707	–	82.07
F3-81	4.16	–
F3-194	5.31	–
F3-814	11.49	–
F3-810	58.48	–
F3-825	60.00	–
F3-328	68.13	–
F3-846	85.53	–
F_1	71.77	79.83
$F_1 - \bar{X}p$	34.28[*]	38.89[*]
B_{F3}[a]	85.53	82.07
$B_{F3} - \bar{X}p$	48.04[*]	41.13[*]
$\bar{X}_{BF3}$[b]	68.04	66.89
$\bar{X}_{WF3}$[c]	6.99	9.17
$\bar{X}_{BF3} - \bar{X}_{WF3}$	61.05[*]	57.72[*]
LSD (5%)	19.41	19.41

[*] Significant at $p = 0.05$.
[a] Best F3 families.
[b] Mean of best F3 families.
[c] Mean of weak F3 families.

Parents of the two crosses ('LR35', 'C149', 'C40') as well as their two F1 hybrids and 16 F3 families derived from F2 plants were studied for their embryogenic response. Pericarps were removed from the seeds before they were surface sterilized by 15 min immersion in a solution of 5% (w/v) calcium hypochlorite and five rinses in sterile distilled water. Seeds were germinated in Magenta boxes containing 75 ml of Murashige–Skoog's culture medium [30] to obtain hypocotyls. The pH was adjusted to 5.7 before autoclaving at 120 °C (1.2 bars) for 20 min. Each Magenta box contained eight seeds of the same genotype. Cultures were maintained at 25 °C under a photoperiod of 16 h, with a light flux of 25 μmol m^{-2} s^{-1} (OSRAM 30 W Nature tubes). Eight days after germination, hypocotyls of plantlets were excised from 1 cm above the crown and were cut into 2 cm-long fragments. The thin epidermal layer was taken from these fragments resulting in 4–5 explants from each fragment. The explants were then transferred to a Fourneau Falcon (120 ml) containing 20 ml of MSB liquid medium [6]. This medium contained Murashige and Skoog's [30] basal salt mixture (4.3 g/l), vitamins (1.0 ml/l), Sucrose (30.0 g/l), casein hydrolisate (2.0 g/l), coconut juice-filter sterilized (20–50 ml/l), NAA (1.0 mg/l), BAP

(1.0 mg/l) and pH was set to 5.7. The Fourneau Falcons were placed on a shaker-stirrer (120 rpm) in darkness at 25 °C, and after 5 days, the swollen epidermal layers were transferred to 250 ml Erlenmeyers containing 100 ml of B590 liquid medium. This medium contained Gamborg et al.'s [31] basal salt mixture (3.1 g/l), vitamin (1.0 ml/l), sucrose (90.0 g/l), with a pH set to 5.7. Cultures were incubated for 8 days in darkness at 25 °C with agitation, and then each 8–10 explants were transferred to 99 mm Petri dishes containing MS120 solid medium and were kept at 25 °C in darkness. MS120 contained Murashige and Skoog's [30] basal salt mixture (4.3 g/l), vitamins (1.0 ml/l), sucrose (120.0 g/l), BAP (0.2 mg/l), phytogel (5.0 g/l) and the pH was set to 5.7. After 2 weeks, the embryogenic explants were counted. The experiment was arranged in a randomized complete block design with three replications and about 20 explants for each replication. Statistical analyses were performed for the number of embryogenic explants per 100 explants plated (EE/E%).

3. Results and discussion

Analysis of variance revealed significant differences among F3 families, F1 hybrids and the three parents for embryogenesis (data not presented). Results concerning mean comparisons are summarized in Table 1. The differences between highly embryogenic parents ('LR35' and 'C40') and the low responding one ('C149') were significant. The performance of the F1 hybrids was significantly superior to that of the mid-parent, implying the existence of heterosis and dominant gene action for this trait. Genes with additive and dominant effects on sunflower embryogenesis have been reported by several authors [18,19,32]. The difference between the best F3 family and the mid-parents was significant. The mean of the best families was also significantly higher than the mean of families presenting low values. The dispersion of positive alleles between parents led to offspring having more extreme values than their parents, so-called transgressive segregation, which implies that embryogenesis in sunflower is polygenic in nature. These results could also imply genes with allelic and/or non-allelic interactions [33,34]. Similar results have been reported in sunflower by Henry et al. [32], Bolandi et al. [18] and Flores-Berrios et al. [20]. In each population, F3 families could be easily assigned into highly and poorly embryogenic groups and the difference among these groups was highly significant.

QTL analysis of somatic embryogenesis of the RILs summarized in Table 2 revealed three QTLs for embryogenesis on linkage groups 5, 10, 13. The positive allele of the QTL on

linkage group 5 (LG5) comes from 'RHA266', whereas the positive QTL allele for both QTLs on LG10 and LG13 come from 'PAC2'. The QTLs on LG5 and LG13 accounted for about 14% of the phenotypic variations, while the QTL on LG10 only accounted for 10% of phenotypic variation (Table 2).

Transgressive segregation was not significant in either population, consequently these three QTLs accounted for most of the embryogenic variation. These QTLs and other background markers together accounted for about 80% of phenotypic variations (TR^2). The new map for LG5 in both F2 populations ('LR35' × 'C149' and 'C149' × 'C40') included six new markers in the QTL region, while the QTL region on LG 13 contained two new markers (Figs. 1–3). There were no new markers added to the QTL region of LG10, so it was discarded from further analysis. Among the 16 F3 families, in 14 cases the phenotypic expression was in accordance with their genotypic constitution, supporting the genotype-phenotype relationship.

Graphical genotyping using GGT32 [35] revealed that C40 and LR35 retained all three positive QTL alleles while C149 lacks all of them. As shown in Figs. 1–3, these crosses have efficiently increased the resolution of QTL mapping in the relevant genome regions. The number of cross-over events and bins in the RIL map in the QTL regions on LG5 and LG13 had been low, but in the new maps, there are several smaller bins in the regions of these QTLs, making it possible to localize the QTLs into smaller intervals. Analysis of the graphical genotypes for these F2 plants indicates that a relatively small segment on LG5 in both crosses contains the QTL(s) related to embryogenesis (Figs. 1 and 2). In the genomic region associated with the QTL on LG5 in the 'LR35' × 'C149' population, the marker situated at 107.6 cM, 'e32m501', has the strongest relation with embryogenesis response (Fig. 1). The same QTL in the 'C149' × 'C40' combination was associated with 'e32m6210' which is located at 69.7 cM (Fig. 2). The lack of locus specific markers like SSRs in this linkage group made it difficult to compare the genomic location of these two markers. Nevertheless, any of these markers could be converted to a SCAR-type marker and efficiently used in marker-assisted programs for selecting highly embryogenic genotypes. For the QTL on LG13, the genomic interval between e38m492 and e40m491 with the range between 61.2 and 72.3 cM, showed the strongest association with embryogenesis (Fig. 3).

Somatic embryogenesis is characterized by the development of a somatic cell into an embryo which, like ordinary zygotic embryos, has a bipolar structure leading to shoot and root. This process is controlled by biological and genetic factors. Takada and Tasaka [36] reported that KNOX genes in Arabidopsis can positively or negatively regulate formation of the shoot apical meristem (SAM) during embryogenesis, and that CUp-shaped Cotyledon (CUC) genes are necessary for KNOX gene expression. Additional genes, including STM, AS1 and AS2, down-regulate the expression of KNOX genes during embryogenesis. Thus, the QTLs found in the current study might contain one or some of these regulating genes. In sunflower, Thomas et al. [7] reported that acquisition of the somatic

Table 2
Chromosomal position, sign and magnitude of additive effects and phenotypic variance explained by QTLs for embryogenic explants/100 explants plated

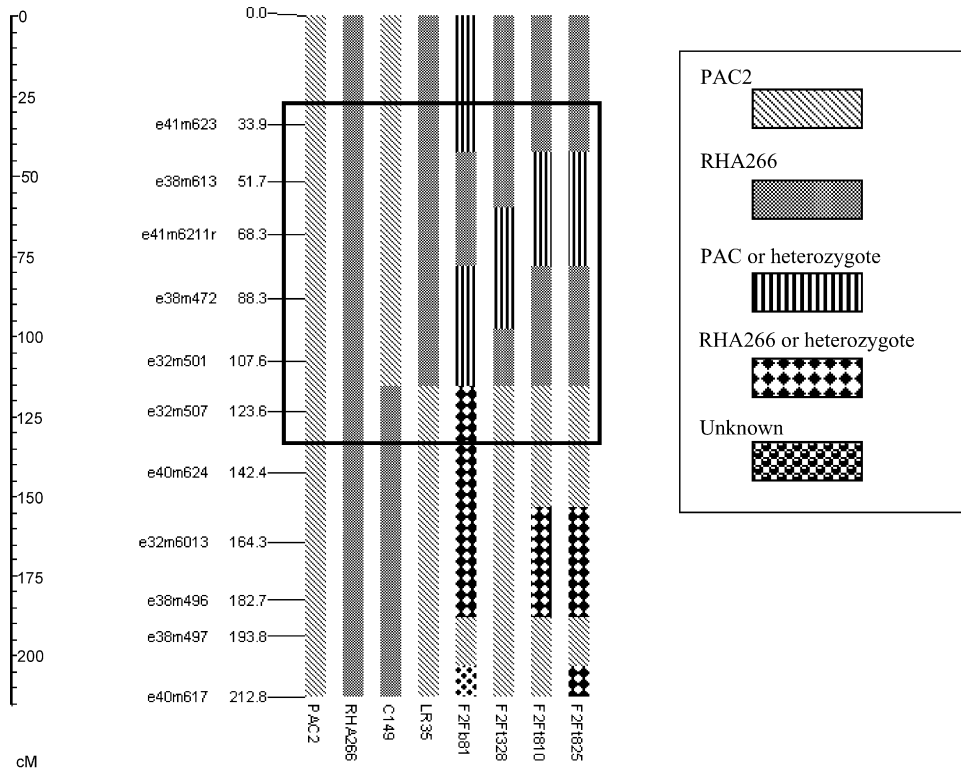| Linkage group	Marker no.	Position (cM)	LOD	Additive effect	R^2	TR^2
5	6	64.51	7.5249	−0.2080	0.1461	0.8329
10	1	0.01	6.4740	0.1774	0.1004	0.8005
13	8	68.52	7.1661	0.2448	0.1407	0.8525

Fig. 1. Comparison of graphical genotypes among selected F$_2$ plants of LR35 × C149 and their parents and grandparents on LG5. The genotypes from left to right are PAC-2, RHA-266, C149, LR35, F$_2$-F$_b$-81, F$_2$-F$_t$-328, F$_2$-F$_t$-810 and F$_2$-F$_t$-825, respectively. F$_t$ shows that the respective F$_2$ has had a high embryogenic F$_3$ family, while F$_b$ represents a poor embryogenic F3 family. The putative region of the QTL is shown in the box.
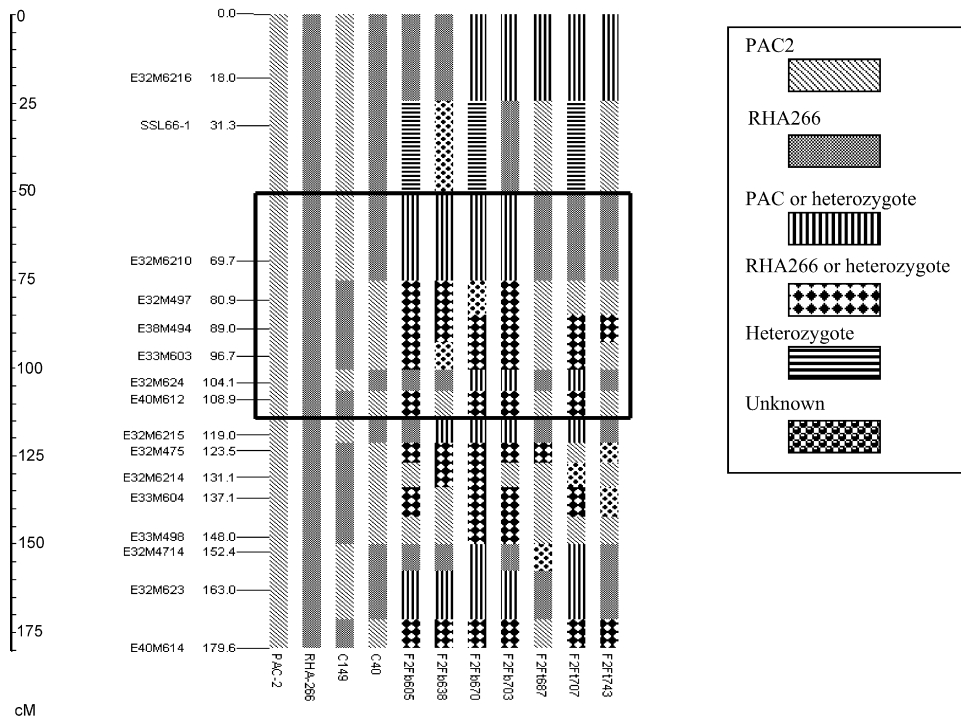

Fig. 2. Comparison of graphical genotypes among selected F$_2$ plants of C149 × C40 and their parents and grandparents on LG5. The genotypes from left to right are PAC-2, RHA-266, C149, C40, F$_2$-F$_b$-605, F$_2$-F$_b$-638, F$_2$-F$_b$-670, F$_2$-F$_b$-703, F$_2$-F$_t$-687, F$_2$-F$_t$-707 and F$_2$-F$_t$-743, respectively. F$_t$ shows that the respective F$_2$ has had a high embryogenic F$_3$ family, while F$_b$ represents a poor embryogenic F$_3$ family. The region of QTL is shown in the box.
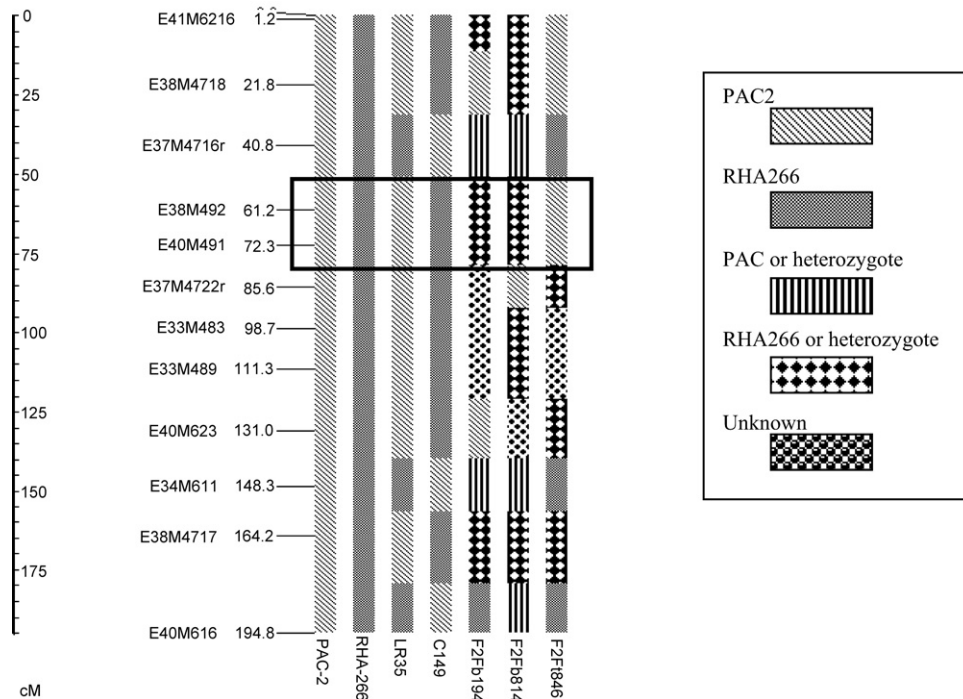
Fig. 3. Comparison of graphical genotypes among selected F2 plants of LR35 × C149 and their parents and grand parents on LG13. The genotypes from left to right are PAC-2, RHA-266, LR35, C149, F2-Fb-194, F2-Fb-814 and F2-Ft-846, respectively. F_t shows that the respective F_2 has had a high embryogenic F_3 family, while F_b represents a poor embryogenic F3 family. The region of QTL is shown in the box.

embryogenesis competence by reactive cells occurs during the first 48 h of culture in a BAP-dependent manner if there is a high accumulation of *SERK* transcripts. They also reported that this acquisition is not affected by the culture conditions, i.e. organogenic or embryogenic conditions used.

Molecular markers closely linked to the QTLs of interest are valuable tools for determining the value of genotypes for traits such as somatic embryogenesis that are difficult or expensive to measure. These markers can be used in marker-assisted backcross breeding programs. They also have the potential to contribute to physical and map based cloning of target genes within the QTL regions. Yu and Pauls [14] in a study on alfalfa identified a RAPD marker that was associated with somatic embryogenesis. Kown et al. [37] reported that a molecular marker *RZ400* was linked to *qAGR-10*, a plant regeneration QTL, and could distinguish between rice lines with poor and good regenerability.

In this study, we have shown that somatic embryogenesis in sunflower is highly genotype-dependent. As well, we showed that this trait is quantitative in nature and identified several genomic regions (QTLs) involved in the phenotype. We efficiently reduced the size of QTL intervals into much shorter regions and identified four markers on linkage groups 5 and 13 related to the trait.

Acknowledgment

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