International Journal of Plant Developmental Biology ©2009 Global Science Books

Genotype Variations of Early Protein Expression During Tomato *in Vitro* Culture

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ABSTRACT

Polymorphism in total protein patterns is an indicator of variability for genes associated with regeneration ability. The objective was to characterize the early expression of proteins involved in tomato organogenesis from leaflet explants of divergent taxonomic genotypes with different regeneration abilities. *Solanum lycopersicum* cv. 'Caimanta' as the highest regenerating genotype, accession LA722 of *S. pimpinellifolium* as the lowest regenerating genotype and F_1 were assayed. *In vitro* culture was performed according to the standard protocol for tomato. Twenty samples of each genotype were analyzed at different days of incubation (from 1 to 10 days). Uncultured explants were the experimental tester. Total proteins were extracted from these samples in phosphate buffer and then separated by SDS-PAGE. Polymorphism was found for polypeptides of 76.6, 55.5, 52.1, 49.7, 44.5, 27.9, 24.7, 24.1, and 19.5 kDa, which accounted for 56% of the total protein patterns. Polymorphic polypeptides between incubation periods were those of 76.6, 49.7, 44.5, and 24.7 kDa. Polymorphic polypeptides between genotypes were those of 52.1, 27.9, 24.1, and 19.5 kDa. Polymorphism of total protein patterns during the first days of incubation, and between divergent taxonomic genotypes with different regeneration ability, indicated variability of genetic expression of the *in vitro* response.

Keywords: organogenesis, plant genetic resources, regeneration ability, *Solanum*

INTRODUCTION

In vitro regeneration of cultivated tomato (*Solanum lycopersicum*) has been a subject of research because of the commercial value of the crop. Numerous studies on plant regeneration from a wide range of tissue and organs of wild type and cultivated tomato germplasm have been conducted (e.g., Faria and Illg 1996; Pratta *et al.* 1997). Dedifferentiation of leaf explants into a callus, either followed or not by shoot formation, has depended on genotype, culture medium and physiological stage of the donor plants. Genetic control of *in vitro* culture traits was investigated in various crops (Kuroda *et al.* 1998; Nestares *et al.* 1998). Pratta *et al*. (1997), utilizing cultivars of *S. lycopersicum* and accessions of the wild taxa *S. lycopersicum* var. *cerasiforme*, *S. pimpinellifolium* and *S. peruvianum* found highly significant differences among genotypes for regeneration ability. Pratta *et al.* (2006) described cv. 'Caimanta' of *S. lycopersicum* as a high regeneration genotype (regeneration percentage >50%) and accession LA722 of *S. pimpinellifolium* as a low regeneration genotype (regeneration percentage <50%). Marchionni Basté *et al.* (2007) evaluated the components of the genetic mean values and variances of the tomato *in vitro* response, and reported a value of 87.93 ± 3.88 for the regeneration percentage in 'Caimanta', 34.44 ± 15.17 in accession LA722, and 76.3 ± 17.9 in their F_1, which did not differ significantly from 'Caimanta', suggesting that dominant alleles determine the high regeneration ability.

Polymorphism in DNA, RNA and protein patterns between genotypes and between different *in vitro* conditions for the same genotype has been considered as a good indicator of genetic variability and differential expression of genes asociated to *in vitro* regeneration abilities in various plants (Komatsuda *et al.* 1993; Martinelli and Gianazza 1995; Taguchi-Shiobara *et al.* 1997; Flores Berrios *et al.* 2000; Vega *et al.* 2007). Studies of polymorphism have improved the knowledge of the genetic control of the trait, finding that genetic mechanisms which control *in vitro* organogenesis are turned on during the first stages of incubation. In tomato, there are insufficient studies on the application of these techniques to *in vitro* regeneration ability: for instance, Koornneeff et *al.* (1993) and Takashina *et al.* (1998) worked with DNA markers, while Torelli *et al.* (1996) studied the RNA transcripts. More recently, Shan *et al.* (2004) reported polypeptide characterization of tomato hypocotyls in *in vitro* culture.

The aim of this work was to characterize variations in protein expression during early phases involved in tomato organogenesis from divergent taxonomic genotypes that have different regeneration ability.

MATERIALS AND METHODS

Plant materials and *in vitro* culture

S. lycopersicum 'Caimanta' as the high regeneration ability genotype and accession LA722 of *S. pimpinellifolium* as the low regeneration ability genotype, together with their F_1 (Caimanta x LA722), were assayed. When plants reached 15 cm in height, leaflets were excised from the third and fourth leaf closer to the apex which served as explants. The *in vitro* protocol described in Marchionni Basté *et al.* (2007) was followed for this experiment. Fifty explants of each genotype were plated. Two complete experiments were carried out independently.

Protein extraction

Two samples per day of incubation from each genotype were analyzed during the first 10 days after plating (total N = 300). Uncul-

Received: 16 December, 2008. Accepted: 6 February, 2009.

Original Research Paper

tured explants (day 0) was considered as the reference material. Each sample was composed by two explants from different individuals of the same genotype (50 mg per explant). They were ground in a pre-chilled mortar with cold acetone. After homogenization, the samples were centrifuged at 4°C for 20 min at 11,500 rpm. The pellet was washed three times with cold acetone and dried at room temperature. Then it was resuspended in 300 µl Buffer B (0.035 M Phosphate Buffer, pH 7.8) containing 35 mM Na_2HPO_4/NaH_2PO_4 pH 7.8, 0.4 M NaCl and 10 mM β-mercaptoethanol.

Protein quantification

After centrifuging for 15 min (10,000 rpm, 4°C), the supernatant was used for calculating the protein content using Bradford's (1976) method. Because of the low protein concentration, extracts were precipitated by 100% TCA. The pellets were washed twice with cold acetone and dry at room temperature and then resuspended in 2% SDS and 0.5% β-mercaptoethanol. The total protein amounts of explants from each genotype at different incubation periods were analyzed by factorial ANOVA. Because of the low number of replications if analysis was made by day, periods composed by two consecutive days were compared in order to increase the statistical significance of the ANOVA.

Protein electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970). The protein samples were mixed with sample buffer consisting of 0.5 M Tris HCl (pH 6.8), 10% glycerol, 4% β-mercaptoethanol, 2% SDS and bromophenol blue as a tracking dye. The preparations were boiled at 90°C for 5 min. The stacking gel consisted of 4% polyacrylamide and the resolving gel had 10% polyacrylamide. The samples were run at constant current of 200 V for 45 min. The gels were stained overnight with Commassie Brilliant Blue (Laemmli 1970) and destained with boiling water, scanned, and analyzed using GelPro Analyzer (MediaCybernetics, Silver Spring, MD, USA). The relative molecular weights were determined using an LMW Calibration kit for SDS Electrophoresis (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Protein patterns analysis

Polymorphism between genotypes and among times of incubation was evaluated by the presence or absence of polypeptides. The protein patterns of F_1 were compared to those of their parents.

RESULTS

The mean values of total protein contents from each genotype at different incubation periods (two days each) are shown in **Fig. 1**. No statistical significance was found for differences among genotypes (F=1.27; *ns*) nor for the interaction (F=0.82; *ns*). Total protein contents were significantly different among periods (F=35.54; p<0.0001).

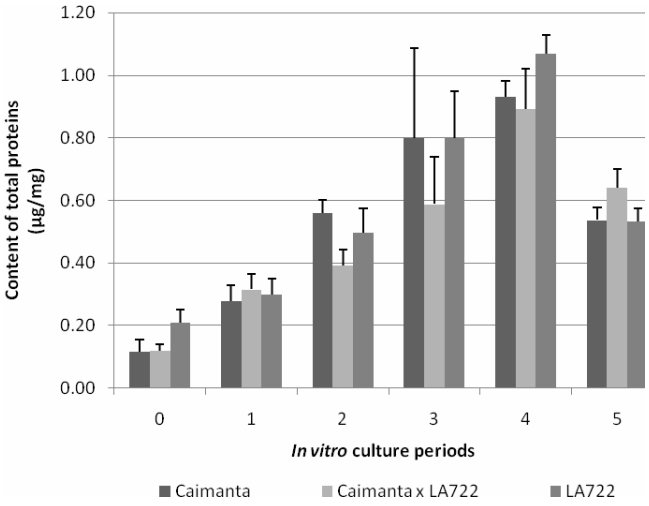

Fig. 1 Content of total protein from each genotype at different incubation periods (two days each). Period 0 = uncultured explant.

A gradual increase of total protein contents of all genotypes was observed until the fourth period (days 7 and 8 of incubation), in which the highest amount of total proteins was found. Then a decrease was detected at the last period (days 9 and 10 of incubation).

SDS-PAGE analysis of total proteins of leaf explants at different days of *in vitro* incubation showed polymorphisms among genotypes and among days of incubation. No visible change in explant morphology was evident at the first two days of *in vitro* culture, but all genotypes presented changes in the polypeptide patterns.

Differences were found between protein patterns from the same genotype at different time of incubation, and from different genotypes at the same period of incubation. The molecular analysis focused on polypeptides which were present in both repetitions. The 16 polypeptides analyzed are shown in **Table 1**.

Whereas polypeptides having molecular weights of 70.2, 66.5, and 46.9 kDa were monomorphic, polypeptides of 80.2, 40.3, 37.6, and 35.0 kDa were monomorphic just during the *in vitro* culture, but polymorphic when considering day 0. Otherwise, more complex polymorphism was found for polypeptides having molecular weights of 76.6, 55.5, 52.1, 49.7, 44.5, 27.9, 24.7, 24.1, and 19.5 kDa, which accounted for 56% of total protein patterns.

Table 1 Protein expression of uncultured explant (day 0) and during the first ten days of *in vitro* culture in the parents-LA722 (P) and 'Caimanta' (C), and their F_1. Presence (+). Absence (-).

PROTEIN	Day 0			Day 1			Day 2			Day 3			Day 4			Day 5			Day 6			Day 7			Day 8			Day 9			Day 10		
(kDa)	P	F_1	C	P	F_1	C	P	F_1	C	P	F_1	C	P	F_1	C	P	F_1	C	P	F_1	C	P	F_1	C	P	F_1	C	P	F_1	C	P	F_1	C
80.2	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
76.6	-	-	-	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	+	-
70.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
66.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
55.5	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
52.1	-	-	-	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	+	-
49.7	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46.9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
44.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	+	-	+	+	+
40.3	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37.6	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
35.0	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
27.9	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-
24.7	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-
24.1	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19.5	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-

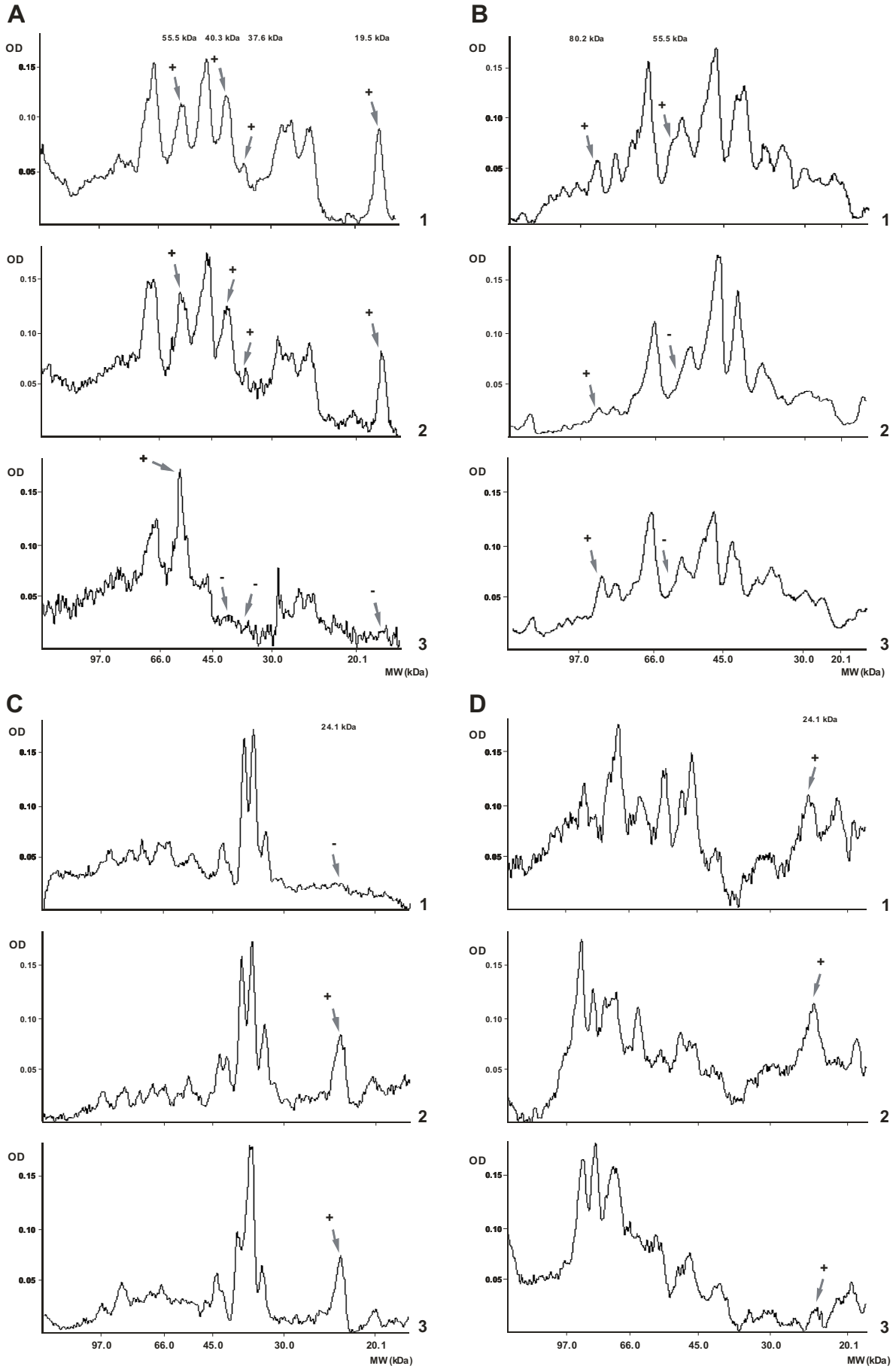

Fig. 2 Laser densitometric scanning maps of total proteins in LA722 (1), F_1 (2), and Caimanta (3) at day 0 (**A**), day 1 (**B**), day 2 (**C**), and day 3 (**D**). Presence (+). Absence (-).

The behavior of the F_1 was like LA722 in the uncultured explants whereas it was more similar to 'Caimanta' during the first days of incubation (**Table 1; Fig. 2**). A differential expression associated with different regeneration abilities of

the genotypes was found. The 55.5 kDa polypeptide was present in all genotypes at day 0 and only in LA722 at day 1. The 24.1 kDa polypeptide was absent in all genotypes at day 0 and present in the genotypes with high regeneration abilities ('Caimanta' and F_1) at day 2. In LA722 it was present just at day 3. This behavior is shown in **Fig. 2** by Laser densitometric scanning maps of total proteins.

DISCUSSION

In vitro regeneration capacity varies among various species within a genus or among cultivars of the same species. In fact, differences in organogenic response and its genetic inheritance have been analyzed in some crops like wheat, barley, corn, alfalfa, and rice, among others (Henry *et al.* 1994; Flores Berrios *et al.* 2000; Fambrini *et al.* 2001). In tomato, Pratta *et al.* (1997) studied regeneration abilities and reported intra- and interspecific variability for the *in vitro* culture responses.

Roberts *et al*. (1989) used a combination of SDS-PAGE and microscopy to characterize stages of white spruce embryo development in order to develop more precise criteria for somatic embryogenesis. Their study revealed that the competence to form somatic embryos is limited to a specific stage of development prior to the accumulation of storage proteins.

The amounts of totals proteins found in this work were similar among genotypes and the highest amount was present at the fourth period (days 7 and 8 of incubation). In concordance with this result, Shan *et al.* (2004) studied different stages of the tomato *in vitro* organogenesis and determined that the highest amounts of soluble proteins were detected during the first 10 days of culture, at the dedifferentiation stage. Branca *et al.* (1994), working with protein electrophoretic patterns of *in vitro* tomato cotyledons in different culture medium compositions, suggested that the increase in protein amount observed over the first 7 days is probably due to plating-induced stress. They also found major patterns changes after seven days of culture. Similar results were found in this work, although differences between genotypes were found even on the first day, increasing polymorphism was detected since day 7 (see **Table 1**).

The equal contents of total proteins indicated that the rate of proteins synthesis and degradation during *in vitro* culture was the same in genotypes with different regeneration abilities, but the molecular polymorphisms among genotypes indicated synthesis and degradation of different proteins.

Buckley and Trigiano (1994) compared the embryogenic potential of *Cercis canadensis* (redbud) ovules cultured during different developmental stages with protein profiles of ovules over time. Differences in staining intensity of six bands were found to be associated with changes in the somatic embryogenic potential of ovules.

An important fact observed in this experiment was that molecular differences were found between days of incubation, which indicated changes in expression due to the *in vitro* conditions. Examples of this behavior are polypeptides of 80.2 and 35.0 kDa which are present in all genotypes only during incubation. They could be specific polypeptides just expressed under the *in vitro* condition. Similar results were found working with tomato hypocotyls by Shan *et al.* (2004), who suggested that different genes would be expressed in a specific time sequence during organogenesis and somatic embryogenesis stages, and that the expression of some genes might inactivate other genes, which would initiate new developmental processes such as dedifferentiation, redifferentiation, and regeneration of complete plants.

A remarkable difference between polypeptides pattern of the uncultured and cultured explants was observed among the three genotypes. Whereas the hybrid showed the same pattern of LA722 in the uncultured explant, at the beginning of incubation it appeared to show a pattern more similar to 'Caimanta', the highest regeneration ability genotype carrying dominant alleles (Marchionni Basté *et al.* 2007). Also differential expression associated with different regeneration abilities of the genotypes was found, such as the 55.5 and 24.1 kDa polypeptides. The first one is present in all the genotypes at the uncultured explant and only in LA722 at day 1. The disappearance of the 55.5 kDa polypeptide would be associated with the appearance of another of 24.1 kDa. In the genotypes with high regeneration abilities, 'Caimanta' and the hybrid, the polypeptide of 24.1 kDa is present at day 2, whereas in the low regeneration ability genotype (LA722) it was detected just at day 3. Summarizing, the absence of the polypeptide of higher molecular mass would be associated with the presence of the lower mass one. This synchronization on the proteomic expression of these two polypeptides took place differentially on the genotypes of high regeneration abilities compared to LA722.

Shan *et al.* (2004) found differences in the protein expression associated with the *in vitro* embryogenic regeneration ability and detected a specific accumulation of a polypeptide of 54.0 kDa on nonembryogenic callus. These results were not corroborated in the experiments due to the use of different explants and genetic materials.

Similar results have been obtained in others species as rice (Chen, 2000), cotyledon of melon (Leshem and Sussex, 1990), sunflower (Vega *et al.* 2007), peanut (Roja Rani *et al.* 2005). All these authors reported differences associated to medium compositions. In melon, the differential protein expression was used as an organogenic marker. Leshem and Sussex (1990) suggested that synthesis of a group of polypeptides having molecular weight of 20-25 kDa in embryogenic calli is essential for redifferentiation and a fall in the synthesis in embryogenic calli could provoke the loss of regeneration ability.

In this work, possible molecular markers of a quantitative trait as *in vitro* regeneration ability were found. These results should be confirmed in a segregating population. Torelli *et al.* (1996), working with differential display, detected specific mRNA transcripts associated with organogenesis of tomato and expressed at early stage of *in vitro* culture. A high regeneration ability QTL on chromosome III was mapped by Koornneef *et al.* (1993) in an interspecific cross by RFLP analysis. Takashina *et al.* (1998) found RAPD and isozyme markers associated with high regeneration ability on wild species *L. chilense*. Three AFLP markers associated with low regeneration ability were reported by Pratta *et al.* (2006) working with recombinant inbred lines derived from an interspecific cross.

Given that many studies had observed changes in genetic expression during the first stage of *in vitro* incubation, proteins present during the first days of *in vitro* culture could become useful markers to predict the future behavior of the explants.

CONCLUSION

Polymorphism of total protein patterns during the first days of incubation, and between genotypes from divergent taxonomic genotypes that have different regeneration ability, indicated variability of genetic expression involved in the *in vitro* response of tomato.

ACKNOWLEDGEMENTS

Thanks to Lic. in Biotech. Valeria A. Martinez for helping in laboratory techniques.

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