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Novel natural genetic variation controlling the competence to form adventitious roots and shoots from the tomato wild relative *Solanum pennellii*

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ABSTRACT

Tomato (*Solanum lycopersicum* L.) is an attractive model to study the genetic basis of adventitious organ formation capacity, since there is considerable natural genetic variation among wild relatives. Using a set of 46 introgression lines (ILs), each containing a small chromosomal segment of *Solanum pennellii* LA716 introgressed and mapped into the tomato cultivar M82, we characterized a high shoot-regeneration capacity for ILs 3-2, 6-1, 7-1, 7-2, 8-2, 8-3, 9-1, 9-2, 10-2 and 10-3, when cotyledon explants were cultivated on medium containing 5.0 μM BAP. F1 seedlings from the crosses 'Micro-Tom × ILs' and 'ILs × ILs' demonstrated that the shoot regeneration capacity of most ILs was dominant and that the regeneration ability of IL8-3 enhanced that of the other ILs in an additive manner. The ILs 3-2, 7-1, 8-3, and 10-2 also exhibited enhanced root formation on MS medium containing 0.4 μM NAA, indicating that these chromosomal segments may contain genes controlling the competence to assume distinct cell fates, rather than the induction of a specific organ. We also performed the introgression of the genes controlling competence into the model system 'Micro-Tom'. The further isolation of such genes will improve our understanding of the molecular basis of organogenic capacity.

1. Introduction

Despite the wide use of adventitious organ formation for biotechnological purposes [1], the molecular basis of this capacity still remains largely unknown [2], though informative results have been obtained using transgenic plants [3–5], mutants [6,7], and gene expression analysis [8–10]. Equally, there is a lack of information about the genetic identity of the natural variation for adventitious organ formation capacity often observed in the wild relatives of cultivated plants [4,11–15]. The study of natural genetic variation for a given developmental process is relevant since it may advance our understanding of how plants adapt to different environments, besides the possibility for further application in biotechnology and agriculture. For instance, natural genetic variation controlling adventitious organ formation *in vitro* may correspond to genes recruited during evolution to improve resources allocation through the formation of supernumerary organs [16], or to cope with stresses, replacing shoots or roots after episodes of fire [17], herbivory [18], or flooding [19].

Studies on the genetic and physiological basis for adventitious organ formation capacity can be ideally pursued on plant model systems, where mutants and other allelic variations can be easily explored [15]. Tomato (*Solanum lycopersicum* L.) presents many attributes of a suitable genetic model [20,21]: it is an autogamous diploid species, with a small sequenced genome (950 Mb) distributed in 12 chromosomes, represented in saturated genetic linkage maps (http://solgenomics.net/), with numerous markers associated with traits of large economic and biological importance, as well as a plethora of well-characterized mutants (http://tgrc.ucdavis.edu/). Besides induced mutations, natural genetic variation can be widely found in wild *Solanum* species from the section *Lycopersicon*, most of which are interfertile and amenable for crossing with the cultivated tomato [22]. These species are valuable sources of quantitative trait loci (QTL), and of allelic variation for major genes [23]. The observation of new phenotypes and identification of novel alleles coming from wild species is facilitated by the use of introgression lines (ILs), which are permanent mapping resource populations [24]. Once identified, the specific effect of a given natural genetic variation can be efficiently

Abbreviations: BAP, benzylaminopurine; CIM, callus-inducing medium; IL, introgression lines; MT, Micro-Tom; NAA, naphthaleneacetic acid; NILs, nearly isogenic lines; RIM, root-inducing medium; *Rg1*, *Regeneration locus 1*; SIM, shoot-inducing medium.

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studied by constructing nearly isogenic lines (NILs) that differ only at a single QTL region or a Mendelian gene [25]. Since the process to obtain NILs may take various generations of backcrossing, the use of a rapid-cycling genotype, such as the tomato cultivar Micro-Tom [26], is highly advantageous. 'Micro-Tom' (MT) is a miniature dwarf determinate tomato cultivar, originally bred for home gardening purposes [27], and later proposed by Meissner et al. [28] as a genetic model system. The small size (8 cm when grown in 50–100 mL pots) and short life cycle (70–90 days from sowing to fruit-ripening) of MT make it a genetic model system comparable to *Arabidopsis*, with the additional advantage of its edible crop status, which brings basic and applied sciences together [29].

Tomato has proved to be an excellent model for studying natural genetic variation controlling *in vitro* regeneration capacity. Among the tomato-related wild species, *Solanum peruvianum* and its sibling species *Solanum chilense* are considered highly organogenic [4,30–32]. The occurrence of *Solanum habrochaites* and *Solanum pennellii* accessions ranging from highly recalcitrant [31,33] to highly organogenic [4,31,33] has also been reported. Other genotypes described by their ability to form shoots *in vitro* includes *Solanum pimpinellifolium* WV700 [34], and the tomato cultivars 'UC82B' [35], 'VFNT Cherry' [36], and 'Lukullus' [37], although the genetic basis of such capacity was only suggested for *S. pimpinellifolium* WV700 [34]. Studying the genetic basis of organogenic capacity in *S. peruvianum*, Koornneef et al. [32] found that this character was associated with two major dominant alleles (named *Rg1* and *Rg2*). *Rg1* is sufficient for shoot initiation in cultured roots, and was mapped to chromosome 3, linked to the *yellow flesh* (*r*) locus [38]. The recessive *r* allele represents a loss of function in the chromoplast-specific phytoene synthase gene [39], conferring yellow color to fruits when introgressed into the *S. lycopersicum* background. It has been hypothesized that other green-fruited species harboring the *r* allele may also have versions of the *Rg1* allele conferring high organogenic capacity [4]. The presence of the *r* allele in the green-fruited species *S. peruvianum* created the opportunity to use it as a morphological marker to introgress *Rg1* into cultivated tomato. Using this procedure, the *Rg1* allele discovered by Koornneef et al. [32] was transferred to the MT [40] and further used to create a NIL that has been proposed as a platform for genetic transformation of the MT model [41], besides to be recently used to study the physiological basis of cell competence [42]. Since previous studies suggested that other loci may control *in vitro* regeneration capacity in tomato [32,34], the identification and mapping of these new loci will provide clues to unravel the signal transduction pathway behind this important developmental process.

Here, we took advantage of the high *in vitro* regeneration capacity of the green-fruited species *S. pennellii* accession LA716 [31], which enabled us to use a collection of 46 ILs, each containing a small chromosomal segment of *S. pennellii* 'LA716' introgressed and mapped into the tomato cultivar M82, to search for natural genetic variation controlling *in vitro* organ formation capacity. We found evidence for superior *S. pennellii* alleles present in at least ten ILs, including IL3-2, which spans the homologous region of the *Rg1* and *r* loci. We also performed the introgression of the selected alleles into the genetic model system 'Micro-Tom', which will allow the creation of NILs necessary for further characterization and isolation of important genes controlling organogenic competence.

2. Materials and methods

2.1. Plant material and breeding

The cultivar 'M82' and the collection of 46 introgression lines (ILs) derived from *S. lycopersicum* cv M82 × *S. pennellii* LA716 [24] were kindly provided by Dr. Roger Chetelat at 'The C. M. Rick Tomato Genetics Resource Center' (http://tgrc.ucdavis.edu/). Tomato (*S. lycopersicum* L.) cv Micro-Tom (MT) and the near-isogenic lines harboring the wild type alleles *Self pruning* (MT-*Sp*), *Uniform ripening* (MT-*U*) and *Dwarf* (MT-*D*) [26] were from the tomato mutant collection maintained at the "Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), Universidade de São Paulo (USP), Brazil (http://www.esalq.usp.br/tomato/). Selected ILs were crossed and backcrossed with MT (as pollen receptor) using the same procedure as previously described for the introgression of the *Rg1* allele into MT [41]. After each backcross, seedlings were evaluated for organogenesis capacity (see below).

2.2. Plant cultivation

Plants were grown in either 150-mL pots (MT) or 10-L pots (ILs) containing a 1:1 mixture of commercial substrate (Plantmax HT, Eucatex, São Paulo; Brazil) and expanded vermiculite, supplemented with 1 g NPK 10:10:10 L^{-1} substrate and 4 g dolomite limestone ($MgCO_3 + CaCO_3$) L^{-1} substrate. Plants were kept in a greenhouse under automatic irrigation (four times a day), at an average mean temperature of 28 °C; 11.5/13 h (winter/summer) photoperiod, and 250–350 μmol m^{-2} s^{-1} PAR by natural radiation reduction with a reflecting mesh (Aluminet – Polysack Industrias Ltda; Leme, SP, Brazil). At flowering stage (about 35 days from sowing), plants were supplemented with NPK (*ca.* 0.2 g/150 mL pot). About 40 days after each crossing, mature fruits were harvested and the seed pulp was removed by fermentation for 12 h using commercial baker's yeast (*Saccharomyces cerevisae*, Fermix, São Paulo; Brazil). Seeds were subsequently washed, air-dried and stored at 10 °C for further use.

2.3. In vitro culture

Seeds from MT, MT-*Sp*, MT-*U* and MT-*D*, 'M82,' *S. pennellii* LA716 and the introgression lines (ILs) were surface-sterilized by shaking in 100 mL of 30% (v/v) commercial bleach (2.7% sodium hypochloride) plus two drops of commercial detergent for 15 min, followed by three rinses with sterile water. The seeds were then germinated on media containing half strength MS salts; half strength B5 vitamins; 15 g L^{-1} sucrose and 6 g L^{-1} agar (Merck, Darmstadt, Germany). Medium pH was adjusted to 5.8 before autoclaving. Approximately 40 seeds were sown per flask containing 30 mL of medium. Cultures were sealed with polyvinyl chloride (PVC) plastic wrap and incubated at 25 ± 1 °C in the dark for 4 d, followed by 4 or 8 d under 16 h photoperiod provided by a 40 W cool white fluorescent tube (*ca.* 45 μmol PAR m^{-2} s^{-1}).

Cotyledons were then isolated from 8- and 12-day-old (after sowing) seedlings. The distal and proximal tips were removed, and the cotyledons were divided transversally in two or three pieces. Explants were placed with the abaxial side down immediately after isolation onto semi solid 'Shoot-inducing medium' (SIM), composed by MS salts with B5 vitamins, 30 g L^{-1} sucrose, 6 g L^{-1} agar, 5 μM benzylaminopurine (BAP) (Sigma, St. Louis, USA), or 'Root-inducing medium' (RIM), which has the same composition of SIM, except by the replacement of BAP with 0.4 μM naphthaleneacetic acid (NAA) (Sigma, St. Louis, USA). During explanting, a Petri dish containing potassium permanganate salts was kept inside the laminar flow hood to avoid ethylene accumulation which can reduce tomato regeneration afterwards [7]. Fifteen cotyledonary explants were cultured per Petri dish (90 × 15 mm), with 6 plates per treatment. Plates were sealed with PVC and maintained under 16 h photoperiod at 25 ± 1 °C for 3 weeks.

After each backcross, seedlings were used as explant (12-day-old cotyledon) for regeneration. Seedlings with cotyledons removed were maintained *in vitro* for later greenhouse

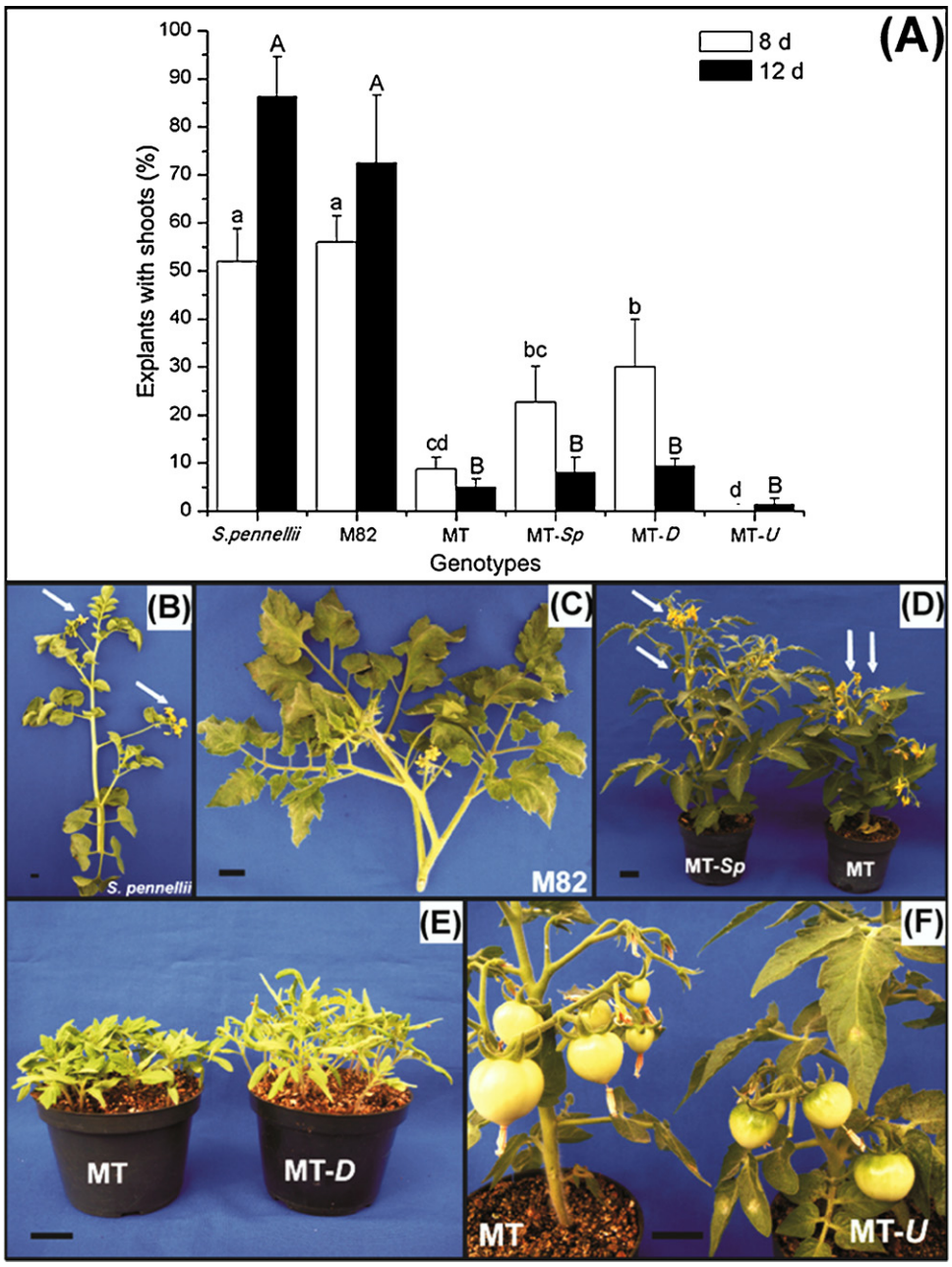

Fig. 1. *In vitro* shoot regeneration capacity of the wild species *S. pennellii* LA716 and various tomato (*S. lycopersicum*) genotypes. (A) 8-day- and 12-day-old seedling cotyledon explants were cultivated during 21 days on MS media with 5.0 μM BAP. The tomato cultivars tested were 'M82' and 'Micro-Tom' (MT), as well as MT near isogenic lines harboring the wild type alleles *Sp* (indeterminate growth), *D* (non dwarf) and *U* (fruits with green shoulders). 'M82' has the mutant alleles *sp* and *u*, while 'MT' harbors the alleles *sp*, *u* and *d*. (B–F) Representative plants of *S. pennellii* (B), 'M82' (C), 'MT-*Sp*' (D), 'MT-*D*' (E) and 'MT-*U*' (F). The arrows are indicating sympodial indexes (number of leaves between two consecutive inflorescences) equal to 2 in *S. pennellii* (B), 3 in MT-*Sp* (D, left) and zero in MT (D, right). The bars depicted with the same lowercase and uppercase letter are not significantly different ($p > 0.05$) according to the unpaired Student's *t*-test ($n = 6$ Petri dishes, each containing 15 cotyledon explants). Scale bar = 2 cm in B–F.

acclimatization of those that presented high organ regeneration, after 21 days of their corresponding cotyledons culture on either SIM or RIM.

2.4. Molecular analysis

Genomic DNA was extracted from 100 mg tomato leaflets using the method described by Fulton et al. [43]. Cleaved Amplified Polymorphic Sequences (CAPS) markers, previously defined in the Tomato-'EXPEN2000' and available at the 'Sol Genomics Network' – SGN (http://solgenomics.net/), were amplified using specific primers (Table S1) for the chromosomal regions spanning the ILs 3-2, 7-1 and 8-3, which presented high frequency of explants forming shoots, with more shoots per explant. Each polymerase chain reaction (25 μL) contained 75 ng plant DNA; 50 mM KCl; 20 mM Tris–HCl; 1.5 mM $MgCl_2$; 0.2 mM dNTPs; 0.25 μM of each primer; and 1 U *Taq* DNA polymerase (Fermentas; Vilnius, Lithuania). The reactions were submitted to the following conditions: 5 min at 94 °C; followed by 40 cycles of 40 s at 94 °C; 30 s at 55 °C; and 1 min at 72 °C; with a final cycle of 5 min at 72 °C. Amplification products were subsequently digested with their respective enzymes (Table S1) at 37 °C for 3 h, and the resulting fragments were separated by 1.5% agarose gel electrophoresis in 1X SB buffer (from 20X SB: 8 g NaOH diluted in 1000 mL water, plus boric acid

until pH 8.0) at 60 V for 3 h, using SYBR Gold (Invitrogen) for staining.

3. Results

3.1. Shoot regeneration capacity of two tomato cultivars, three monogenic mutations and S. pennellii

We evaluated the *in vitro* regenerating capacity of *S. pennellii* (Fig. 1), a green-fruited species, morphologically distinct from the cultivated tomato (Fig. 1B and C). The improved *in vitro* shoot formation capacity of *S. pennellii* was evident when compared to the cultivar MT for both 8- and 12-day-old cotyledon explants cultivated on 5.0 μM BAP (Fig. 1A). We also observed a high shoot formation capacity in the cultivar 'M82', when compared to MT (Fig. 1A). Since the regeneration capacity of MT is considered to be equivalent to that of most tomato cultivars [4], we evaluated if the known mutations present in 'M82' would have some impact in the shoot formation capacity. Like many other processing tomato cultivars, 'M82' harbors the recessive alleles *uniform fruit* (*u*) and *self-pruning* (*sp*), which confer the absence of green shoulders in fruits [44] and determinate growth habit [45], respectively. Since these same mutations are present in MT [29], it is unlikely that they are associated with the high shoot formation phenotype of 'M82'. Accordingly, NILs containing the wild type alleles *Sp* (Fig. 1D) and *U* (Fig. 1F) introgressed into the MT background presented a regeneration capacity not significantly different from the control MT in both 8- and 12-day-old cotyledon explants (Fig. 1A). Considering that the small plant size of MT is caused by the recessive allele *dwarf* (*d*) [46], a brassinosteroid-related mutation [47], a NIL harboring the wild type allele *D* (Fig. 1E) was also tested. Comparing MT-*D* with MT, a significant difference in shoot formation was found only in 8-day-old explants. This indicated that the *d* allele is unlikely to explain the regeneration differences when comparing MT and M82, especially for 12-day-old explants.

3.2. Shoot regeneration capacity of S. pennellii-derived introgression lines

Despite the fact that more contrasting regeneration capacity was observed when comparing *S. pennellii* with MT, rather than with 'M82' (Fig. 1A), the cross 'M82' × *S. pennellii* would produce a population of plants suitable to study the segregation of loci controlling *in vitro* shoot formation capacity if such loci were complementary between the two parentals. This appeared to be the case, since the population of ILs harboring small segments of *S. pennellii* introgressed into 'M82' showed considerable variation for the capacity to form shoots in 12-day-old cotyledon explants cultivated *in vitro* (Fig. 2). Among the lines evaluated, ILs 2-1, 3-1, 6-3 and 7-5 presented the lowest ability to form shoots *in vitro* (Fig. 2), when compared to both parentals. Such ILs may contain loci where *S. pennellii* alleles are inferior for shoot regeneration capacity than those of 'M82'. These transgressive phenotypes might be the product of epistatic interactions into the 'M82' background, or the effect of *S. pennellii* alleles *per se* [48]. On the other hand, the ILs 3-2, 6-1, 7-1, 7-2, 8-2, 8-3, 9-1, 9-2, 10-2 and 10-3 are likely revealing superior alleles present in *S. pennellii* controlling the capacity to form shoots *in vitro*.

Using the concept of bin mapping created for this same IL population [49], it was possible to classify the chromosomal regions which most probably harbor the alleles for high shoot formation capacity into the bins 3C (IL3-2), 6A (IL6-1), 7H (IL7-1 and IL7-2), 8F (IL8-2 and IL8-3), 9DE (IL9-1 and IL9-2) and 10F (IL10-2 and IL10-3). Among the ILs with the highest frequency of explants forming

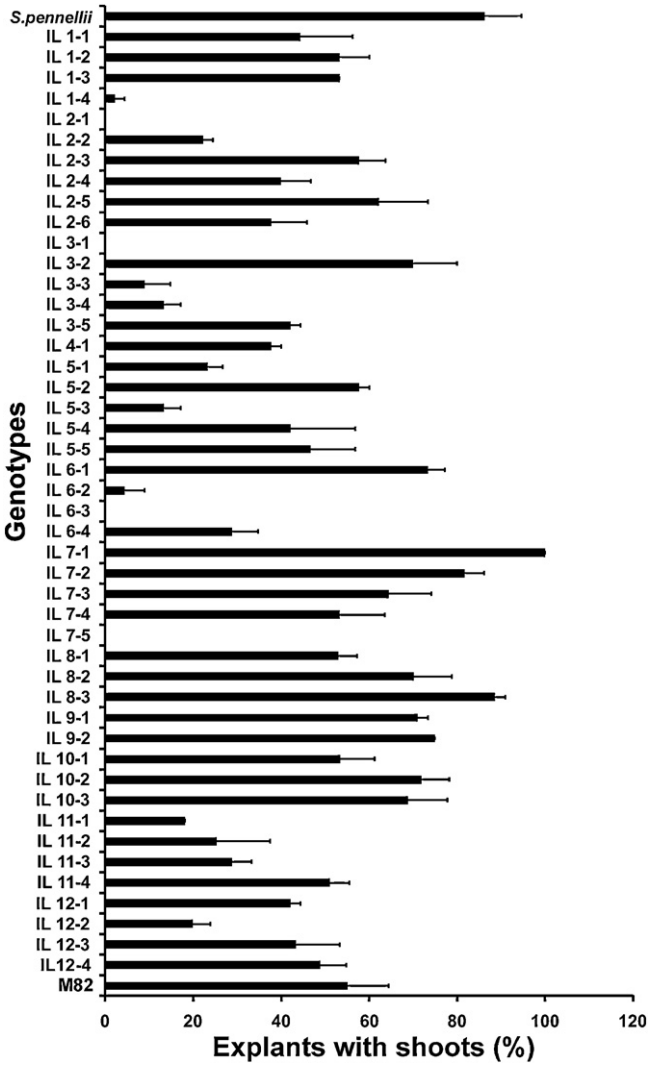

Fig. 2. *In vitro* shoot regeneration capacity of 12-day-old seedling cotyledon explants from tomato introgression lines (IL) containing *S. pennellii* LA716 chromosomal segments into the cv M82 genetic background. The numbers following each IL represent the chromosome and the segment, respectively. Cotyledon explants were cultivated on MS media with 5.0 μM BAP for 21 days. Error bars represent mean ± SE, $n = 6$ Petri dishes each containing 15 cotyledons.

shoots, ILs 3-2, 7-1 and 8-3 also presented more shoots formed per explant, resembling the parental *S. pennellii* (Fig. 3A, C–E).

We further produced F1 plants by crossing MT with the ILs correspondent to bins 3C, 6A, 7H, 8F, 9DE and 10F, to test if the alleles improving regeneration ability in these bins are dominant or recessive. The regeneration capacity of F1 seedlings from the crosses MT × M82 and MT × IL10-2 was not significantly different to that of the parental MT (Fig. 4A), suggesting that the high regeneration ability of M82 and IL10-2 is recessive. On the other hand, the significant high regeneration ability, when compared to MT, of F1 seedlings from the cross between MT with ILs 3-2, 6-1, 7-1, 7-2 and 8-3 (Fig. 4A) indicated that the shoot regeneration capacity of these ILs are dominant. The dominant nature of the regeneration ability of such ILs indicates that they are harboring alleles present in the segments introgressed from *S. pennellii*; and not in the M82 background, whose high organogenic capacity is recessive (Fig. 4A). We also tested the shoot regeneration ability of F1 seedlings from various combinations of IL × IL (Fig. 4B). The most prominent results from such crosses were the negative effect of the combination of ILs 3-2 and 6-1, and the trend of IL8-3 to improve the regeneration capacity in different combinations (Fig. 4B).

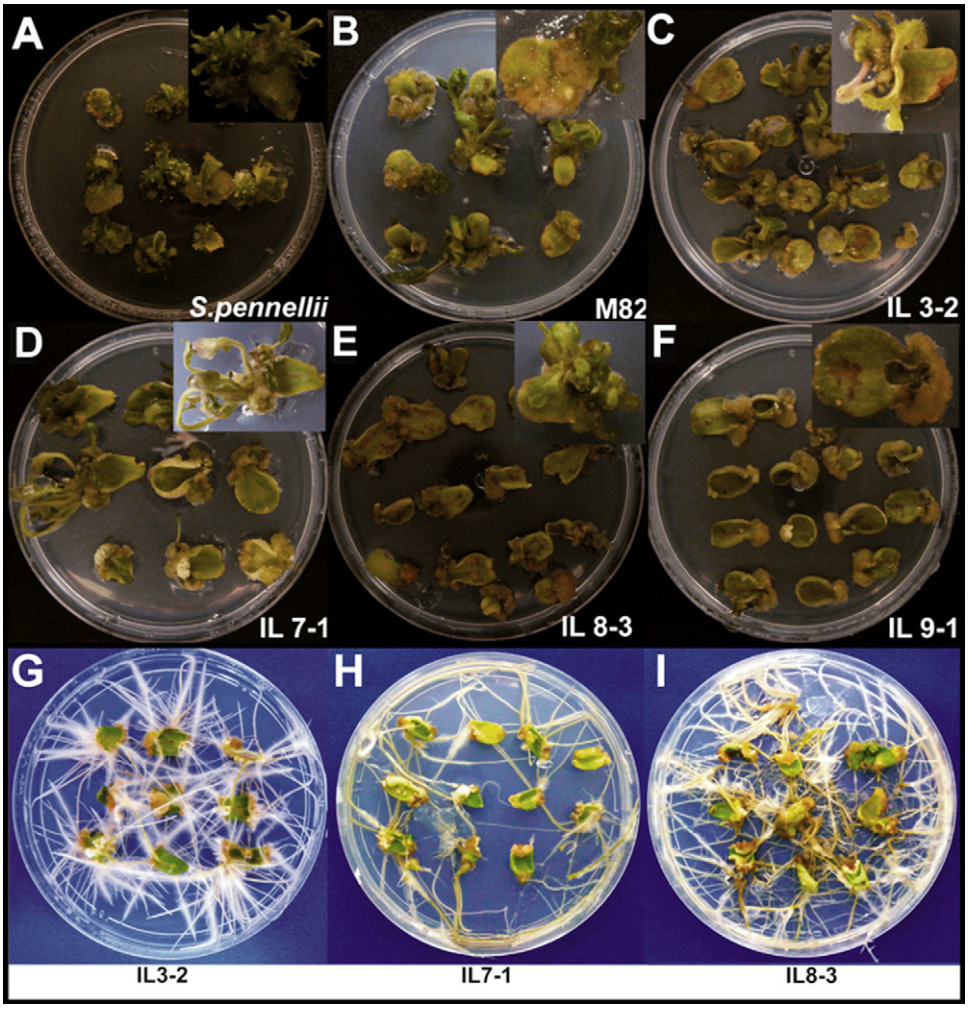

Fig. 3. Aspect of shoot and root regeneration from selected genotypes. Twelve-day-old cotyledon explants from different genotype were cultivated on MS media with 5.0 μM BAP (A–F) or MS media with 0.4 μM NAA for 21 days (G–I).

3.3. Root regeneration capacity of the introgression lines presenting high shoot regeneration

We further tested the capacity of the ILs previously selected for high shoot regeneration on SIM to form roots on RIM (0.4 μM NAA). The significantly higher root formation capacity of ILs 3-2, 8-3, 10-2 and 7-1, when compared to M82 (Fig. 4C), indicated that the alleles present in these ILs might be controlling the formation of both shoot and roots. On the other hand, the alleles from ILs 6-1 and 9-1 appeared to be specifically improving shoot formation, but not the ability to form roots (Fig. 4C).

3.4. The introgression of S. pennellii loci improving both root and shoot formation in vitro

To create NILs and further investigate the effect of the alleles present in ILs 3-2, 7-1 and 8-3, which are dominant and control both root and shoot formation capacity, we started their introgression through successive backcrosses (BC) into the model system MT, evaluating the regeneration capacity of seedlings. In the first backcross generation (BC_1 and BC_1F_2), we were able to select seedlings from different crosses presenting high shoot formation capacity when compared to mean values exhibited by the parental MT (Fig. 5). Considering the introgression of the high regeneration capacity of IL3-2, the seedlings that demonstrated the highest regeneration were #21, #23 and #24 (Fig. 5A). To determine the presence of introgressed chromosome fragments of *S. pennellii*, seedlings were analyzed using CAPS markers. The CAPS marker T1388, previously selected based on the existence of polymorphism between *S. pennellii* and cultivated tomato in the region of IL3-2 (see Table S1), showed that seedlings #23 and #24 are homozygous for *S. pennellii* and MT alleles, respectively, while seedling #21 is heterozygous (Fig. S1). Consistently, adult plant #23 displayed yellow fruits (not shown), which evidenced the presence of the recessive *r* allele from *S. pennellii*, that maps to the same region [49]. From the cross 'MT' × IL7-1, five BC1 seedlings (#5, 8, 14, 17 and 23) were selected presenting high shoot regeneration capacity, whereas one (#6) displayed low capacity (Fig. 5B), and all survived acclimatization. Analyzing these seedlings with the CAPS markers CT114 and T1255 (Table S1) indicated that seedling #6 is homozygous for the MT allele (Fig. S1C and D), whereas the seedlings with high regeneration ability were mostly heterozygous for the *S. pennellii* allele, at least for the marker CT114 (Fig. S1D), with the exception of seedling #14, which was homozygous for the MT allele. As for the cross with IL8-3, two seedlings with high shoot regeneration ability (#16 and 17) were selected in BC1 (Fig. 5C). Only seedling #17 survived acclimatization, and proved to be homozygous for MT allele for the markers T1359 and TG294 (Fig. S1B and D). Although we cannot exclude the possibility that high regenerating seedlings homozygous for MT markers maybe harboring M82 alleles instead of *S. pennellii* alleles, this is probably not the case in the BC1 generations used for the introgression of IL7-1 and 8-3 alleles, since

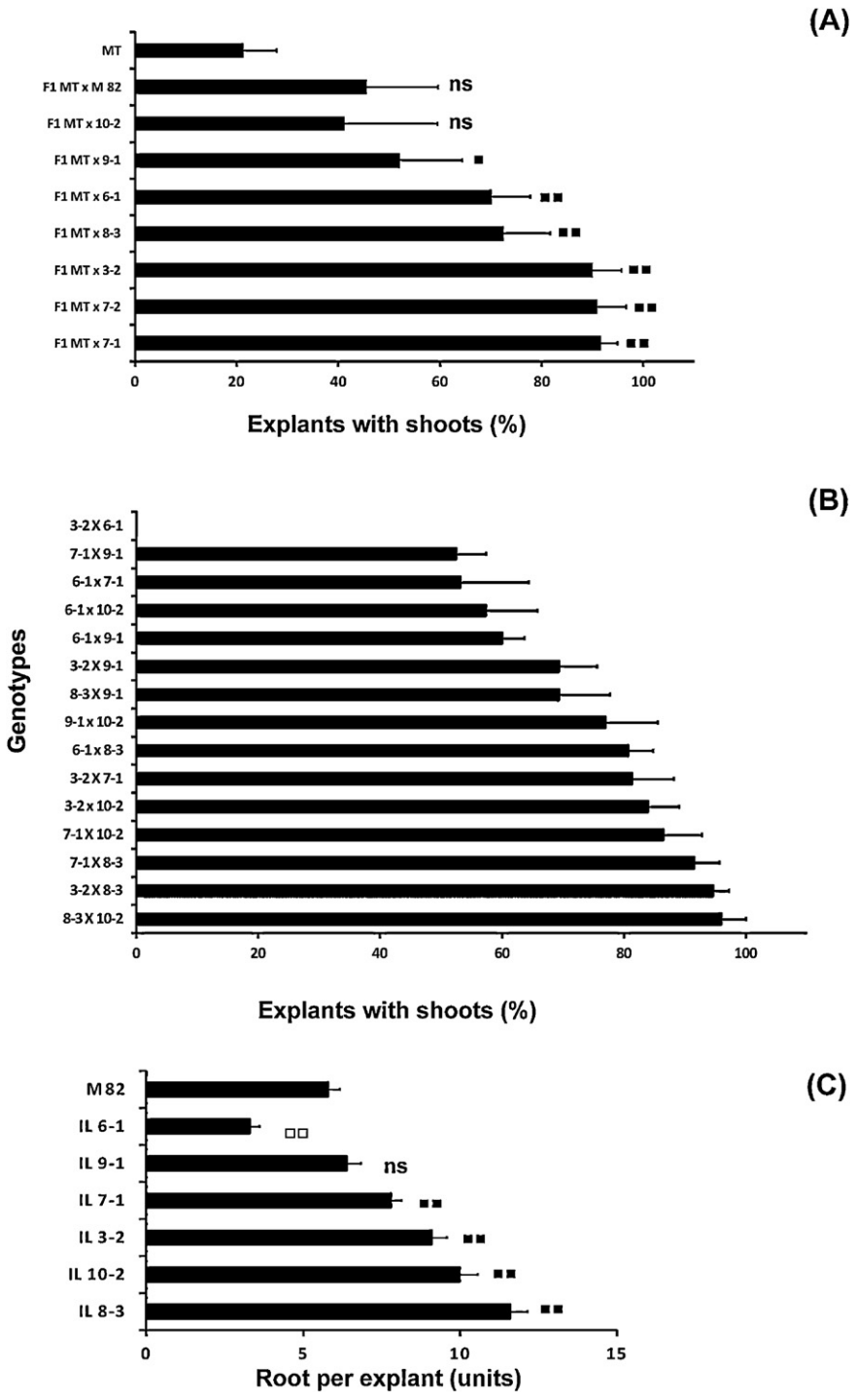

Fig. 4. Shoot and root regeneration capacity of selected introgression lines (ILs) and their derived crosses. (A) Shoot formation in cotyledonary explants of F1 seedlings derived from the cross Micro-Tom (MT) × ILs. (B) Shoot formation in cotyledonary explants derived from different combination of IL × IL crosses. (C) Number of roots formed per cotyledonary explant derived from selected genotypes. Cotyledon explants from 12-day-old seedlings were cultivated on MS media with 5.0 μM BAP or 0.4 μM NAA for 21 days. Error bars represent mean ± SE, $n = 6$ Petri dishes each containing 15 cotyledon explants. Squares represent values statistically significantly higher (filled) and lower (open) than MT at $p < 0.01$ (two squares) or $p < 0.05$ (one square), according to Student's t-test. "ns" means non-significant.

the regeneration capacity of M82 is recessive (Fig. 4A). Therefore, plant #14 derived from IL7-1, and plant #17 derived from IL8-3, may represent narrower chromosomal segments of the *S. pennellii* introgressed into the MT genome.

The selected BC1 and BC1F2 plants derived from crosses with ILs 3-2, 7-1 and 8-3 were further backcrossed with MT and the capacity to regenerate roots *in vitro* was tested in advanced generations (Fig. 6). The presence of high root-regenerating plants in BC2 and BC3 confirms that the plants previously selected in the screening for high shoot formation capacity (Fig. 5) were harboring the alleles present in the chromosomal segments from ILs 3-2, 7-1 and 8-3. Additionally, the high shoot formation capacity was also reconfirmed in pooled BC3F2 and BC1F2 seedlings derived from selected individual BC3 high root-forming seedlings (IL7-1 and 3-2) and BC1 high shoot-forming seedlings (IL8-3), respectively (Fig. S2). These results are also consistent with the fact that such alleles appeared

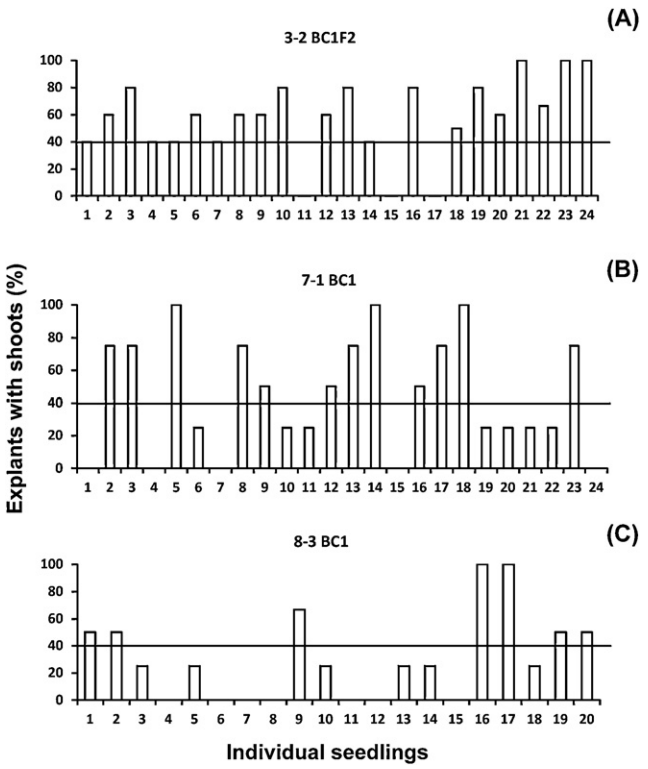

Fig. 5. Screening of regenerating seedlings from early backcrosses, using 'Micro-Tom' (MT) as the recurrent parental, of selected ILs. (A) Shoot regeneration capacity of BC1F2 seedlings derived from the cross MT × IL3-2. (B) Shoot regeneration capacity of BC1 seedlings from the cross MT × IL7-1. (C) Shoot regeneration capacity of BC1 seedlings from the cross MT × IL8-3. Twelve-day-old seedling cotyledonary explants were cultured on MS media with 5.0 μM BAP for 21 days. The horizontal lines represent the average regeneration capacity of the control MT.

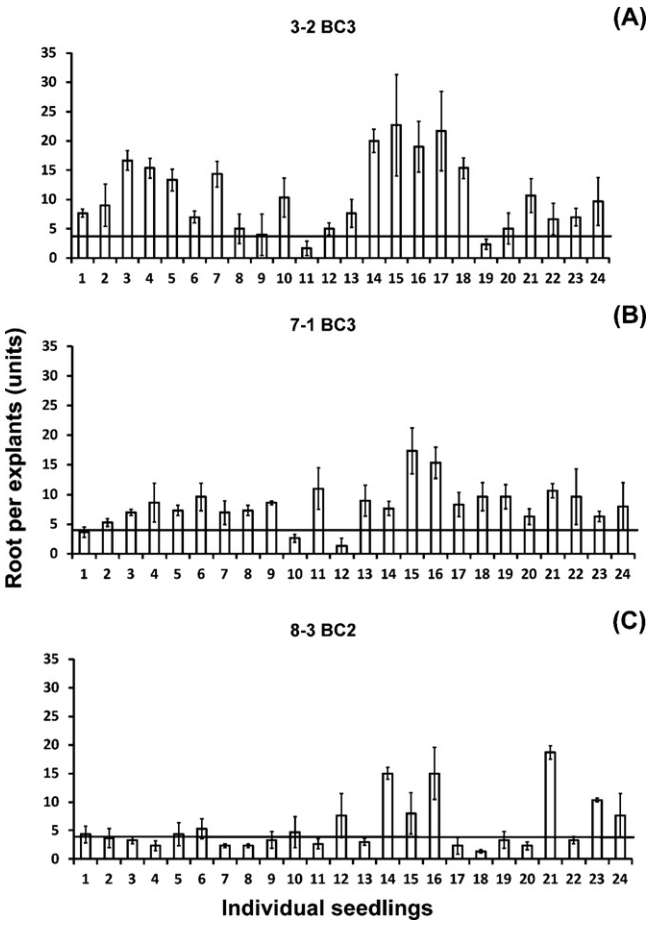

Fig. 6. Screening of regenerating seedlings from advanced backcrosses, using 'Micro-Tom' (MT) as the recurrent parental, of selected ILs. (A) Root regeneration capacity of BC3 seedlings derived from the cross MT × IL3-2. (B) Root regeneration capacity of BC3 seedlings from the cross MT × IL7-1. (C) Root regeneration capacity of BC2 seedlings from the cross MT × IL8-3. Twelve-day-old seedling cotyledonary explants were cultured on MS media with 0.4 μM NAA for 21 days. The horizontal lines represent the average regeneration capacity of the control MT.

to be dominant (Fig. 4A) and to be able to improve both shoot (Figs. 2 and 4A) and root formation *in vitro* (Fig. 4C). The plants presenting the highest root regeneration capacity in the BC3 generation from ILs 3-2 (plants #14-18) and 7-1 (plants #15 and 16), and in the BC2 generation from 8-3 (plants #14, 16 and 21) can be used for further backcrosses and screening of shoot or root formation capacity until the BC6F2, when homozygous plants could be selected through observation of their derived seedlings (BC6F3). Such BC6Fn plants will be considered NILs to MT, consisting in a valuable tool to perform comparative studies (*e.g.* phenotype observation and gene expression analysis) to unravel the physiological basis of cell competence.

4. Discussion

In the present work, we identified six chromosomal segments (bins 3C, 6A, 7H, 8F, 9DE and 10F) whose alleles from the tomato wild relative *S. pennellii* improve organogenesis *in vitro*. These segments, which may represent QTLs [25] for *in vitro* regeneration capacity, were here named as *RG3C*, *RG6A*, *RG7H*, *RG8F*, *RG9DE* and *RG10F* after Liu et al. [49]. In a recent paper, Trujillo-Moya et al. [50] developed two mapping populations (F2 and BC1) derived from a cross between the tomato cultivar Anl27, with low regeneration ability, and a high regeneration accession of *S. pennellii* (PE-47). They identified six QTLs controlling shoot formation on five chromosomes (1, 3, 4, 7 and 8) in the BC1 population. Although Trujillo-Moya et al. [50] used different methods and genotypes, and did not assessed the capacity to form roots, the QTLs that they identified at chromosomes 3, 7 and 8 may correspond to those here identified. Regarding the presence of a QTL at chromosome 3 controlling regeneration, it is interesting to note that Koornneef et al. [32] found that the high organogenic capacity of *S. peruvianum* was associated with two major dominant alleles at two distinct loci (named *Rg1* and *Rg2*), with the *Rg1* further mapped to chromosome 3, close to the *yellow flesh* (*r*) locus [38]. The *RG3C* here described and *Rg1* are likely to be allelic, since both, together with the *r* locus, all mapped to the same 3C bin [49]. Some of the other loci described here might correspond to *Rg2* [32], which, to the best of our knowledge, has not been mapped. The name *Rg2* was also given to describe a dominant allele controlling the high capacity to regenerate shoots from root explants of *S. chilense* [51], a sibling species of *S. peruvianum*. Since this *S. chilense* gene was mapped in the same position of *Rg1* (and *RG3C*) on chromosome 3 [51], it is most likely to be another allele for the same *Rg1/RG3C* locus, as also suggested by Trujillo-Moya et al. [50]. In this regard, *S. chilense*, *S. peruvianum*, *S. habrochaites* (formerly known as *Lycopersicon hirsutum*) and *S. pennellii* are all green-fruited species harboring the *r* allele, and probably linked to a high-regeneration allele at the *Rg1/RG3C* locus, since all species exhibit the capacity to regenerate shoots from root explants [4,30], an ability absent in species with red fruits [4,38,52].

The dominant behavior of the alleles improving *in vitro* regeneration from wild tomato related species described here and by others [32,34] suggests that cultivated tomato may have corresponding non-functional or weak alleles achieved or retained

during domestication. In the case of *RG3C*, it should be noticed that it is linked to the *yellow flesh* (*r*) locus, which is usually undesired by growers and breeders, who normally select for red tomatoes. Thus, although *S. peruvianum* had been crossed with cultivated tomato to improve disease resistance for a long time [53], the selection for red fruits have probably caused an early elimination of the *Rg1* allele in most modern cultivars. Moreover, it has been reported that the *Rg1* allele, and maybe other loci improving shoot formation *in vitro*, produces a pleiotropic phenotype of highly branching plants [40]. The common practice of weekly side-branch elimination to improve tomato production [54] may also have lead to some indirect negative selection against alleles that contribute to improve *in vitro* (and *ex vitro*) shoot formation in most tomato cultivars, although side-branch elimination is less important in the case of determinate cultivars (*e.g.* cv 'M82') used for industrial processing (manufacturing ketchup and sauces).

4.1. The possible gene function for the loci controlling in vitro organogenesis

The loci *RG3C*, *RG7H*, *RG8F* and *RG10F* here described as controlling elevated *in vitro* shoot formation capacity, also enhanced root formation in adequate media (Fig. 5). The other two loci, *RG6A* and *RG9DE*, seemed to be specific for shoot formation capacity. Christianson and Warnick [55] divided the process of *in vitro* organogenesis in the following stages: (1) dedifferentiation; (2) acquisition of competence; (3) induction; (4) determination; (5) differentiation; and (6) formation of the organ. In this pathway, the stage of acquisition of competence is likely to be a general process, necessary for both shoot and root formation [56,57], whereas induction requires specific auxin-to-cytokinin balance leading to shoot or root formation [58], but not both organs. Based on this concept, we here propose that *RG3C*, *RG7H*, *RG8F* and *RG10F* are probably affecting the step of acquisition of competence, whereas *RG6A* and *RG9DE* are likely affecting the stage of shoot induction (Fig. 7). One important corollary from Christianson and Warnick [55] model is that *competence* may be opposite to *determination*, since an explant highly committed ('determined') to a particular developmental pathway will probably be more recalcitrant ('non-competent') to assume a different fate [59]. A classic study of cell determination was presented by Tran Thanh Van [60], who demonstrated that epidermal explants of tobacco flower stalks tend to continue to form new flowers *in vitro*, even in a non-inducing medium. These concepts may help further elucidation of the genetic identity of *RG3C*, *RG7H*, *RG8F* and *RG10F*, since they could represent genes arresting the specification of cell fates, or increasing the population of indeterminate cells (stem cells) in a given explant [61]. Noteworthy, hormonal measurements showed that *Rg1* does not confer an increase in the endogenous levels of cytokinin, although it increases shoot production *in vitro* [62]. Since genes leading to cytokinin accumulation will favor a cytokinin-to-auxin balance, inducing shoots but not roots [3,6,63], the results from Boiten et al. [62] are consistent with the idea that *Rg1*/*RG3C* is controlling competence to form both shoots and roots, instead of the specific induction of shoots.

Among the regulatory genes identified and annotated at the "Sol Genomics Network" (http://solgenomics.net/) for the bins here identified as putatively controlling competence, members of the *GRAS* (bins 3C, 7H and 8F) and *NAC* families (bins 3C, 8F and 10F) transcription factors were detected (see Supplementary Tables S2–S5). Previous studies suggested these transcription factors as controlling competence, since the *GRAS* genes *SCARECROW* (*SCR*) and *HAIRY MERISTEM* (*HAM*) are associated with the formation of stem cell niches [61], and the maintenance of indeterminacy [64] in both shoot and root meristems. Interestingly, one of the *GRAS* genes present in bin 7H corresponds to the mutant *lateral suppresser*

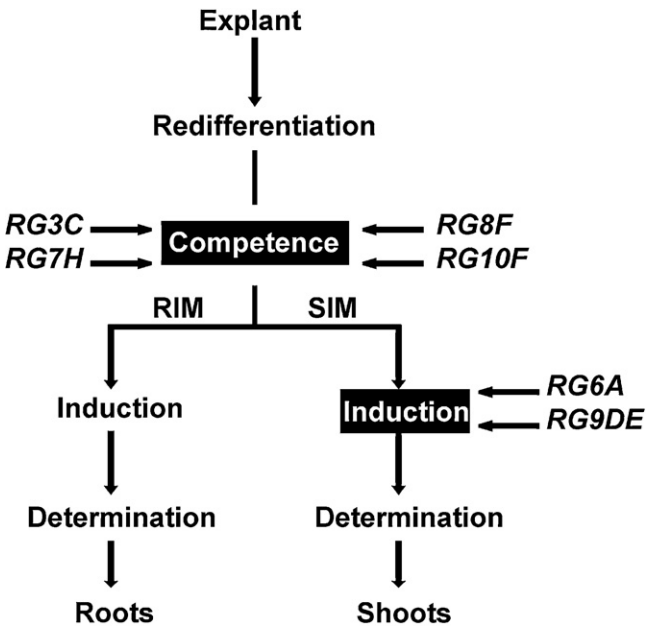

Fig. 7. A working model for the position of putative alleles presented by selected introgression lines (ILs) in a scheme modified from Christianson and Warnick [55]. The alleles improving both shoot and root formation capacity are probably affecting the unspecific step of acquisition of competence, which is shared by both organs [57]. On the other hand, alleles improving only shoot regeneration capacity are probably affecting this specific induction step. The nomenclature for the alleles was after their probable position in the bin mapping [49].

(*Solyc07g066250*), which fails to form axillary meristem [65]. In addition, the expression of the *NAC* gene *CUP SHAPED COTYLEDON* (*CUC2*) marks the site of both shoot and lateral root primordia formation, indicating that this gene may be a marker of cell competence [66].

As for the identity of *RG6A* and *RG9DE*, their specific role in the induction of *in vitro* shoots may suggest candidate genes controlling auxin-to-cytokinin balance, or genes associated with shoot meristem identity. It is well established that genetic differences leading to plants overproducing cytokinin increase *in vitro* shoot formation capacity [3,6,63]. Additionally, the expression of genes controlling cytokinin response or shoot meristem identity, such as the *ARABIDOPSIS RESPONSE REGULATOR5* (*ARR5*) and the homeoboxes *SHOOT MERISTEMLESS* (*STM*) and *WUSCHEL* (*WUS*), correlate with adventitious shoot formation, and may also serve as markers for this capacity [5,8–10]. Interestingly, no cytokinin-related genes and neither homeoboxes are represented in the list of genes annotated in the bins 6A and 9DE (see Supplementary Tables S6 and S7). In contrast, both bins contain genes associated to gibberellin (GA) and abscisic acid (ABA) signaling and metabolism, including ABA receptor (*PYL4* in bin 6A, and *PYL6* in bin 9DE), and gibberellin 20-oxidase (*GA20ox2* in bin 6A and *GA20ox1* in bin 9DE) genes. Among the GA-related genes in bin 9DE stands *SPINDLY* (*SPY*), a negative regulator of GA response. In arabidopsis, reduced levels or sensitivity to GA is associated to an enhanced *in vitro* shoot formation [67].

4.2. Micro-Tom as a model system to study in vitro regeneration capacity

The production of NILs for *RG6A* and *RG9DE*, as well as the effort described here to produce NILs harboring *RG3C*, *RG7H*, *RG8F* (Figs. 5 and 6; Fig. S2), will help further studies involving hormonal dosages and the analysis of expression of relevant candidate genes, such as those present in the bins (Tables S2–S7), or in their further narrowed regions. Moreover, the use of NILs, which isolate

a single QTL region, transformed the task of QTL cloning into the similar one performed for simple Mendelian traits [25]. Such NILs are preferable to be created into a reference low-regenerating genotype. In this work, the genotype chosen was 'Micro-Tom' (MT), due to its small size and rapid life cycle [29], and the availability of mutants affecting hormonal metabolism and sensitivity in the same genetic background [26]. Such plethora of hormonal mutants, together with other mutants affecting developmental process in MT (www.esalq.usp.br/tomato and http://tomatoma.nbrp.jp/), will allow the approach of double mutant analysis [68], once NILs affecting *in vitro* regeneration become available in the same MT genetic background.

4.3. Implications for tomato biotechnology and breeding

Regardless of the genetic identity of the loci described here, the usefulness of genes improving *in vitro* regeneration ability is evident, not only for tomato, but for most crop species. Although *Agrobacterium*-mediated plant transformation has long been established for tomato [69], this procedure is being continuously improved, with the contribution of alleles enhancing *in vitro* regeneration [41]. The additive effect observed here in the 'IL × IL' crosses suggests that pyramiding distinct alleles into a single genotype may contribute for further improvement of *in vitro* regeneration traits in tomato. Moreover, until today, the important breeding tool of double haploid production [70], and thus reverse breeding [71], is not yet available in tomato. The main barrier for haploid production in tomato stands in the low regeneration ability of anthers when cultivated in various media [72]. Since some of the alleles described here are likely to be controlling the ability to assume different cell fates (competence), they may be useful to improve different regeneration systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2012.11.005.

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