BIOLOGIA PLANTARUM 50 (3): 421-424, 2006

BRIEF COMMUNICATION

Variability for the *in vitro* culture response in tomato recombinant inbred lines

G.R. PRATTA*,**, R. ZORZOLI**[1], L.A. PICARDI** and E.M. VALLE*

*IBR - CONICET, Fac. Cs. Bioq. Farm., UNR, Suipacha 531 S2002LRK Rosario, Argentina**
*CIUNR, Cátedra de Genética, Facultad de Ciencias Agrarias UNR, CC 14 S2125ZAA Zavalla, Argentina***

Abstract

The aim of this work was to estimate genetic variability for *in vitro* culture response of recombinant inbred lines (RILs) of the genus *Lycopersicon*. The callus percentage (C), the regeneration percentage (R) and the productivity rate (PR) were evaluated 45 d after culture initiation in a set of 16 elite tomato RILs and their parents. The narrow sense heritability (h^2) values were 0.38 ± 0.04 for C, 0.46 ± 0.04 for R, and 0.28 ± 0.03 for R, while the genetic correlation (r_g) values were -0.96 ± 0.07 between C and R, 0.81 ± 0.14 between PR and R, and -0.79 ± 0.16 between PR and C. Three AFLP markers associated to the *in vitro* traits were identified.

Additional key words: amplified fragment length polymorphism (AFLP), callus production, *Lycopersicon esculentum*, *Lycopersicon pimpinellifolium*, plant breeding, shoot formation.

Intra and interspecific variability for callus proliferation and shoot regeneration has been widely reported in the genus *Lycopersicon* (Tal *et al.* 1977, Pratta *et al.* 1997). Dedifferentiation of leaf explants into a callus, either followed or not by shoot formation, was dependent on genotype, culture medium and physiological stage of the donor plants. Although genetic control of *in vitro* culture traits was investigated in various crops (Kuroda *et al.* 1998, Nestares *et al.* 1998, Ogburia 2003/4) there is not enough information about the inheritance of callus production and shoot formation in the cultivated tomato (*Lycopersicon esculentum* Mill.) (Frankerberger *et al.* 1981, Pratta *et al.* 2003). Reports on molecular markers associated to the tomato *in vitro* culture responses are even scarcer. Koornneef *et al.* (1993) mapped a high regeneration QTL to chromosome III in an interspecific tomato cross by RFLP analysis. Torelli *et al.* (1996) detected by the differential display technique, some specific mRNA transcripts expressed during the earlier incubation period of tomato explants that were associated to the shoot formation capacity, while Takashina *et al.* (1998) found at least two RAPD and one isoenzymatic markers linked to the high regeneration capacity of the wild *L. chilense*.

The objective of this research was to evaluate both the *in vitro* callus production and shoot formation, and to detect cosegregating AFLP markers in a set of elite tomato genotypes. Our ultimate goal was to contribute to the knowledge of the inheritance of *in vitro* culture response of the tomato.

Seeds of 16 elite tomato recombinant inbred lines (RILs) and their parents (*Lycopersicon esculentum* (L.) Mill. cv. Caimanta and *L. pimpinellifolium* Mill. LA722, included as testers) were sown in seedling trays under greenhouse conditions at the field station "José F. Villarino" (Facultad de Ciencias Agrarias UNR, Zavalla, Argentina, 33°S and 61°W). RILs were the F_7 filial generation (more than 99 % homozygotes) from the interspecific cross Caimanta × LA722. They were developed through a breeding program to obtain long shelf life and high mass tomato genotypes through divergent and antagonic selection. *In vitro* culture was performed according to Pratta *et al.* (2003). A completely randomized design was used, in which the individual plants (N = 6 per RIL) were the replications. Six to ten explants were tested per plant, but some losses were produced during incubation. The final number of tested plants and explants is shown in Table 1.

Received 24 August 2004, *accepted* 2 July 2005.
Abbreviations: C - callus percentage; h^2 - narrow sense heritability; PR - productivity rate; R - regeneration percentage; r_g - genetic correlation; RILs - recombinant inbred lines.
[1] Author for correspondence; fax: (+54) 341 4970080, e-mail: rzorzoli@fcagr.unr.edu.ar

Callus percentage [C = (number of explants that only produced callus)/(total number of explants) × 100], regeneration percentage [R = (number of explants that differentiated into shoot primordia)/(total number of explants) × 100] and productivity rate [PR = (total number of developed shoots)/(total number of explants)] were evaluated 45 d after culture initiation. The normal distribution of each *in vitro* trait was tested according to Shapiro and Wilk (1965). This test showed that only C and R were normally distributed (W = 0.91, and W = 0.95, ns). The [log (PR + 0.001) + log (PR + 2)] was calculated to obtain an adequately normal distribution (W = 0.89, $P < 0.05$). Thus, analyses on PR were made with these transformed values because a normal distribution was necessary to appropriately apply a quantitative genetic approach. Mean values of C, R, and PR were classified by the Duncan multiple range test, and the narrow sense heritability (h^2) and the genetic correlation (r_g) among traits were estimated by *ANOVA* and *ANCOVA*, respectively (Kearsey and Pooni 1996).

Three plants per genotype were randomly chosen for molecular marker analyses. Total genomic DNA was extracted from young leaves with the DNeasy kit (*Qiagen*®, Frankfurt, Germany). The standard AFLP protocol was followed, and selectively PCR amplification was made with three pairs of MseI +3 and EcoRI +3 primer's combinations selected in a previous experiment (Pratta *et al.* 2002):
A combination, 5'-GATGAGTCCTGAGTAACTA-3'/ 5'-GACTGCGTACCAATTCAGA-3',
B combination, 5'-GATGAGTCCTGAGTAACAT-3'/ 5'-GACTGCGTACCAATTCAGC-3',
N combination, 5'-GATGAGTCCTGAGTAACAT-3'/ 5'-GACTGCGTACCAATTCATC-3'.
Amplified fragments were separated on a 60 g dm^{-3} acrylamide/bis-acrylamide (20:1), 7.5 M urea and 1× TBE gel, and stained with silver nitrate (Blears *et al.* 1998). Polymorphism among RILs was detected as presence/ absence of each fragment, and the Mendelian segregation 1:1 was verified by the χ^2 test for polymorphic fragments. Associations between Mendelian segregating fragments and the *in vitro* culture traits were assessed by the single point analysis method of *ANOVA* (Tanksley 1993).

In vitro callus and shoot primordia proliferation were observed in all genotypes, but regeneration of fully developed shoots was detected just in some of them. Differences among genotypes were detected for C, R and PR (Table 1). Comparisons among parents were also made by the Student *t*-test to avoid the effects of the large common error used by the Duncan test, and significant differences for C, R and PR were found ($P < 0.05$). General mean values of RILs were 62.00 for C, 36.00 for R and 0.42 for PR. Some RILs (1, 8, 10 and 12) had more extreme values than Caimanta and LA722. Pratta *et al.* (1997) concluded that dedifferentiation, *e.g.* callus production, appeared to be a common fact among *Lycopersicon* genotypes whereas shoot formation was restricted to certain ones, as also found in this set of elite RILs. Halámková *et al.* (2004) reported similar results for barley genotypes. Though callus production previous to

Table 1. The callus percentage (C), the regeneration percentage (R), and the productivity rate (PR) of the 16 elite tomato recombinant inbred lines (RILs) and their parents (Caimanta and LA722). Values are means ± standard errors. Different letters indicate significant differences ($P < 0.05$) among genotypes by the Duncan multiple range test. *N* - number of plants per genotype, *n* - total number of cultured explants per genotype.

Genotype	*N*	*n*	C	R	PR
Caimanta	6	38	31.64 ± 10.36 ^{cd}	68.36 ± 10.36 ^{ab}	1.16 ± 0.62 ^{a}
LA722	6	36	58.73 ± 6.58 ^{bc}	41.27 ± 6.58 ^{c}	0.56 ± 0.23 ^{ab}
RIL 1	6	50	73.23 ± 9.59 ^{ab}	26.77 ± 9.59 ^{cde}	0.62 ± 0.24 ^{ab}
RIL 3	6	30	31.95 ± 11.47 ^{cd}	68.05 ± 11.47 ^{ab}	1.13 ± 0.65 ^{a}
RIL 4	6	44	65.70 ± 7.61 ^{abc}	34.30 ± 7.61 ^{cde}	0.18 ± 0.09 ^{ab}
RIL 5	6	41	48.43 ± 9.51 ^{bc}	51.57 ± 9.51 ^{bc}	0.75 ± 0.33 ^{ab}
RIL 6	6	26	61.67 ± 11.45 ^{abc}	38.33 ± 11.45 ^{bcd}	0.40 ± 0.14 ^{ab}
RIL 7	5	36	46.80 ± 16.03 ^{bcd}	53.20 ± 16.03 ^{bc}	0.85 ± 0.73 ^{ab}
RIL 8	6	29	69.45 ± 16.34 ^{ab}	13.89 ± 9.04 ^{de}	0.00 ± 0.00 ^{c}
RIL 9	6	45	67.89 ± 7.25 ^{ab}	23.41 ± 9.64 ^{cde}	0.35 ± 0.19 ^{b}
RIL 10	6	32	80.29 ± 10.89 ^{ab}	16.93 ± 9.63 ^{de}	0.00 ± 0.00 ^{c}
RIL 11	6	30	32.23 ± 4.98 ^{cd}	67.77 ± 4.94 ^{ab}	0.85 ± 0.26 ^{ab}
RIL 12	6	29	93.89 ± 3.89 ^{a}	3.33 ± 3.33 ^{e}	0.00 ± 0.00 ^{c}
RIL 13	6	27	84.26 ± 8.65 ^{a}	15.74 ± 8.65 ^{de}	0.18 ± 0.13 ^{bc}
RIL 14	5	35	63.14 ± 13.87 ^{abc}	36.86 ± 13.87 ^{cd}	0.32 ± 0.13 ^{b}
RIL 15	6	43	74.03 ± 8.23 ^{ab}	25.97 ± 8.23 ^{cde}	0.12 ± 0.09 ^{bc}
RIL 16	6	28	16.03 ± 7.76 ^{d}	83.97 ± 7.76 ^{a}	1.13 ± 0.46 ^{a}
RIL 18	6	35	84.03 ± 10.78 ^{a}	15.97 ± 10.78 ^{de}	0.13 ± 0.10 ^{bc}

shoot formation generally increases the rates of somaclonal variation, Bhatia and Ashwath (2004) concluded that genetic fidelity of tissue cultured *Lycopersicon* plants can be maintained if appropriate protocols are used.

The h^2 values were 0.38 ± 0.04 for C, 0.46 ± 0.04 for R, and 0.28 ± 0.03 for PR ($P < 0.01$ in all cases). For R, half of the phenotypic variation was due to additive variance as indicated by the h^2 values. Instead, a greater amount of dominance and/or epistatic variance was found for C and PR. Previously, Frankerberger *et al.* (1981) reported the absence of non-additive effects for the shoot-forming capacity in a diallel cross among selected tomato genotypes but in those experiments wild germplasm was not included. Pratta *et al.* (2003) performed a diallel analysis among cultivated and wild genotypes of the *in vitro* culture response and reported that additive and non-additive effects were involved in the expression of callus production and shoot formation, the latter effects being more important on the productivity rate. Accordingly, this trait had the lowest h^2 value in the present report. The r_g values were -0.96 ± 0.07 between C and R, 0.81 ± 0.14 between PR and R, and -0.79 ± 0.16 between PR and C ($P < 0.01$ in all cases). All genetic correlations were high and significant, indicating that pleiotropy and/or close linkage are involved in the expression of these *in vitro* traits.

As expected, no difference among the three plants within the same genotype was observed for the AFLP profiles. A total of 97 fragments were selectively amplified with the three pairs of primer's combinations. Fragments were identified with a letter indicating the corresponding combination and a number indicating the relative position on the gel. Of the 97 selectively amplified fragments, 70 (72 %) were polymorphic among lines, while just 31 of these polymorphic fragments (44 %) adjusted to the Mendelian segregation 1:1 ($P > 0.05$, ns). Even though the number of RILs was low, checking for segregation minimized the effects of linkage drag that could be caused by selection for fruit shelf life and mass. Three fragments (A8, N4 and B16) are analyzed below. It was reported that the low number of RILs prevented the construction of a reliable map to localize the fragments (Flores Berrios *et al.* 2000). Therefore, associations between a given AFLP fragment and each *in vitro* culture trait were assessed by presence *vs*. absence of a fragment as the variation source and the different traits as the dependent variable. When a one-way *ANOVA* model was significant, the fragment appeared to correlate with the particular *in vitro* trait. The A8 fragment was simultaneously associated to C and R. Mean values of the group of RILs defined by the presence of A8 were 70.25 for C and 27.49 for R. Mean values of the group of RILs defined by the absence of A8 were 48.37 for C and 50.17 for R. The fragments N4 and B16 were associated to R and PR, respectively. Mean value of R was 24.39 in the group of RILs defined by the presence of N4, and 47.60 in the group of RILs defined by the absence of this marker. For PR, mean values were 0.25 and 0.70 in the groups of RILs defined by the presence or the absence of B16, respectively. All these mean values were significantly different from the general mean of C, R and P ($P < 0.05$). Flores Berrios *et al.* (2000) also found that either common or singular AFLP markers were associated to two closely related *in vitro* traits (the number of shoots per total explants and the number of shoot per regenerating explants) in a set of sunflower RILs.

The relatively low proportion of phenotypic variance explained by the AFLP marker (A8: 0.26 for C and 0.24 for R, N4: 0.22 for R and B16: 0.33 for PR) indicates that additive gene effects are not predominant in the expression of the traits, which is consistent with the moderate to low h^2 values of these traits. Flores Berrios *et al.* (2000) also suggested such a relationship. The common AFLP marker A8 with pleiotropic and opposite mean effects on the callus and the regeneration percentages agrees to the high and negative genetic correlation between them. The other marker affecting R (N4) was not associated with C or PR in this set of elite RILs. Pratta *et al.* (2003) proposed that different genes underlie C, R and PR.

Previously reported molecular markers were associated with increasing the *in vitro* shoot formation (Koornneef *et al.* 1993, Torelli *et al.* 1996, Takashina *et al.* 1998). The AFLP markers detected in the present research were associated with a reduction in the regeneration capacity of the RILs. It is worthy to note that *L. pimpinellifolium* had already been classified as a recalcitrant species (Tal *et al.* 1977, Pratta *et al.* 1997). A8 and B16 markers were present in LA722 (the low regeneration capacity parent) and absent in Caimanta (the high regeneration capacity parent), while the opposite was found for N4. Thus both parents carry genes reducing shoot formation, accounting for the finding that some RILs had either higher or lower values than their parents. Recombination of those genes in segregating generations could produce the accumulation of the 'reducing shoot formation' alleles in some RILs and 'increasing shoot formation' alleles in others, resulting in the extreme phenotypes for the *in vitro* culture response.

References

Bhatia, P., Ashwath, N.: Comparative performance of micropropagated and seed-grown tomato plants. - Biol. Plant. **48**: 625-628, 2004.

Blears, M.J., De Grandis, S.A., Lee, H., Trevors, J.T.: Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications. - J. ind. Microbiol.

Biotechnol. **21**: 99-114, 1998.

Flores Berrios, E., Gentzbittel, L., Kayyal, H., Alibert, G., Sarrafi, A.: AFLP mapping of QTLs for *in vitro* organogenesis traits using recombinant inbred lines in sunflower (*Helianthus annuus* L.). - Theor. appl. Genet. **101**: 1299-1306, 2000.

Frankerberger, E.A., Hasegawa, P.M., Tigchelaar, E.C.: Diallel analysis of shoot-forming capacity among selected tomato genotypes. - Z. Pflanzenphysiol. **102**: 233-242, 1981.

Halámková, E., Vagera, J., Ohnoutková, L.: Regeneration capacity of calli derived from immature embryos in spring barley cultivars. - Biol. Plant. **48**: 313-316, 2004.

Kearsey, M.J., Pooni, H.S.: The Genetical Analysis of Quantitative Traits. First Edition. - Chapman & Hall, London 1996.

Koornneef, M., Bade, J., Hanhart, C., Horsman, K., Schel, J., Soppe, W., Verkek, R., Zabel, P.: Characterization and mapping of a gene controlling shoot regeneration in tomato. - Plant J. **3**: 131-141, 1993.

Kuroda, S., Kato, H., Ikeda, R.: Heterosis and combining ability for callus growth rate in rice. - Crop Sci. **38**: 933-936, 1998.

Nestares, G., Zorzoli, R., Mroginski, L.A., Picardi, L.A.: Cytoplasm effects on the regeneration ability of sunflower. - Plant Breed. **117**: 188-190, 1998.

Ogburia, M.N.: Somatic embryogenesis, plantlet regeneration and micropropagation of cultivars and F_1 hybrids of *Manihot esculenta*. - Biol. Plant. **47**: 429-432, 2003/4.

Pratta, G., Cánepa, L.N., Zorzoli, R., Picardi, L.A.: Diallel analysis of *in vitro* culture traits in the genus *Lycopersicon*. - HortScience **38**: 110-112, 2003.

Pratta, G., Zorzoli, R., Picardi, L.A.: Intra and interspecific variability of *in vitro* culture response in *Lycopersicon* (tomatoes). - Braz. J. Genet. **20**: 75-78, 1997.

Pratta, G., Zorzoli, R., Picardi, L.A., Valle, E.M.: Tomato recombinant inbred lines characterized by AFLP markers - Basic appl. Genet. **15**: 134, 2002.

Shapiro, S.S., Wilk, M.B.: An analysis of variance test for normality (complete samples). - Biometrika **52**: 591-611, 1965.

Takashina, T., Suzuki, T., Egashira, H., Imanishi, S.: New molecular markers linked with the high shoot regeneration capacity of the wild tomato species *Lycopersicon chilense*. - Breed. Sci. **48**: 109-113, 1998.

Tal, M., Dehan, K., Heikin, H.: Morphogenetic potential of cultural leaf sections of cultivated and wild species of tomato. - Ann. Bot. **41**: 937-941, 1977.

Tanksley, S.D.: Mapping polygenes. - Annu. Rev. Genet. **27**: 205-233, 1993.

Torelli, A., Soragni, E., Bolchi, A., Petrucco, S., Ottonello, S., Branca, C.: New potential markers of *in vitro* tomato morphogenesis identified by mRNA differential display. - Plant mol. Biol. **32**: 891-900, 1996.