University of São Paulo
"Luiz de Queiroz" College of Agriculture

Characterization of natural genetic variations affecting tomato cell competence to assume different developmental fates

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Thesis presented to obtain the degree of Doctor in Science. Area: Plant Physiology and Biochemistry

Piracicaba
2016

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Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA - DIBD/ESALQ/USP

Pinto, Maísa de Siqueira
Characterization of natural genetic variations affecting tomato cell competence to assume different developmental fates / Maísa de Siqueira Pinto. - - Piracicaba, 2016.
99 p. : il.

Tese (Doutorado) - - Escola Superior de Agricultura “Luiz de Queiroz”.

1. Linhagens de Introgressão 2. Regeneração 3. *Solanum lycopersicum* 4. *Solanum pennelli* I. Título

CDD 635.642
P659c

To my parents, who have raised me to be the person I am today. Thank you for all the unconditional love, guidance, and support that you have always given me!

ACKNOWLEDGEMENT

I would like to thank all the people who contributed in some way to the work described in this thesis.

I would like to express my sincere gratitude to my advisor, Prof. Lázaro Peres, for the continuous support of my Ph.D study and related research, for his confidence, motivation, and immense knowledge.

My sincere thanks also goes to Dr. Vagner Benedito for having received me in his laboratory at West Virginia University in the United States, during my internship abroad and have given me access to the laboratory and research facilities. Thank you for the attention, teaching and help.

To all the teachers from Plant Physiology and Biochemistry program, thank you for the scientific teachings, in special to Prof. Ricardo Kluge for the knowledge transmitted to me during my teacher training.

I greatly thank my labmates, Ariadne Felício, Guilherme Oliveira, Ignácio Achon, Diego Sevastian, Mariana Azevedo, Jonata Freschi, João Pedro Bernardes, Ivan Sestari, Lucas Cutri, Agustin Zsögön, Joni Lima, Lílian Ellen Pino and James Gattward for the stimulating discussions and for all the fun we have had in the last four years. I specially thank Marcela Notini, Eloísa Vendemiatti, Frederico de Jesus, Stevan Bordignon and Mateus Vicente for the friendship and conviviality outside the laboratory.

I also thank the labmates I had during my internship abroad, Lucas Maia, Samuel Chaves, Lina Yang and Christina Wyman. I specially thank Roshan Abeyratne for the immense help during the experiments in West Virginia University.

To my friends and roommates Tânia Batista, Cibele Teixeira, Marcela Nunes de Freitas and Luiza Zazycki, thank you for listening, offering me advice, and supporting me through this entire process, your friendship was fundamental during the stressful and difficult moments, but also during the joy ones.

I would like to thank Cassia Figueiredo and Francisco Vitti for their daily assistance, making possible the development of our experiments.

I truly thank “Luiz de Queiroz” College of Agriculture for the opportunities granted to me along these years, and the Plant Physiology and Biochemistry program, specially Maria Solizete Granziol Silva for the help, support and availability whenever necessary.

I thank the governmental institutions CAPES (Coordination for the Improvement of Higher Education Personal) and Fapesp (São Paulo Research Foundation) for the financial support, which made my studies possible in Brazil and United States.

My heart-felt gratitude to my family: my parents Márcia and Guilherme and my sister Patrícia, for their love and support throughout my life and my studies.

Finally, thanks to the God, for His blessings throughout my life and for the guidance during the development of this work.

"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales." - Marie Curie

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RESUMO

Caracterização de variações genéticas naturais em tomateiro controlando a competência celular para assumir diferentes vias de desenvolvimento

O estudo de variações genéticas naturais afetando a capacidade de organogênese *in vitro* em tomateiro (*Solanum lycopersicum*) é promissor devido a existência de uma série de espécies selvagens relacionadas ao tomateiro, que apresentam alta capacidade organogênica *in vitro*. A caracterização de tais variações é relevante não apenas com o objetivo de manipulação do desenvolvimento vegetal, mas também com o intuito de entender o significado ecológico e evolutivo de tal característica. O objetivo desse trabalho foi caracterizar três loci de tomateiro, cujos alelos vindos de seu parente selvagem *S. pennellii* aumentam a capacidade de regeneração de gemas caulinares e radiculares *in vitro*, e analisar o envolvimento de tais loci na fase de aquisição de competência para regeneração. Nós apresentamos no primeiro capítulo a caracterização genética e fisiológica dos loci *Rg3C*, *Rg7H* e *Rg8F*. Os alelos de *S. pennellii* foram introgredidos na cultivar modelo Micro-Tom (MT), criando as linhagens quase isogênicas (*Near Isogenic Lines – NILs*) MT-*Rg3C*, MT-*Rg7H* e MT-*Rg8F*. No segundo capítulo nós analisamos comparativamente as *NILs* MT-*Rg3C* e MT-*Rg1*. Uma vez que *Rg1* foi proposto como gene chave na aquisição de competência, e assim como *Rg3C* está localizado no cromossomo 3, acredita-se que *Rg3C* seja provavelmente ortólogo ao gene *Rg1* de *S. peruvianum*. Após a introgressão dos loci na cultivar MT, as *NIL*s, assim como esperado, apresentaram alta taxa de regeneração tanto de gemas caulinares, quanto de radiculares *in vitro*, confirmando que os loci foram devidamente introgredidos. A análise do tempo de aquisição de competência e indução, juntamente com a caracterização molecular das *NIL*s, indicam que os genes localizados nos loci *Rg3C*, *Rg7H* e *Rg8F* afetam a regeneração *in vitro* através de rotas distintas. Enquanto *Rg3C* diminui o tempo necessário tanto para a aquisição de competência quanto para indução de gemas caulinares, os outros dois loci parecem influenciar apenas a aquisição de competência, no caso de *Rg8F*, ou a indução de gemas caulinares, no caso de *Rg7H*. Além disso, apesar de MT-*Rg3C* apresentar alta ramificação, MT-*Rg7H* e MT-*Rg8F* não diferiram de MT nesse aspecto, o que evidencia que a formação de gemas caulinares *in vitro* não está necessariamente relacionada ao aumento da ramificação. As análises comparativas entre MT-*Rg3C* e MT-*Rg1* indicam fortemente que *Rg1* e *Rg3C* sejam dois alelos de um mesmo gene controlando a alta capacidade de regeneração. Através do cruzamento dos dados de mapeamento disponíveis para esses dois alelos foi possível diminuir o número de genes candidatos à *Rg1*/*Rg3C* para apenas 27 genes, que são apresentados nesse trabalho.

Palavras-chave: Linhagens de Introgressão; Regeneração; *Solanum lycopersicum*; *Solanum pennellii*

ABSTRACT

Characterization of natural genetic variations affecting tomato cell competence to assume different developmental fates

The study of natural genetic variations affecting organogenic capacity in tomato (*Solanum lycopersicum*) is attractive due to the existence of several tomato wild relatives with enhanced organogenic capacity. The characterization of such variations is relevant not only in order to manipulate plant development, but also to understand its ecological and evolutionary significance. The objective of this work was to characterize three tomato loci whose alleles from the wild relative *S. pennellii* enhance *in vitro* shoot and root regeneration, and analyze their involvement in the acquisition of competence phase. In the first manuscript, we report the genetic and physiological characterization of the loci *Rg3C*, *Rg7H* and *Rg8F*. The *S. pennellii* alleles were introgressed into the tomato genetic model cv. Micro-Tom (MT), creating the near isogenic lines (NILs) MT-*Rg3C*, MT-*Rg7H* and MT-*Rg8F*. In the second manuscript we present a comparative analysis between the Near-Isogenic Lines (NILs) MT-*Rg3C* and MT-*Rg1*. Since *Rg1* was proposed to be a key gene in the acquisition of competence, and was mapped in the chromosome three, it is believed that *Rg3C* is probably equivalent to the *Rg1* allele from *S. peruvianum*. After the introgression of the loci into the MT background, the NILs presented enhanced regeneration of both roots and shoots, confirming that the loci were successfully introgressed. The analysis of the time for acquisition of competence and induction, together with the molecular characterization of the NILs, indicate that the genes present in the loci *Rg3C*, *Rg7H* and *Rg8F* affect *in vitro* regeneration by distinct pathways. While *Rg3C* decreased the time required for both acquisition of competence and induction, the other loci seem to influence only the time of acquisition of competence, in the case of *Rg8F*, or the time of induction, in the case of *Rg7H*. Additionally, although MT-*Rg3C* has an enhanced shoot branching phenotype, MT-*Rg7H* and MT-*Rg8F* did not differ from MT in this trait. This indicates that enhanced *in vitro* shoot formation in tomato is not necessarily related to a deleterious high branching phenotype. Comparative analyses of MT-*Rg1* and MT-*Rg3C* strongly indicate that *Rg1* and *Rg3C* are alleles of a same gene controlling regeneration capacity. Integrating *Rg1* and *Rg3C* mapping information, we were able to narrow the number of candidate genes for *Rg1/Rg3C* to only 27, which were also analyzed and discussed.

Keywords: Introgression lines; Regeneration; *Solanum lycopersicum*; *Solanum pennellii*

1 INTRODUCTION

Plants' ability to form new shoots and roots after wounding have been explored, by agriculture and biotechnology, since long ago (SUSSEX, 2008). Due to their incredible plasticity, it was always thought that all plant cells are totipotent, in other words, have the ability to form an entire plant from a single or few non-zygotic cells (HABERLANDT, 1902). However, recent studies showed that, at least in the case of organogenesis in Arabidopsis, specific cells, which behaves like stem cells, are the responsible for the plants incredible regeneration ability (ATTA et al., 2009; SUGIMOTO; JIAO; MEYEROWITZ, 2010).

In roots and hypocotyls, the cells, from which new organs (roots or shoots) regenerate, are the xylem pole pericycle cells (ATTA et al., 2009; CHE; LALL; HOWELL, 2007; GORDON et al., 2007; SUGIMOTO; JIAO; MEYEROWITZ, 2010). In other organs, pericycle-like cells, that share expression of at least one reporter gene with xylem pole pericycle cells, are the cells responsible for new organs regeneration (SUGIMOTO; JIAO; MEYEROWITZ, 2010). Since in plants these adult stem cells are very accessible, that may be the reason of their uncommon regeneration capacity (SUGIMOTO; GORDON; MEYEROWITZ, 2011).

The events involved in plant regeneration can be divided in the following phases. In the first phase, the explant acquires the competence or ability necessary to follow a new developmental pathway. Next, the competent cells are inducted, by the medium composition, to undergoes a multistep process that will culminate in the determination of the cells to follow a specific developmental fate. In this phase, the medium composition determines the organ that will be formed, or shoots or roots. In the last phase, the cells already determined, will develop to form the new organ, independent of the medium composition (CHRISTIANSON; WARNICK, 1985).

The developmental fate that the explants will follow after acquisition of competence is determined by the hormones present in the inducing medium. The most known hormones for acting in determining the regeneration fate are auxin and cytokinin. The ratio between these two hormones determines if the explants will form shoots or roots. A high auxin/cytokinin ratio in the medium leads to the formation of roots, while a low auxin/cytokinin ratio leads to the formation of shoots (SKOOG; MILLER, 1957). Endogenous auxin and cytokinin level also strongly influences the regeneration capacity. Hence, Arabidopsis mutants with superexpression of the gene of auxin biosynthesis *YUCCA* or the gene of cytokinin

biosynthesis *IPT*, have a high regeneration of roots or shoots, respectively, even in medium without this hormones (SUN et al., 2003; ZHAO et al., 2001, 2013).

In Arabidopsis, the organogenic process happens by a two-step protocol. In the first step, in a Callus-Inducing Medium (CIM) enriched with auxin, the explant acquires the competence necessary to respond to the induction signal. In the second step, if transferred to a medium rich in cytokinin (SIM – Shoot inducing medium), the explants will be induced to form shoots. Conversely, if transferred to a medium rich in auxin (RIM – Root inducing medium), the explants will be induced to form roots (CARY; CHE; HOWELL, 2002; VALVEKENS et al., 1988). However, the regeneration of many important crops don`t follow the above steps, making difficult the development of a general plant regeneration protocol (DUCLERCQ et al., 2011).

1.1 Molecular mechanisms of shoot regeneration

The molecular path of shoot regeneration in the last years has been gradually revealed. The regeneration process is mostly known in Arabidopsis. In Arabidopsis radicular explants, shoot regeneration starts from the pericycle cells, and the first steps resemble a lateral root formation, including the genes involved in the process (MOTTE et al., 2014).

During CIM incubation, the founder cell specification occurs in the pericycle cells, and the auxin signaling is the responsible to initiate the organogenic callus formation. Since shoot and lateral root formation in intact roots share their initial developmental stages, many auxin mutants that lack the capacity to form lateral roots efficiently also have a diminished regeneration capacity (PÉRET et al., 2009)

During the formation of lateral roots in Arabidopsis, a local auxin maximum in the pericycle cells are responsible to specify the pericycle cells into founder cells. The lateral root formation begins with the pericycle cells division driven by the local auxin maxima (DUBROVSKY et al., 2008). This event seems to be essential also for shoot regeneration, since the ablation of the pericycle cells prevents shoot regeneration (CHE; LALL; HOWELL, 2007). PIN auxin efflux carrier also seems to play an important role during the first steps of shoot regeneration. These transporters are important in generating an auxin gradient, and the inhibition of the polar auxin transport was shown to stimulate organogenic callus formation (PERNISOVÁ et al., 2009).

After the auxin signaling in the pericycle cells, Auxin Response Factors (ARFs) mediate auxin-dependent response by activating the expression of several genes involved in

the lateral root formation process. The mutation of several of these genes also alters the regeneration phenotype of these mutants, which is an evidence of the overlap of the early events in lateral root formation and shoot regeneration (MOTTE et al., 2014). Among the genes with known function in these early events are the transcription factor *GATA23*, which is known to be involved in the founder cell-specification, and the gene BODENLOS (BDL)/IAA12-MONOPTEROS (MP)/ARF5, which are necessary for root organogenesis after the first cell divisions (MOTTE et al., 2014).

Many genes involved in lateral root development are also expressed during Arabidopsis explants incubation on CIM and callus formation (SUGIMOTO; JIAO; MEYEROWITZ, 2010; MOTTE et al., 2014). In addition, cytokinin biosynthesis genes are expressed in this phase, marking the influence of this hormone important even in the early phases of regeneration. In Arabidopsis root explants, acquisition of competence takes 48 hours of CIM incubation. This time is necessary to induce the expression of genes involved in the regeneration process during CIM incubation, although the time of CIM incubation is also important for the expression of genes expressed later, during SIM incubation (CHE; LALL; HOWELL, 2007).

Markers of the acquisition of organogenic competence should be expressed during CIM incubation, as is the case of the gene *CUC2,* whose transcripts accumulate after two days of CIM incubation at sites with the potential to form shoots (MOTTE et al., 2011). The cytokinin receptor *AHK4* might also be an acquisition of competence marker. During CIM incubation, localized *AHK4* expression identifies sites of future cytokinin-induced *WUS* transcription during the subsequent incubation on SIM (GORDON et al., 2009). The role of *PLETHORA* (*PTL*) genes in the regeneration process has been also recently described. These genes act in the competence establishment by activating root stem cells regulators, and also by acting in the regulation of *CUC* genes during the regeneration process (KAREEM et al., 2015). The genes *ACR4* and *IAA20* that are highly upregulated after 48 hours of incubation in CIM, but not yet after 24 hours, are also candidate genes to control acquisition of competence (MOTTE et al., 2014).

In the next step of regeneration, during SIM incubation, cytokinin is the responsible to form a shoot stem cell niche (GORDON et al., 2009). In this step, the genes involved in the cytokinin uptake, transport, biosynthesis and degradation are very important, since the cytokinin has to reach the founder cells (CORTIZO et al., 2009). Also, the auxin-cytokinin crosstalk is especially important to the expression of key genes that will determine the organization of the developing shoot meristem (MOTTE et al., 2014).

The overexpression of phosphate-isopentenyl transferase (IPTs), a key enzyme in cytokinin biosynthesis (TAKEI; SAKAKIBARA; SUGIYAMA, 2001), allowed the regeneration of shoots on callus even without exogenous cytokinin addition to the medium (SUN et al., 2003), and the loss of function of this gene diminishes regeneration capacity (CHENG et al., 2013).The loss of function of histidine kinases genes (*AHKs*), responsible for cytokinin perception, reduces or completely extinguishes *in vitro* shoot formation (MOTTE et al., 2013). Also, overexpression of CYTOKININ INDEPENDENT KINASE (CKI), involved in cytokinin signaling, results in shoot regeneration independent of the presence of cytokinin in the medium (HWANG; SHEEN, 2001).

Many genes with known function in the formation of the shoot apical meristem *ex vitro,* are also expressed during *in vitro* shoot regeneration (MOTTE et al., 2014). The *WUSCHEL-CLAVATA* (*WUS-CLV*) mechanism () with a known role in the shoot apical meristem (SAM) formation, is also of great importance for shoot regeneration. The gene *WUS* has two main roles in shoot regeneration: it is involved in cell respecification (GORDON et al., 2007), and it is expressed in the regions that where likely to develop shoots, marking the beginning of shoot meristem formation (CHENG et al., 2013). Following the *WUS* expression, *CLV3* is expressed in the apex of organ primordia during their conversion to shoot meristem (CHATFIELD et al., 2013).

SHOOT MERISTEMLESS (*STM*) also acts together with *WUS* in the meristem formation and maintenance (LENHARD; JÜRGENS; LAUX, 2002). In Arabidopsis, the timing of *STM* expression marks the timing when the explants can be transferred from SIM to hormone free medium without affecting their regeneration capacity (ZHAO; FISHER; AUER, 2002), thus, marking the end of the induction phase and the shoot determination (MOTTE et al., 2014).

Some MicroRNAs also have a differential expression in regenerative and recalcitrant callus. *miR165* and *miR166* were shown to be important during shoot induction and development (JUNG; PARK, 2007; ZHANG; ZHANG, 2012).

1.2 Molecular mechanisms in root regeneration

Although shoot induction usually does not occur in hormone free medium, this is not true for root induction. An explanation for this maybe the fact that in leaf explants cultivated with no additional hormones in the medium, wounding induces the production of free auxin,

which is then highly concentrated in the procambium stem cells. It was recently proposed that the procambium cells may serve as a pericycle-like cells in aerial organs (LIU et al., 2014).

Lateral root and adventitious root formation, after their initiation, are rather similar developmentally. Although, in the first step of cell transition, adventitious root formation requires the expression of the genes *WUSCHEL RELATED HOMEOBOX11* (*WOX11*) and *WOX12*, whereas lateral root initiation does not. Also, initiation of adventitious roots shares similar regulatory mechanisms with that of callus. Whether leaf explants produce adventitious roots or callus mainly depends on free auxin levels. The auxin maximum induces *WOX11* expression, and its action redundantly with *WOX12* mediates the cell fate transition from procambium cells to root founder cells (LIU et al., 2014).

Age is critical for the regeneration of adventitious roots from leaf explants. The decreased regeneration ability of older leaves is probably a result of insufficient free auxin level in their tissues, or inefficient polar auxin transport in older leaves (CHEN et al., 2014).

In addition to the auxin-mediated cell transition pathway, another cell fate transition pathway has been proposed recently. This new pathway involves a *NAC* transcription factor that seems to function in regulating the cellular environment in both mesophyll and competent cells for promotion of root tip emergence. The *NAC* pathway is independent of the auxin-mediated *WOX11* pathway. The *NAC* expression is induced in the wounded sites and promotes cysteine endopeptidases (*CEP*s) expression. *CEP*s were shown to act in the degradation of cell wall extensin (EXT) proteins (HELM et al., 2008). EXP are basic components of the cell wall whose genes expression are induced by wounding (MERKOUROPOULOS; SHIRSAT, 2003). Upregulation of *CEP*s is probably related to the degradation of EXTs that promote wound healing and might be a barrier for the emergence of regenerated root tips. There is the hypothesis that the *NAC1-CEP* pathway antagonizes *EXT*-mediated wound healing, and this allows the emergence of regenerated root tips (CHEN et al., 2016)

1.3 Tomato *in vitro* regeneration

As discussed above, most that is known regarding *in vitro* root and shoot regeneration came from studies using the plant model Arabidopsis. However, since Arabidopsis is different in many aspects from the most economically important crops, the use of other species as plant models to study the regeneration process may be useful to increase our knowledge about this process.

In this aspect, tomato is a great model to study plant regeneration, since many tomato related wild species have an enhanced *in vitro* regeneration (PERES et al., 2001). In the last few years, studies involving these wild species, mainly *S. peruvianum* and *S. pennellii* allowed some aspects of tomato *in vitro* regeneration to be unraveled (ARIKITA et al., 2013; AZEVEDO, 2012; KOORNNEEF et al., 1987; LOMBARDI-CRESTANA et al., 2012; PINO et al., 2010)

S. peruvianum high organogenic capacity is related mainly to two dominant alleles called *REGENERATION 1 (Rg1)* and *REGENERATION 2 (Rg2)* (KOORNNEEF et al., 1987). The presence of *Rg1* is sufficient to form shoots *in vitro* from roots explants. *Rg1* was mapped in the chromosome three (KOORNNEEF et al., 1993), between the genes *BETA-CAROTENE HYDROXYLASE* (*CrtR-b*) (GALPAZ et al., 2006) and *PHYTOENE SYNTHASE* (*PSY1*) (BARTLEY et al., 1991; FRAY, GRIERSON, 1993). *S. peruvianum* harbors the recessive allele *yellow flesh* (*r*) of the gene *PSY1*. This allele represents a loss of function that gives the yellow color to the fruit when introgressed into the *S. lycopersicum* background (KOORNNEEF et al. 1987).

The presence of the allele *r* in these species made possible its utilization as a morphological marker for the introgression of the *Rg1* allele into cultivated tomato. Using this approach this allele was introgressed into the cultivar Micro-Tom (MT) background, producing a genotype with high regeneration capacity and a dwarf phenotype (LOMBARDI-CRESTANA, 2012). MT-*Rg1* was suggested to be a valuable tool for genetic transformation of the model MT (PINO et al., 2010).

In MT background, these allele, besides increasing shoot formation, also enhances *in vitro* root formation, when cultivated in RIM (LOMBARDI-CRESTANA et al., 2012). Moreover, *Rg1* was also capable of revert the low *in vitro* regeneration in the mutant *procera*, which has a constitutive gibberellin response, due to a point mutation in the DELLA gene *LeGAI* (JASINSKI et al., 2008). The double mutant *proRg1* had a higher number of both root and shoot *in vitro* than had the mutant *procera*. The recovery of the low organs formation of the mutant *procera* by *Rg1* demonstrates the occurrence of epistasis between these two mutations (LOMBARDI-CRESTANA et al., 2012), which is indicative that these two alleles are in a signal transduction pathway that converge at some point. Also, *Rg1* was capable of rescue the *ex vitro* low lateral shoot formation in the mutant *lateral suppressor*. Since the acquisition of competence is probably a common event for both roots and shoots formation *in vitro*, the high capacity to form both roots and shoots *in vitro* make of *Rg1* a good candidate to be controlling acquisition of competence.

In Arabidopsis the main goal of CIM pre incubation is to obtain organogenic callus with primordia that have the competence to form organs (MOTTE et al., 2014). In tomato, although, the acquisition of competence can be achieved without CIM pre incubation, it was demonstrated that two days of incubation on RIM before SIM increases shoot regeneration in MT explants (PINO et al., 2010).

Acquisition of competence in MT takes 2-3 days on SIM incubation, but takes only 1-2 days in MT-*Rg1*. The early acquisition of competence of MT-*Rg1* is an additional evidence that this allele is probably enhancing root and shoot regeneration acting in this developmental (AZEVEDO et al., 2012).

Considering the possibility that other wild species harbouring the allele *r* probably would harbor the genes related to the high organogenic capacity (PERES et al., 2001), *in vitro* organogenic capacity of the specie *Solanum pennellii* was tested, confirming that this specie also owns a high organogenic capacity (ARIKITA et al., 2013).

Since previous studies suggested that other loci than *Rg1* would be controlling organogenic capacity in tomato, the utilization of an introgression line collection, composed by 50 ILs (introgression lines), each one harbouring a small segment of a certain chromosome of *S. pennellii* 'LA716' introgressed and mapped into M82 cultivar, made possible the identification of six introgression lines (*Rg3C*, *Rg7H*, *Rg8F*, *Rg9DE*, *Rg10F*, *Rg6A*) harbouring a locus for high organogenic capacity. Four of these ILs (*Rg3C*, *Rg7H*, *Rg8F*, *Rg10F*) have high formation of both root and shoot *in vitro*, an evidence that probably these alleles are involved in the acquisition of competence phase. The other two alleles (*RG9DE*, *RG6A*) enhance only *in vitro* shoot formation, probably affecting the organogenic induction phase (ARIKITA et al., 2013).

As described above, in the last few years many studies have collaborated to increase the understand regarding plant in vitro regeneration. Although, most discoveries are related to the induction phase of the regeneration process in Arabidopsis, while the phase of acquisition of competence remains poorly understood. Thus, the results of this work are divided into two manuscripts. In the first one, after the complete introgression of the alleles Rg3C, Rg7H, Rg8F from the wild relative S. pennellii, which confer enhanced in vitro shoot and root regeneration, into Micro-Tom (MT), we present a phenotypic and molecular characterization of these genotypes, called MT-*Rg3C*, MT-*Rg7H* and MT-*Rg8F*. In the second one, we present a comparative analysis between the two Near-Isogenic Lines (NILs), MT-*Rg1* and MT-*Rg3C*, contributing to the assertion that *Rg1* and *Rg3C* are two alleles of the same gene, and propose a list of 27 genes candidates to *Rg1/Rg3C*. With this work we aim to collaborate

to increase our understanding about plant *in vitro* regeneration, especially the acquisition of competence phase.

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2 GENETIC AND PHYSIOLOGICAL CHARACTERIZATION OF THREE NATURAL ALLELIC VARIATIONS AFFECTING ORGANOGENIC CAPACITY IN TOMATO (*Solanum lycopersicum* CV. MICRO-TOM).

Abstract

Beyond the importance of postembryonic organogenesis for plant biotechnology, the capacity of continuous organ initiation and growth in these sessile organisms probably evolved to confer advantage in oscillating environmental conditions. Thus, the study of natural variations affecting organogenic capacity is not only relevant to manipulate plant development, but also to understand its ecological and evolutionary significance. Here, we report the genetic and physiological characterization of three tomatoes (*Solanum lycopersicum*) loci whose alleles from the wild relative *S. pennellii* enhance *in vitro* shoot and root regeneration. The *S. pennellii* alleles were introgressed into the tomato genetic model cv. Micro-Tom (MT), creating the near isogenic lines (NILs) MT-*Rg3C*, MT-*Rg7H* and MT-*Rg8F*. We evaluated the time taken for shoot induction and acquisition of competence by quantifying organogenesis after transferring explants, respectively, from shoot-inducing medium (SIM) to basal medium (BM) and from root-inducing medium (RIM) to SIM. In these two events, we monitored the expression patterns of key genes related to shoot regeneration, such as *SHOOT MERISTEMLESS* (*STM*), *CUP-SHAPED COTYLEDON 2* (*CUC2*) and *WUSCHEL* (WUS). MT-*Rg3C* and MT-*Rg7H* started shoot induction 48h and 24h, respectively, earlier than MT and MT-*Rg8F* while MT-*Rg3C* acquired competence 24h before MT. Since acquisition of competence is a common pathway for both root and shoot formation, at least one gene located in each of the MT-*Rg3C* and MT-*Rg8F* introgressed regions must be involved in the production of undifferentiated cells able to undertake different fates. MT-*Rg7H*, which presented enhanced expression of the shoot-related genes *WUS* and *STM*, seems to specifically affect shoot induction. Phenotypic characterization of greenhouse-growing plants showed that the *Rg3C* region induced increased branching *ex vitro*, when comparing MT- *Rg3C* to MT. On the other hand, the normal branching development observed in MT-*Rg7H* and MT-*Rg8F* indicates that adventitious *in vitro* shoot formation and *ex vitro* axillary bud formation/outgrowth are induced by different genetic pathways, which has practical implications to breeding. We further discuss the evolutionary and ecological significance of alleles enhancing organogenesis in tomato.

Keywords: Regeneration; *Solanum pennellii*; Competence; Introgression lines

2.1 Introduction

The remarkable ability of plants to develop new and adventitious organs after embryonic growth is a feature with great ecological and evolutionary significance. Since plants are sessile organisms, continuous organ formation and growth allow them to use newly formed roots and shoots to foraging ecological resources, such as light and nutrients. *De novo* organogenesis was also an important trait during the domestication of vegetatively propagated crops (HARLAN, 1992), and later, the possibility to regenerate new organs *in vitro* from plant

tissues was essential for the development of biotechnological tools (GERSZBERG et al., 2015). Currently, *in vitro* regeneration is a *sine qua non* in most protocols developed to obtain transgenic plants (SUSSEX, 2008). In the last years, however, the molecular pathway to shoot regeneration has been gradually revealed (MOTTE et al., 2014). Nonetheless, this process, as much as its elusive steps (e.g., the acquisition of organogenic competence) are not fully understood yet.

Skoog and Miller (1957) were among the first to master plant *in vitro* regeneration. They showed the influence of plant hormones and their interactions in adventitious organs formation. Thus, the ratio of auxin to cytokinin in the *in vitro* medium is crucial to determine the kind of organ formed: a high auxin/cytokinin ratio induces root development while a low ratio induces shoot formation. An intermediate ratio is expected to induce cell multiplication leading to the formation of a tissue called callus. The *in vitro* plant regeneration process can be divided in three phases: acquisition of competence, induction and determination. During acquisition of competence, the tissues develop characteristics to respond to the induction phase, when hormones in the medium will direct the cell fate to forming either roots or shoots. In the third phase, the cells are already determined to follow a developmental fate and will differentiate and develop visible roots or shoots regardless of the medium composition (CHRISTIANSON; WARNICK, 1985).

Probably all explants have a natural organogenic capacity to form roots or shoots, although some seem to have a genetic or developmental blockage that prevents the explant to acquire the competence necessary to respond to hormonal stimuli in the medium (AUER et al., 1999, CHRISTIANSON; WARNICK, 1985, 1988; GILISSEN et al., 1996). Many studies related to *in vitro* organogenesis are focused in overcoming this blockage, allowing the explant to acquire the competence necessary to induce a given organ (CARY; CHE; HOWELL, 2002). *In vitro* organogenesis may occur directly, without previous callus formation, or indirectly, with callus formation occurring before the induction of shoots or roots. Protocols for indirect organogenesis are usually divided into two steps. First, the explant acquires the competence necessary to respond to the induction signal on a callus-inducing medium (CIM) rich in auxin. After acquiring competence, organ induction and formation will occur on a medium rich in cytokinin (Shoot-inducing Medium – SIM) or again auxin (Root-inducing Medium – RIM) (CARY et al., 2001; CARY; CHE; HOWELL, 2002; VALVEKENS; MONTAGU; LIJSEBETTENS, 1988). However, many important crops do not follow this two-step organogenesis process, making it difficult to develop a general regeneration protocol.

Until recently, callus was thought to be a mass of undifferentiated cells formed by the dedifferentiation of somatic cells. However, now callus is actually seen as organized and differentiated tissues that harbor specialized cells, called stem cells (SUGIMOTO; GORDON; MEYEROWITZ, 2011). These stem cells have the capacity to develop shoots and roots, and are regarded as pluripotent. In Arabidopsis roots cultivated *in vitro*, pericycle cells develop a cellular mass that resembles the lateral root meristem, which will form a callus and then the new organs (ATTA et al., 2009). The same developmental pattern was observed in other explants, such as cotyledons and petals, which have pericycle-like cells around the vascular tissue that are responsible for new organ initiation by proliferating after the hormonal balance stimulus (SUGIMOTO; JIAO; MEYEROWITZ, 2010). The corollary is that *in vitro* root and shoot developmental pathways branch off from the same initial genetic-physiological mechanism. On CIM, auxin stimulus is perceived by the pericycle (or pericycle-like) cells triggering competence acquisition and is identical for both, root and shoot formation (LOMBARDI-CRESTANA et al., 2012; MOTTE et al., 2011). Therefore, the genes controlling this process should affect both organogenesis pathways, and their expression are expected to be induced by auxin (CHE; LALL; HOWELL, 2007).

It is conceivable that the genes and signals involved in the acquisition of competence should act upstream the genes specifying organ development, which will act in the induction phase. Therefore, in order to identify key regulator of acquisition of competence, genes expressed a step before the commitment to organogenesis should be identified and characterized (SANTOS et al., 2009). Another approach is to search for and study genotypes (induced mutants and natural genetic variations) with high or low organ formation rates on both, SIM and RIM (ARIKITA et al., 2013; LOMBARDI-CRESTANA et al., 2012). Identifying these factors is an important step to further our understanding the organogenesis mechanisms, particularly the acquisition of competence phase (KOORNNEEF et al., 1993; LOMBARDI-CRESTANA et al., 2012).

Here, we genetically and physiologically characterized three tomatoes (*Solanum lycopersicum*) loci using near-isogenic lines (NILs) whose alleles from the wild relative *S. pennellii* confer enhanced *in vitro* shoot and root regeneration in our model system, cv. Micro-Tom (MT). One of the loci (*Rg7H*) is specifically linked to shoot induction, which is consistent to an enhanced expression of the shoot-related genes *WUSCHEL* (*WUS*) and *SHOOT MERISTEMLESS* (*STM*) in the NIL harboring the *S. pennellii* allele. The other two *pennellii* loci (*Rg3C* and *Rg7H*) enhanced the capacity to form both roots and shoots on RIM and SIM, respectively. This suggests that these loci control acquisition of competence, *i.e*. the

production of uncommitted cells able to undertake different developmental fates. We also demonstrated that *in vitro* adventitious shoot formation as well as *ex vitro* axillary bud formation/outgrowth are induced by different genetic pathways. These results open the possibility to create tomato varieties and hybrids with improved capacity of *in vitro* regeneration and production of transgenic plants without undesired traits, such as increased number of side shoots (suckers). We further discuss the evolutionary and ecological significance of alleles enhancing organogenesis in tomato.

2.2 Material and Methods

2.2.1 Plant material

The introgression of the *S. pennellii* loci *Rg7H, Rg8F e Rg3C* into the MT cultivar was carried out as described previously (CARVALHO et al., 2011; PINO et al., 2010). Briefly, pollen was collected from parent plants and used to fertilize emasculated MT flowers. Since part of the introgression process had already been carried out in previous work, MTx*Rg8F* BC1, MTx*Rg7H* BC3 and MTx*Rg3C* BC3 were used as parents (ARIKITA et al., 2013). After each backcrossing, seeds were germinated *in vitro* and their cotyledons were used to select plants according by their shoot regeneration capacity on shoot-inducing medium (SIM - see item 2.3). Plants used as explant source were kept *in vitro* after cotyledon excision and the ones with high shoot regeneration rates were selected for the next backcross cycle (Figure 2.1). This process was repeated up to the sixth generation (BC6). After BC6F2 the resulting genotypes were considered as near-isogenic lines (NILs) (CARVALHO et al., 2011; STAM; ZEVEN, 1981).

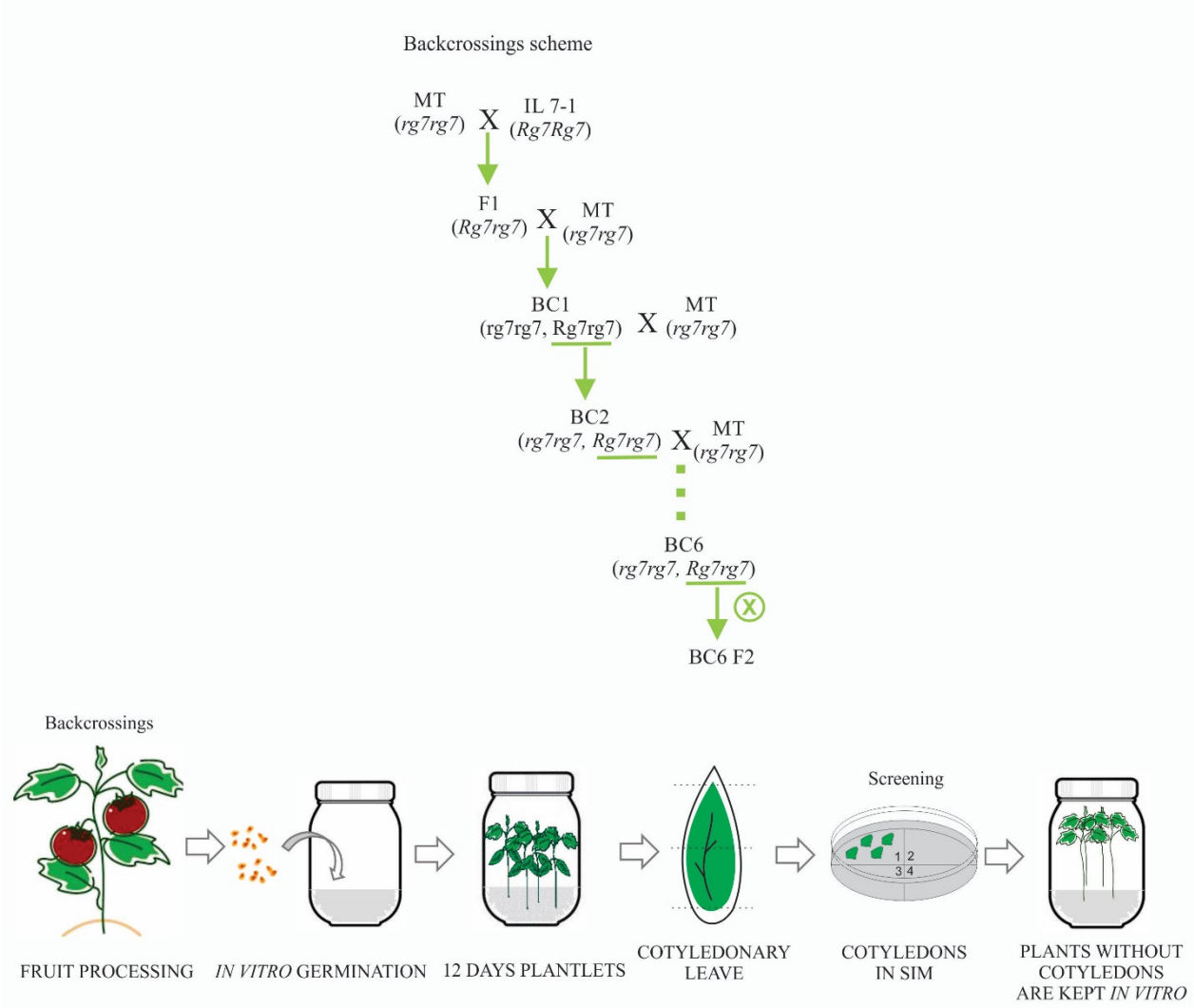

Figure 2.1 - Backcross scheme and screening process. *Rg7H* is used as an example, and the same method was used for introgression of *Rg3C* and *Rg8F* loci into *S. lycopersicum* cv. Micro-Tom

2.2.2 Growth conditions

Plants were grown in a glasshouse at the Laboratory of Hormonal Control of Plant Development, ESALQ-USP (Piracicaba, SP, Brazil). The growth conditions were mean temperature of 28 ºC, 11.5 h/13 h (winter/summer) photoperiod, 250–350 µmol photons m^{-2} s^{-1} PAR irradiance, attained by a reflecting mesh (Aluminet, Polysack Indústrias Ltda, Leme, Brazil), and automatic irrigation to field capacity four times a day. Seeds were germinated in bulk in 350 mL pots with a 1:1 mixture of commercial potting mix Basaplant® (Base Agro, Artur Nogueira, SP, Brazil) and expanded vermiculite supplemented with 1 g L^{-1} 10:10:10 NPK and 4 g L^{-1} dolomite limestone ($MgCO_3$ + $CaCO_3$). Upon appearance of the first true leaf, seedlings of each genotype were individually transplanted to pots containing the soil mix described above, except for that NPK supplementation was increased to 8 g L^{-1}.

2.2.3 *In vitro* tissue culture

Seeds from *Solanum pennellii* LA716, *S. lycopersicum* cv. Micro-Tom and the lines *MT-Rg3C*, *MT-Rg7H*, *MT-Rg8F* were surface-sterilized by soaking in 20 mL 30% (v/v) commercial bleach (2.7% sodium hypochlorite) 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 (MURASHIGE; SKOOG, 1962), half strength B5 vitamins (GAMBORG; MILLER; OJIMA, 1968), 30 g L^{-1} sucrose, and 2.3 g L^{-1} phytagel. 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 days, followed by 8 days under 16-h photoperiod.

Cotyledons were collected from 12-day-old (after sowing) seedlings. The distal and proximal tips were removed, and the cotyledons were divided transversally into two or three pieces. Explants were placed with the abaxial side down, immediately after isolation, onto semi-solid medium composed by MS salts with B5 vitamins, 30 g L^{-1} sucrose, 2.3 g L^{-1} agar, and 5 µM benzylaminopurine (BAP) for Shoot-Inducing Medium (SIM) or 0.4 µM naphthalene acetic acid (NAA) for Root-Inducing Medium (RIM). Sixteen cotyledonary explants were cultured per Petri dish (90 × 15 mm), with 10 plates per treatment for the lines and 6 plates for the double mutants. Plates were sealed with PVC film and maintained under 16-h photoperiod at 25 ± 1°C. After 9 days, the number of roots per explant on RIM was evaluated, and after 21 days, the percentage of explants with shoots on SIM was recorded.

2.2.4 Determination of the timing required for acquisition of competence and shoot induction

Shoot induction timing was determined by the progressive transfer of MT, MT-*Rg3C*, MT-*Rg7H*, MT-*Rg8F* and *S. pennellii* cotyledonary explants from SIM to the Basal Medium (BM – MS salts with B5 vitamins, 30 g L^{-1} sucrose, 2.3 g L^{-1} agar) and varying the number of days the explants remained in each medium (Figure 2.2). After a total of 21 days of *in vitro* cultivation, the percentage of explants with shoots was determined.

	DAYS							
SIM	3	4	5	6	7	8	9	21
BM	18	17	16	15	14	13	12	0

Figure 2.2 - Gradual medium substitution scheme used to determine the beginning of the *in vitro* shoot induction phase. SIM – Shoot-Inducing Medium. BM – Basal Medium

Timing for acquisition of competence was determined by the progressive transfer of MT, MT-*Rg3C*, MT-*Rg7H*, MT-*Rg8F* and *S. pennellii* cotyledonary explants from RIM to SIM, varying the number of days the explants remained in each medium, up to 8 days. After that, all explants were transferred to BM, up to complete 21 days of cultivation (Figure 2.3). After 21 days, the percentage of explants with shoots or roots was determined.

	DAYS				
RIM	0	1	2	3	8
SIM	8	7	6	5	0
BM	13				

Figure 2.3 - Gradual medium substitution scheme used to determine the beginning of the acquisition of competence phase. SIM – Shoot-Inducing Medium. RIM – Root-Inducing Medium. BM – Basal Medium

2.2.5 Phenotypic characterization and productivity traits

Phenotypic characterization of all plant genotypes was performed at 50 days after germination (dag): branching index plant height at the primary shoot, number of leaves up to the first inflorescence, and lobule number in the fourth leaf.

The branching index represents the sum of the length of lateral branches divided by the main stem length (MORRIS et al., 2001).

In the individual axis branching analysis, each line represents a leaf axis and each column, an individual plant. Grey squares represent an empty axis, white squares the presence of one or two leaves, light green squares the presence a side shoot and dark green squares the presence of a well-developed side shoot (more than 10 cm). C1 and C2 represent cotyledonary leaves (Analysis adapted from BUSCH et al., 2011).

Productivity performance of plants was assessed at 90 dag. The following parameters were determined: number of fruits per plant, total fruit weight per plant (yield), total soluble solids content in fruits (Brix), frequency of green and ripe fruits, and number of seeds per

fruit. Total soluble solids content was assessed using a digital refractometer (PR-101, Atago, Tokyo, Japan) in fruits from 10 different plants per genotype.

2.2.6 Expression analysis of marker genes for *in vitro* shoot regeneration

To evaluate the expression pattern of key genes involved in *in vitro* shoot regeneration, an *in vitro* shoot regeneration assay (as described in item 2.3) was established using cotyledons of the following genotypes: MT, MT-*Rg3C*, MT-*Rg7H* and MT-*Rg8F*.

During the 3-week cultivation period, samples for gene expression analysis were collected at 0, 1, 2, 6 and 21 days. Acquisition of competence for regeneration occurs at days 1-2, and induction of shoot development is expected to start around day 6. Eight explants of each genotype were collected each day and immediately frozen with liquid nitrogen, with samples kept at -80°C until RNA extraction.

Total RNA was isolated from *in vitro* explants using *mir*Vana™ miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. RNA was quantified in a Thermo Scientific NanoDrop 2000 spectrophotometer and RNA integrity was examined by gel electrophoresis. Total RNA was treated with TURBO DNA-*free*™ Kit (Life Technologies), and then used for cDNA synthesis using SUPERSCRIPT III 1ST Strand Synthesis kit (Life Technologies) according to the manufacturer's instructions. Quantitative RT-PCR (qPCR) data were normalized using the Pfaffl method (PFAFFL, 2001) and the constitutive glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and elongation factor 1 alpha (*EF-1 alpha*) housekeeping genes were used as internal controls.

We used three biological repetitions, composed by 8 explants each, and three technical repetitions. For the statistical analysis the data was transformed using Log2.

We analyzed the expression of genes considered markers of *in vitro* shoot regeneration, based on their known essential roles on *in vitro* regeneration: *GOB* (Solyc07g062840), *WUSCHEL* (Solyc02g083950), *Let6* (Solyc02g081120), and *ARF5* (Solyc04g081240) (MOTTE et al., 2014).

2.3 Results

2.3.1 *In vitro* and molecular characterization of MT-*Rg3C*, MT-*Rg7H* and MT-*Rg8F*

Three alleles were independently introgressed from *S. pennellii* into the cv. Micro-Tom. The loci are located on chromosomes 3 (Figure 2.4), 7 (Figure 2.5), and 8 (Figure 2.6). As described in Arikita et al. (2013), the region defining the *Rg3C* locus was named after the bin 3C, which has 601 genes in bin 3C of chromosome 3. Previously, Koornneef et al. (1993) localized *Rg1*, which is probably allelic to *Rg3C* (ARIKITA et al., 2013), between the markers *white flower* (*wf*) and *yellow flesh* (*r*), thus reducing the list to 181 candidate genes. Bins 7H and 8F contain 257 and 202 genes, respectively. After introgression of the *Rg3C*, *Rg7H* and *Rg8F* loci into Micro-Tom, we analyzed these genotypes for root and shoot regeneration capacity on RIM and SIM medium, respectively.

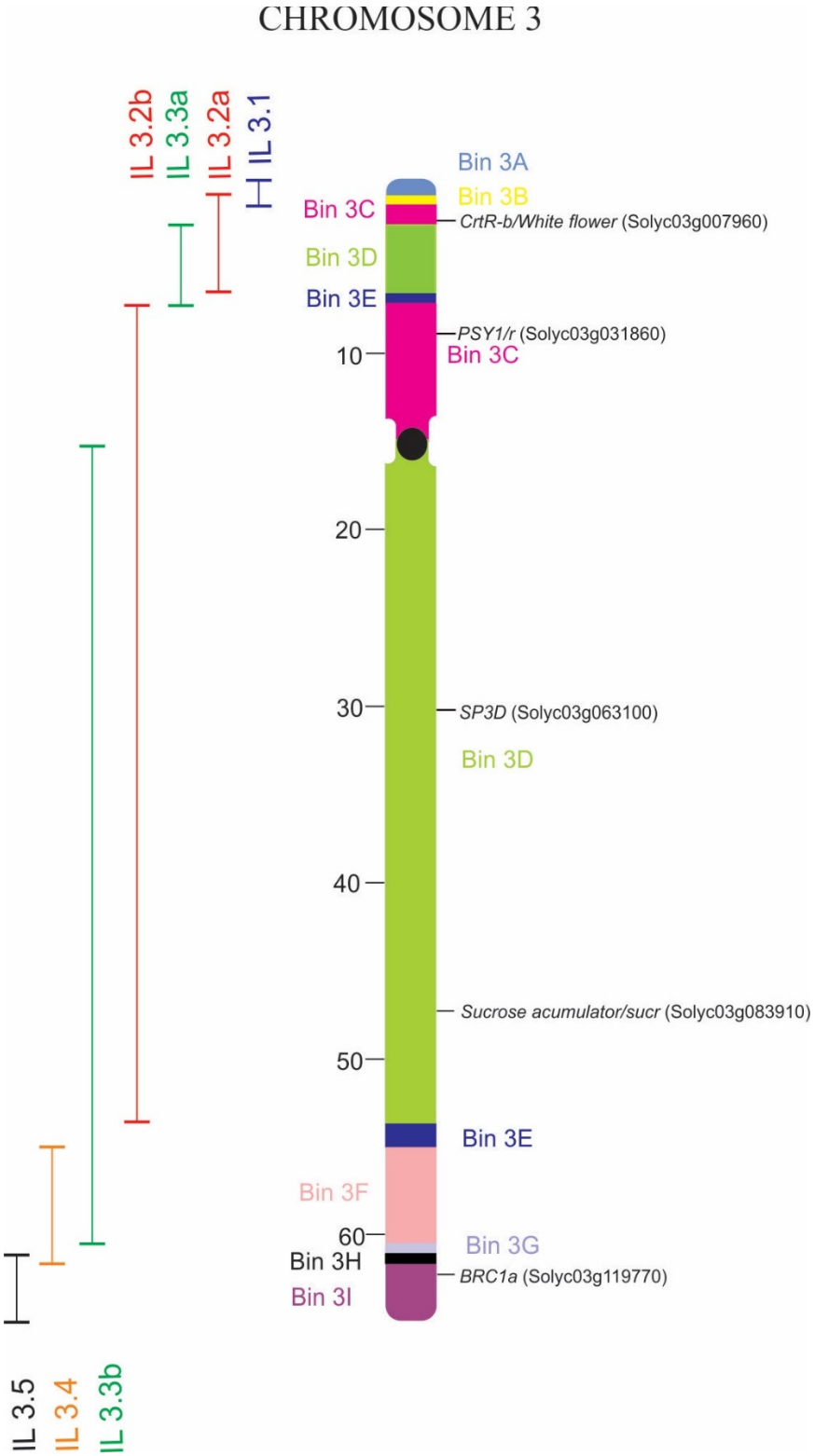

Figure 2.4 - Scheme of tomato chromosome 3, indicating the introgression lines, bins and main marker genes available in distinct introgression lines. Bin 3C (pink) represents the segment from *S. pennellii* where the locus *Rg3C* is located. Image designed from the data presented in Chitwood et al. (2013) and Tomato genome consortium (2012)

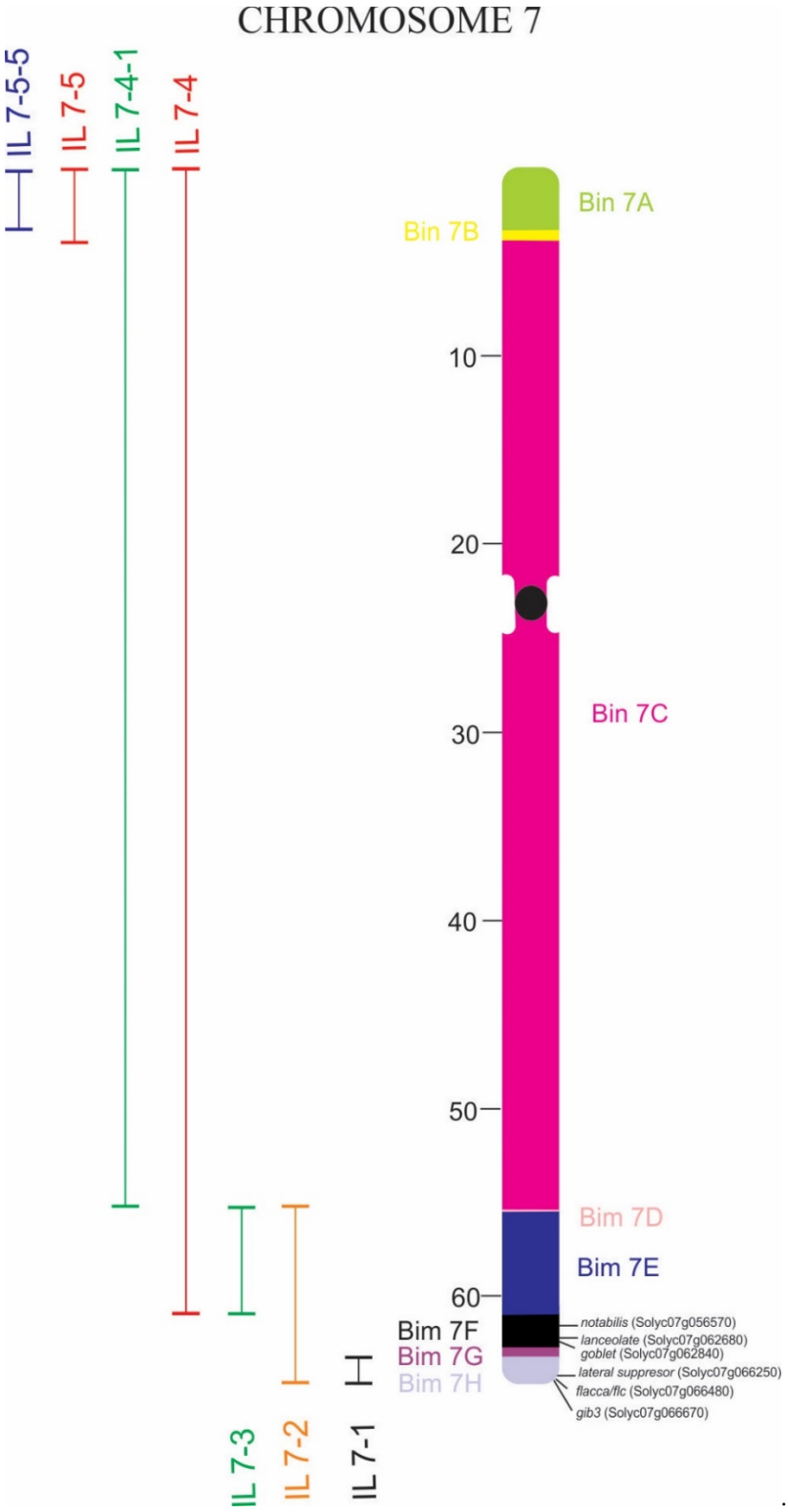

Figure 2.5 - Scheme of tomato chromosome 7, indicating the introgression lines, bins and main marker genes available in distinct introgression lines. Bin 7H (lilac) represents the segment from *S. pennellii* where the locus *Rg7H* is located. Image designed from the data presented in Chitwood et al. (2013) and Tomato genome consortium (2012)

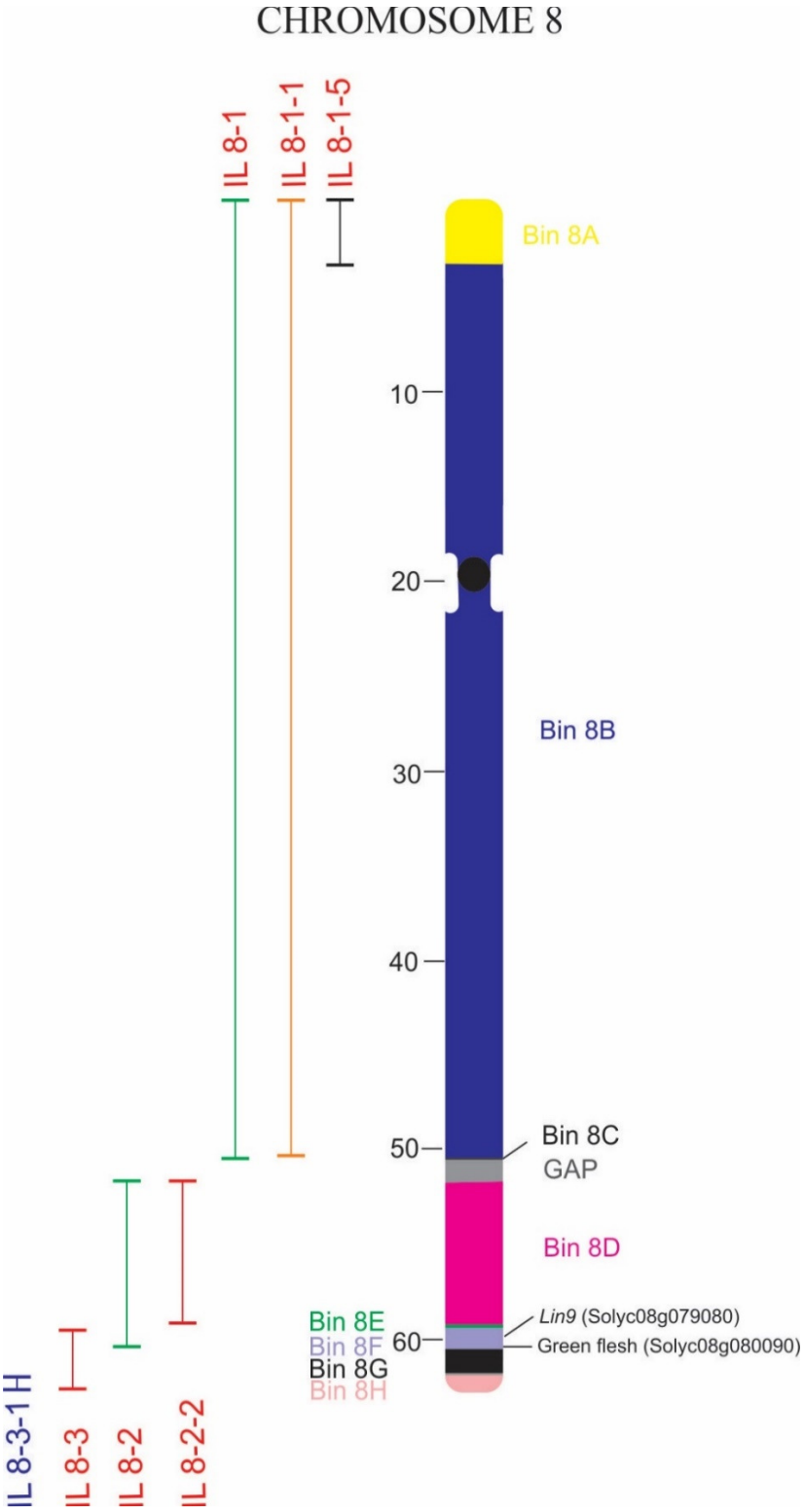

Figure 2.6 - Scheme of tomato chromosome 8, indicating the introgression lines, bins and main marker genes available in distinct introgression lines. Bin 8F (lilac) represents the segment from *S. pennellii* where the locus *Rg8F* is located. Image designed from the data presented in Chitwood et al. (2013) and Tomato genome consortium (2012)

Root development in cotyledonary explants was significantly higher in all three NILs than in MT (Figure 7), confirming that *S. pennellii* locus had been introgressed into MT.

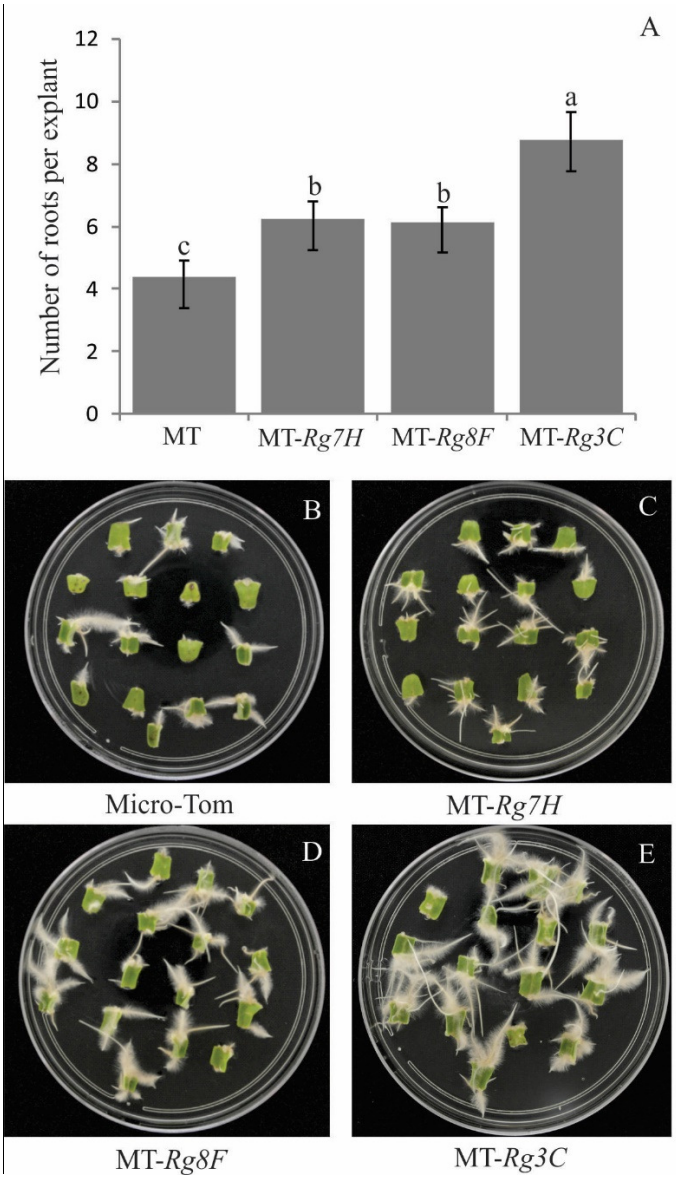

Figure 2.7 - *In vitro* root regeneration of the genotypes Micro-Tom (MT), MT-*Rg7H,* MT-*Rg8F* and MT-*Rg3C*. (A) 12 day-old seedling cotyledon explants were cultivated during 9 days on MS medium with 0.4 µM NAA. (B-E) Aspect of root regeneration from selected genotypes. Bars depicted with same letter are not significantly different ($p>0.05$) according to the unpaired Student's *t*-test (n=10 Petri dishes, each containing 16 explants)

Regarding shoot formation on cotyledonary explants, all three genotypes shower significantly higher proportion when compared to MT (Figure 2.8). This result correlates with root formation (Figure 2.7) and further confirms that the *S. pennellii* loci were correctly introgressed into MT.

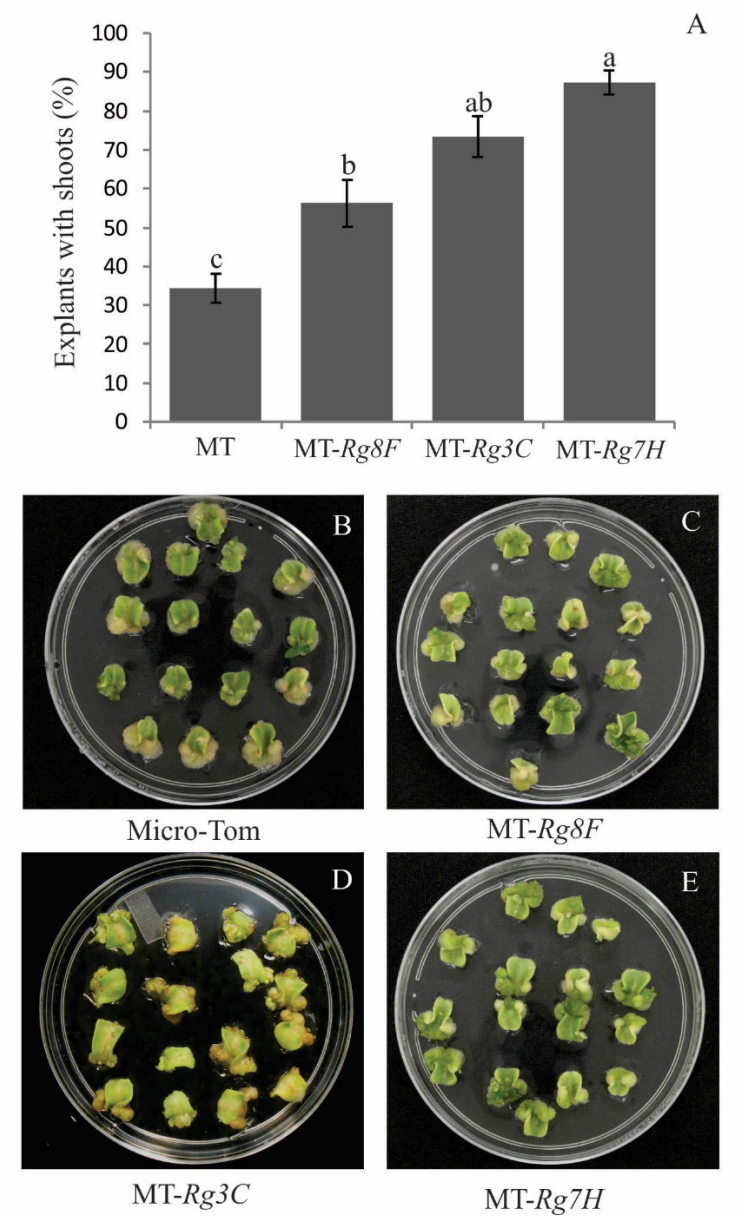

Figure 2.8 - *In vitro* shoot regeneration of the genotypes Micro-Tom (MT), MT-*Rg8F*, MT-*Rg3C* and MT-*Rg7H*. (A) 12- day-old seedling cotyledon explants were cultivated during 21 days on MS medium with 5.0 µM BAP. (B-E) Aspect of shoot regeneration from selected genotypes. Bars depicted with the same letter are not significantly different ($p>0.05$) according to the unpaired Student's *t*-test (n=10 Petri dishes, each containing 16 explants)

These results indicate that the three loci may be involved in the acquisition of competence step, since all *S. pennellii* alleles increased both root and shoot organogenesis. However, it is interesting to note that the NIL with the highest root formation was MT-*Rg3C* while that with the highest shoot formation was MT-*Rg7H*. Therefore, the specific differences in shoot and root formation among the NILs may indicate that other determinants, such as genes dosage or presence of strong or weak alleles in each locus, are also influencing the regeneration processes. Alternatively, besides the acquisition of competence, some loci may also be affecting the step of shoot (e.g., *Rg7H*) as well as root induction (e.g., *Rg3C*). To test

this hypothesis, we evaluated the time required for acquisition of competence and shoot induction for each NIL.

First, we determined the time required for the shoot induction phase to start in each NIL by gradually transferring the explants from SIM to BM. Among all NILs, MT-*Rg3C* required less time to start the shoot induction phase. Hence, just three days on SIM were enough for MT-*Rg3C* to develop shoots after transferring to BM. MT-*Rg7H* also showed early shoot induction when compared to MT, since shoots developed on BM after just four days of initial incubation on SIM, while MT-*Rg8F* and MT required at least five days (Figure 2.9).

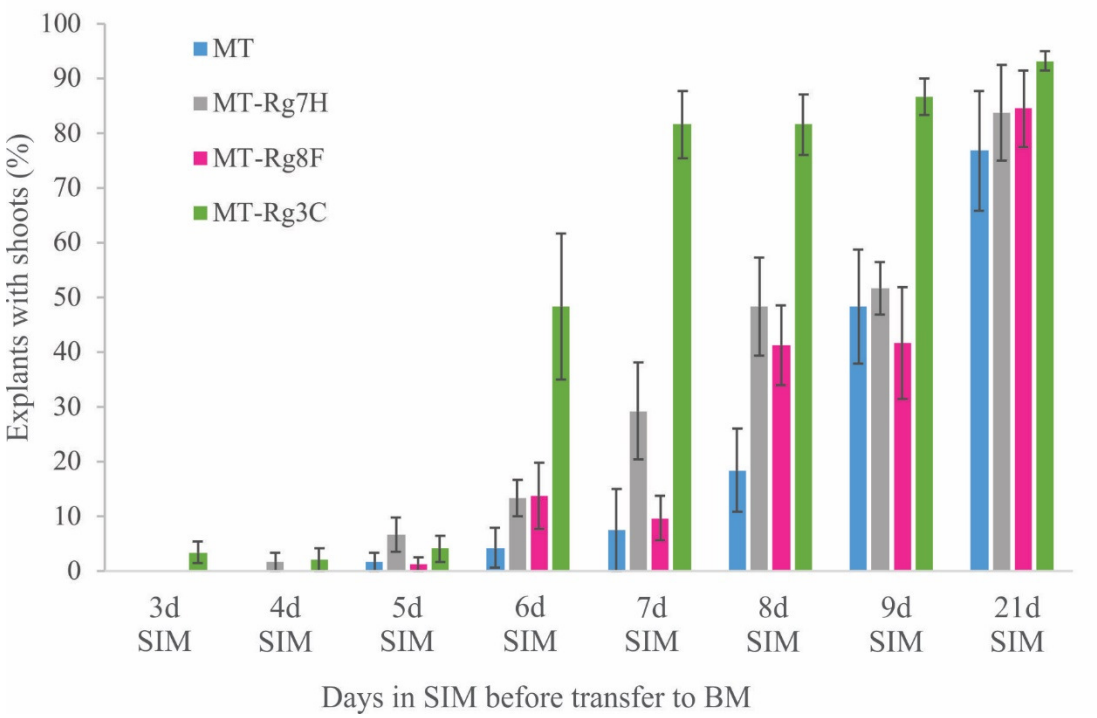

Figure 2.9 - Number of days required on SIM for induction of shoots in cotyledonary explants of MT, MT-*Rg3C*, MT- *Rg7H*, MT-*Rg8F*. After each incubation time on SIM, explants were transferred to and remained on BM until completing 21 days of *in vitro* cultivation. Presence of shoots in the explants was evaluated after completion of the cultivation period. Eight-day-old seedlings were used as cotyledonary explant source. Error bars represent mean ± SE (n=5 Petri dishes, each containing 16 explants)

Since acquisition of competence precedes the *in vitro* induction of shoots and roots, we further determined for each NIL the time required to start the acquisition of competence phase. This has already been determined for MT, which takes 2-3 days, and for MT-*Rg1*, which takes 1-2 days (AZEVEDO, 2012). Taking this into account, the first days on SIM were gradually substituted for RIM for a total of 8 days. All treatments were transferred to MB at the end of four days to complete the 21-day incubation.

For MT, the first two days on SIM can be replaced by RIM without affecting the formation of shoots or roots in the explants (Figure 2.10A). The same was observed for MT-*Rg7H* (Figure 2.10B). However, for MT-*Rg3C* and MT-*Rg8F*, only the first day on SIM can be replaced by RIM without leading to root formation in the explant (Figure 2.10 C-D).

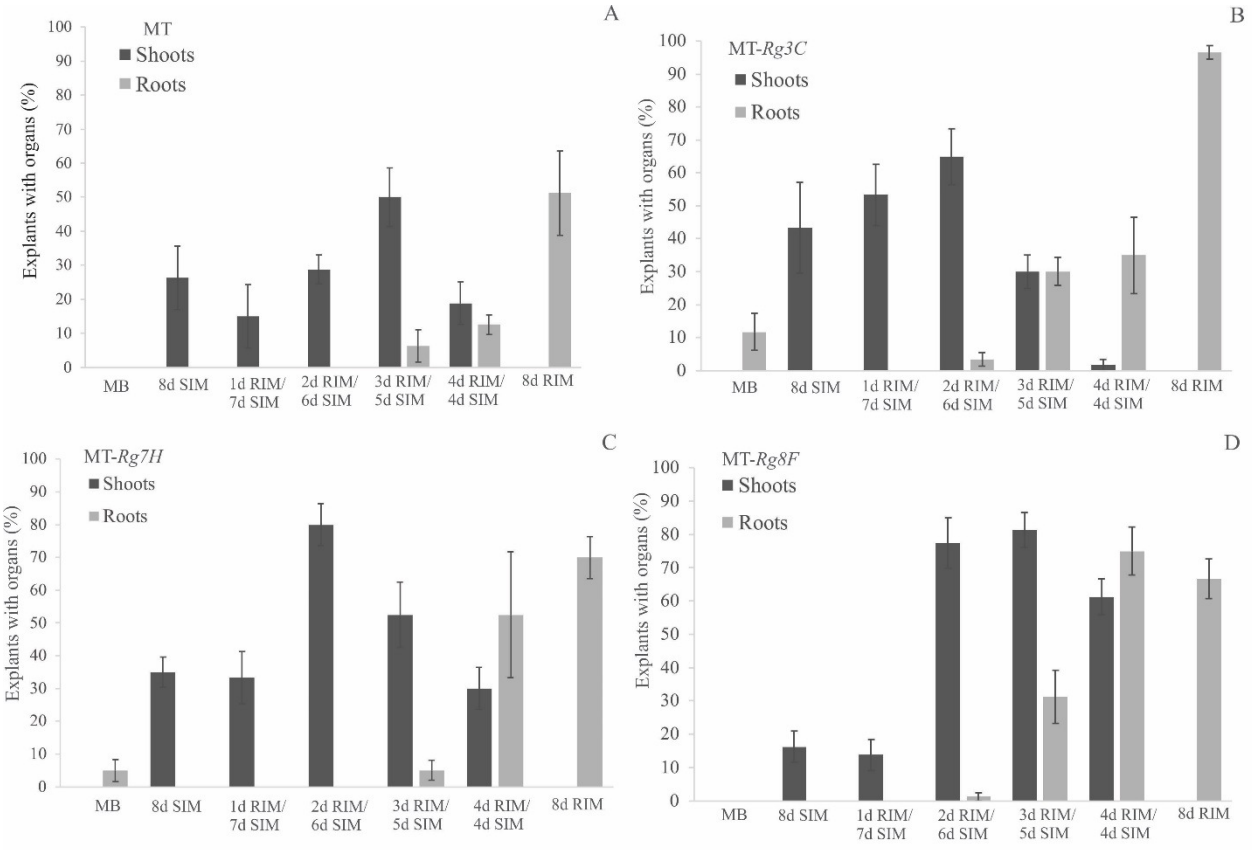

Figure 2.10 - Time required to acquire competence to form new organs (roots or shoots) in cotyledonary explants of MT (A), MT-*Rg7H* (B), MT-*Rg8F* (C) and MT-*Rg3C* (D). RIM was gradually substituted by SIM and after eight days, all treatments were substituted by MB to complete 21 days *in vitro*. Eight-day-old seedlings were used as cotyledonary explant source. Error bars represent mean ± SE (n=5 Petri dishes, each containing 16 explants)

In order to better understand the molecular process of *in vitro* regeneration of the genotypes under study here and the relationship of alleles in these loci with some important genes related to shoot regeneration process, we analyzed the expression of the genes *GOB* (Solyc07g062840), *WUSCHEL* (Solyc02g083950), *Let6* (Solyc02g081120) and *ARF5* (Solyc04g081240) in key days of the shoot regeneration process. The expression of the housekeeping genes used for normalization of gene expression (*GAPDH* and *EF-1 alpha)* followed the same pattern in the five days where RNAs were sampled for all NILs (Figure S1).

Before the incubation of the explants on SIM (Day 0), MT-*Rg8F* was the NIL with higher expression of *GOB* in the cotyledonary explants, followed by MT-*Rg3C*. The expression in MT-*Rg7H* explants, on the other hand, didn`t differ from MT before SIM treatment (Figure 2.11A).

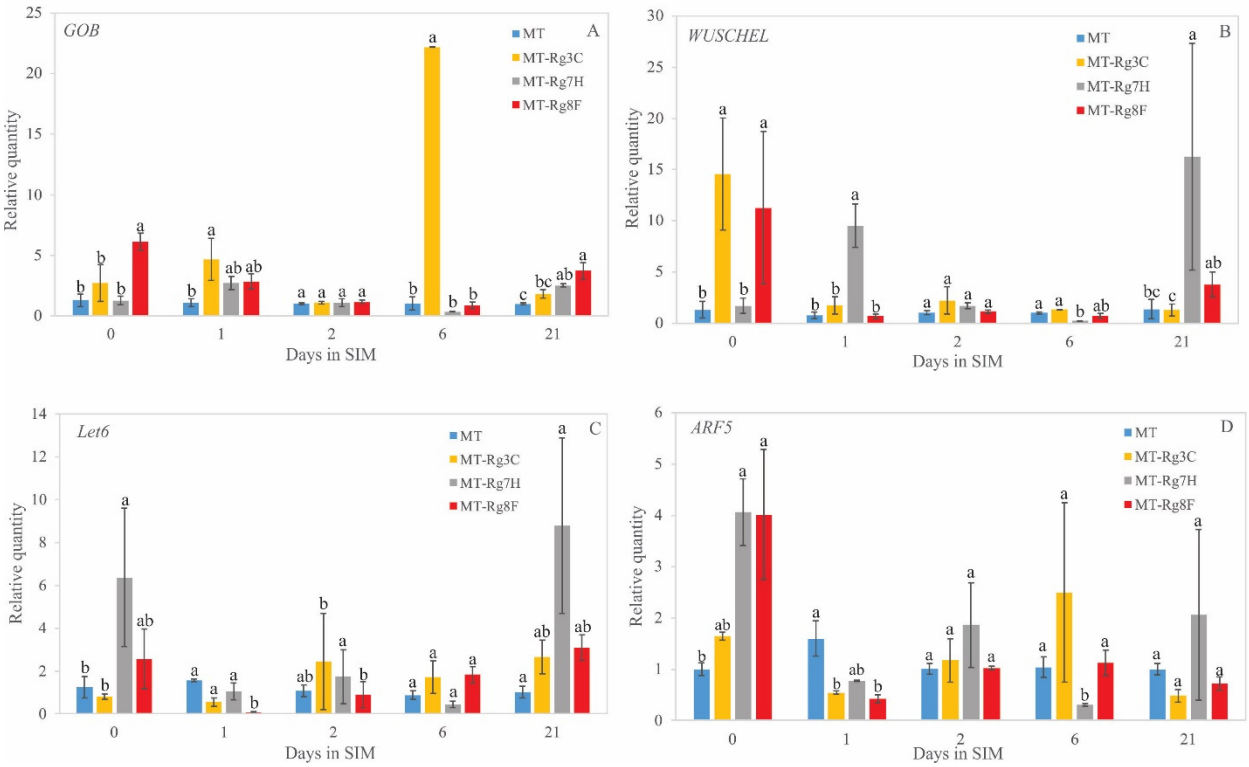

Figure 2.11 - Relative expression of genes related to shoot meristem formation during *in vitro* shoot regeneration for MT, MT-*Rg3C*, MT-*Rg7H* and MT-*Rg8F* genotypes. (A) Relative expression of *GOB* (A), *WUSCHEL* (B), *Let6* (C) and *ARF5* (D). Eight-day-old seedlings were used as cotyledonary explant source. Error bars represent mean ± SE (n=3 biological repetitions, each composed by 8 explants and three technical repetition)

GOBLET (*GOB*) is a specific marker of leaf development (BERGER et al., 2009). After just one day of incubation on SIM, the expression of *GOB* in all NILs was higher than in MT. On the second day, however, none of them differed from MT. On day six, there was a dramatic increase of *GOB* expression in MT-*Rg3C* when compared to the other genotypes. At the end of incubation on SIM (Day 21), the expression of *GOB* in MT-*Rg8F* and MT-*Rg7H* explants was higher than in MT, but MT-*Rg3C* explants did not show differential expression compared to MT (Figure 2.11A).

WUSCHEL (WUS) expression marks the onset of shoot meristem development. *WUS* expression in cotyledonary explants before SIM incubation was not different from MT in MT-*Rg7H* and MT-*Rg8F* explants, but it was higher in MT-*Rg3C* explants on day 0. However,

after SIM incubation, no differences were observed between the expression of *WUS* in MT-*Rg3C* and MT explants (Figure 2.11B).

Despite *WUS* expression in MT-*Rg7H* explants had not differed from MT before incubation, just one day of incubation on SIM was enough to triple *WUS* expression in relation to MT explants in the same day of incubation. After just another day of incubation on SIM (Day 2), *WUS* expression in MT-*Rg7H* explants was the similar to MT explants again, remaining unchanged at day six of incubation. At the end of the incubation period on SIM (Day 21), the *WUS* expression in MT-*Rg7H* was again higher than MT. Interestingly, WUS expression was never higher in MT-*Rg8F* in comparison to MT explants (Figure 2.11B).

The most pronounced difference in *Let6* expression, a marker of lateral shoot development, when compared to MT, was observed in MT-*Rg7H* explants. *Let6* expression in MT-*Rg7H* explants was higher than in MT at, 0, 2 and 21 days of incubation. On days 1 and 6, *Let6* expression in MT-*Rg7H* did not differ from that of MT explants (Figure 2.11C).

In MT-*Rg8F* explants, the expression of *Let6* did not differ from MT, except for after one day on SIM, when it was lower than in MT. In all days of analysis, *Let6* expression in MT-*Rg3C* explants did not differ when compared to MT (Figure 2.11C).

Although *ARF5* expression was higher in MT-*Rg8F* explants than in MT before the incubation on SIM (day 0), both genotypes showed equivalent expression onwards. No differences in *ARF5* expression were observed for all days of analysis in MT-*Rg3C* explants when compared to MT. For MT-*Rg7H* explants, on the other hand, *ARF5* expression was higher than MT on the days 0 and 2, but it did not differ from MT on the other days of analysis (Day 1 and 21) (Figure 2.11D).

2.3.2 Phenotypic characterization of developmental parameters

Genes related to *in vitro* organogenesis capacity may affect other horticultural traits, as already observed in MT-*Rg1* (LOMBARDI-CRESTANA et al., 2012). Therefore, a phenotypic characterization of MT plants harboring the alleles *Rg3C*, *Rg7H* and *Rg8F* was carried out in comparison with the recurrent genotype, MT.

Branching is one of the most evident characteristics observed in plants harboring the *Rg1* allele (LOMBARDI-CRESTANA et al., 2012). Thus, we analyzed branching in the NILs MT-*Rg3C*, MT-*Rg7H* and MT-*Rg8F*. MT-*Rg3C* plants showed expressively higher branching index and pattern than MT (Figure 2.12A-D). The enhanced branching of MT-*Rg3C* is so

evident that we can observe shoot formation even in the cotyledonary axil (Figure 2.12B and 2.12E).

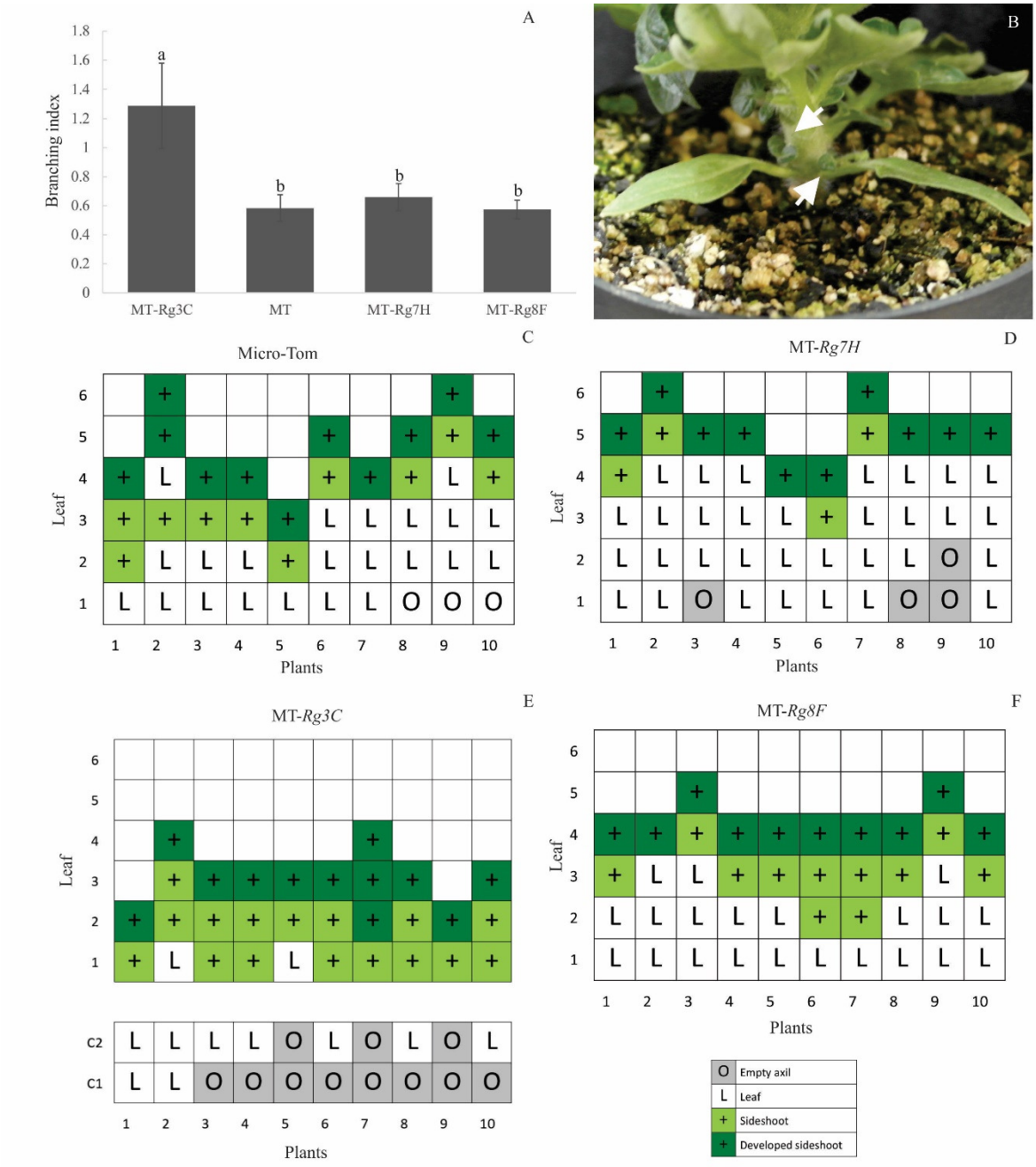

Figure 2.12 - Branching phenotypes of MT, MT-*Rg7H*, MT-*Rg3C* and MT-*Rg8F* plants. (A) Branching index of MT, MT-*Rg7H*, MT-*Rg3C* and MT-*Rg8F* measured in 60-day-old plants. (B) Shoot formation in MT-*Rg3C* axil in 60-day-old plant (C-F) Individual axis branching representation. Each line represents a leaf axis and each column, an individual plant. Grey represents an empty axis, white the presence of one or two leaves, light green the presence a side shoot and dark green the presence of a well-developed side shoot. C1 and C2 represent cotyledonary leaves (Figure adapted from Busch et al., 2011). Error bars represent the mean±SE, n=10. Different letters indicate significant differences at P ≤0.05 (unpaired Student's *t*-test). The branching index represents the sum of the length of lateral branches divided by the main stem length (MORRIS et al., 2001)

MT-*Rg7H* and MT-*Rg8F* branching index and pattern did not differ from MT (Figure 12). Shoot formation in the cotyledonary axils was not observed in these NILs. Besides branching, the other phenotypes that differed in the NIL MT-*Rg3C* were only plant height and leaf number in the main shoot, when comparing to MT (Figure 13).

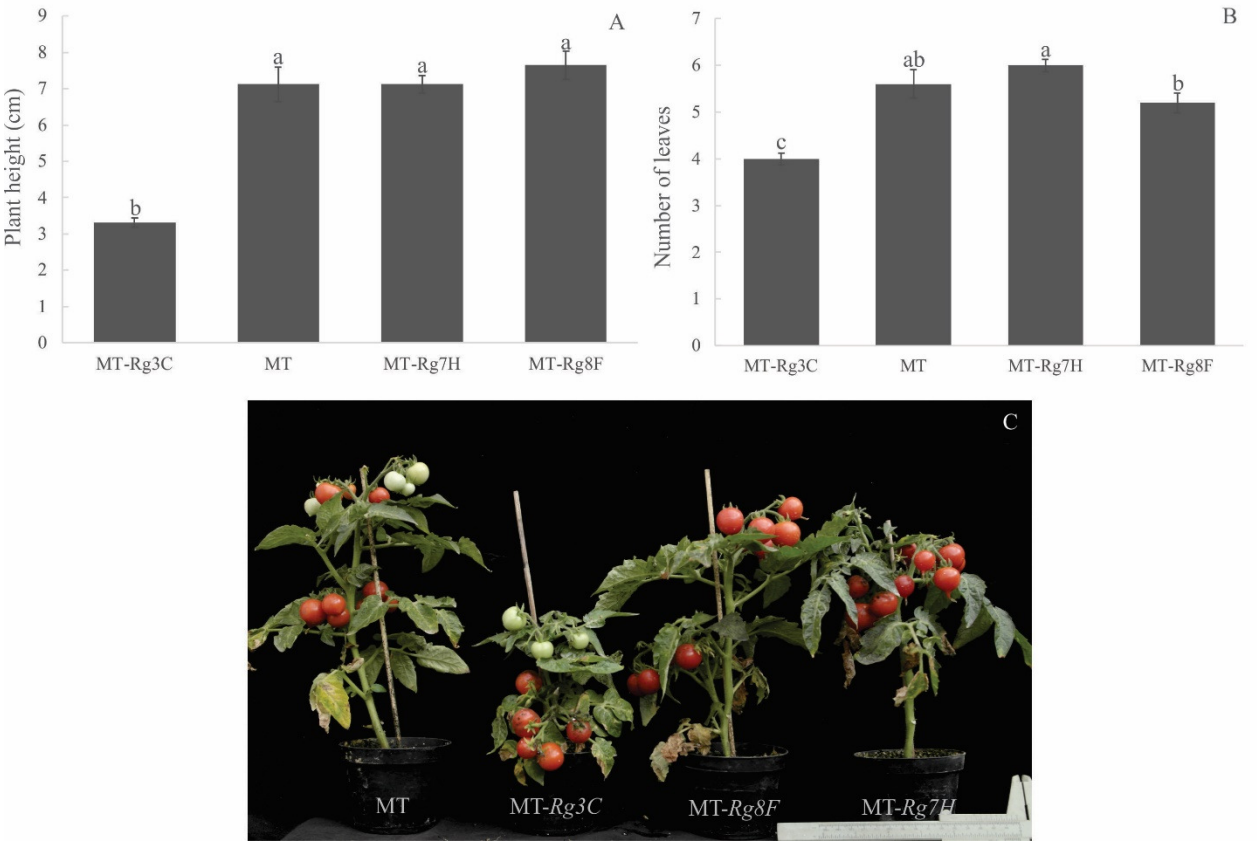

Figure 2.13 - Growth phenotypes of MT, MT-*Rg3C*, MT-*Rg8F* and MT-*Rg7H* plants. (A) Plant height at the first inflorescence of 60-day-old plants. (B) Number of leaves at the first inflorescence of 60-day-old plants. (C) MT, MT-*Rg3C*, MT-*Rg8F* and MT-*Rg7H* representative 90-day-old plants. Error bars represent the mean ±SE, n=10. Different letters indicate significant differences at P ≤0.05 (unpaired Student's *t*-test)

Plants harboring the *Rg3C* allele were significantly shorter than the other NILs and MT, and also developed fewer leaves in the main shoot (Figure 2.13A and 2.13B). An interesting phenotype observed in MT-*Rg8F* and MT-*Rg3C* plants was the number of leaf lobes, which was significantly higher and lower in the respective NILs when compared to MT. MT-*Rg7H* plants did not differ from MT in this trait (Figure 2.14).

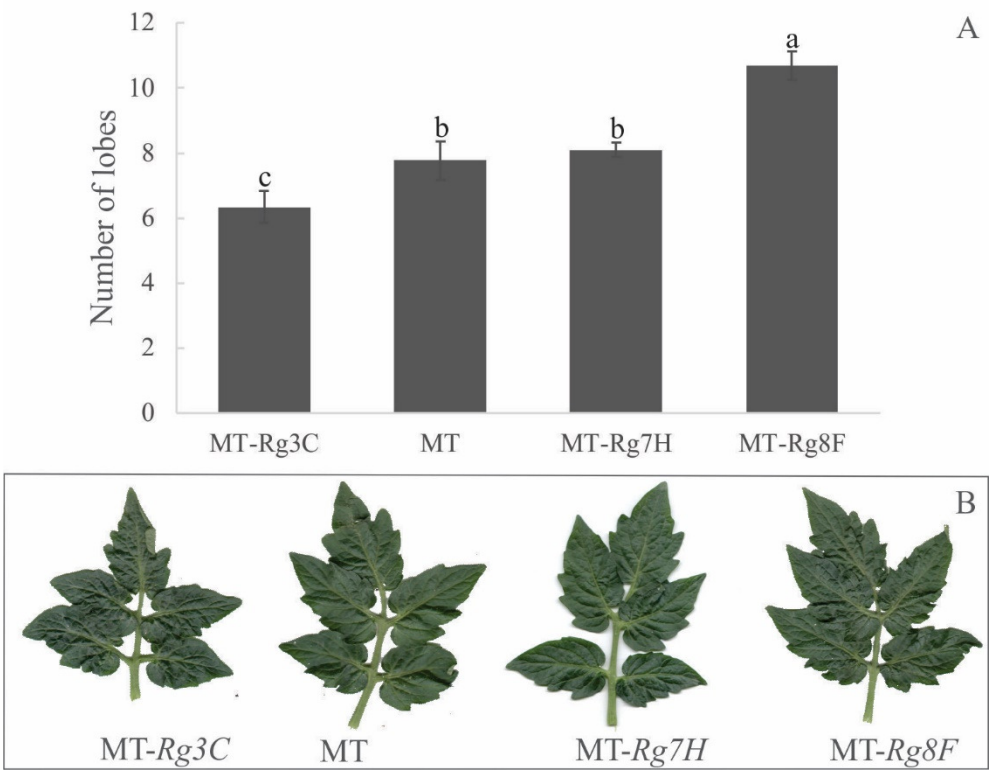

Figure 2.14 - Leaf architecture of MT, MT-*Rg3C*, MT-*Rg8F* and MT-*Rg7H* plants. (A) Number of lobes in the fourth leaf measured in 60-day-old plants. (B) Aspect of the fourth leaf of MT, MT-*Rg3C*, MT-*Rg8F* and MT-*Rg7H* showing increased lobe development in leaflets of MT-*Rg8F*, and reduction in *Rg3C* compared to MT. Error bars represent the mean±SE, n=10. Each repetition consists of the average number of leaflet lobes of the fourth leaf. (60-day-old plants). Different letters indicate significant differences at $P \leq 0.05$ (unpaired Student's *t*-test)

Since MT-*Rg3C*, MT-*Rg7H* and *MT-Rg8F* are good candidate genotypes for tomato transformation protocols, given their higher regeneration rather, we evaluated whether they present reduced productivity or any yield penalty. The total number of fruits was higher in MT-*Rg3C* and MT-*Rg7H* than MT. Besides that, MT-*Rg7H* had the highest fruit fresh matter and the highest frequency of ripe fruits (Figure 2.15).

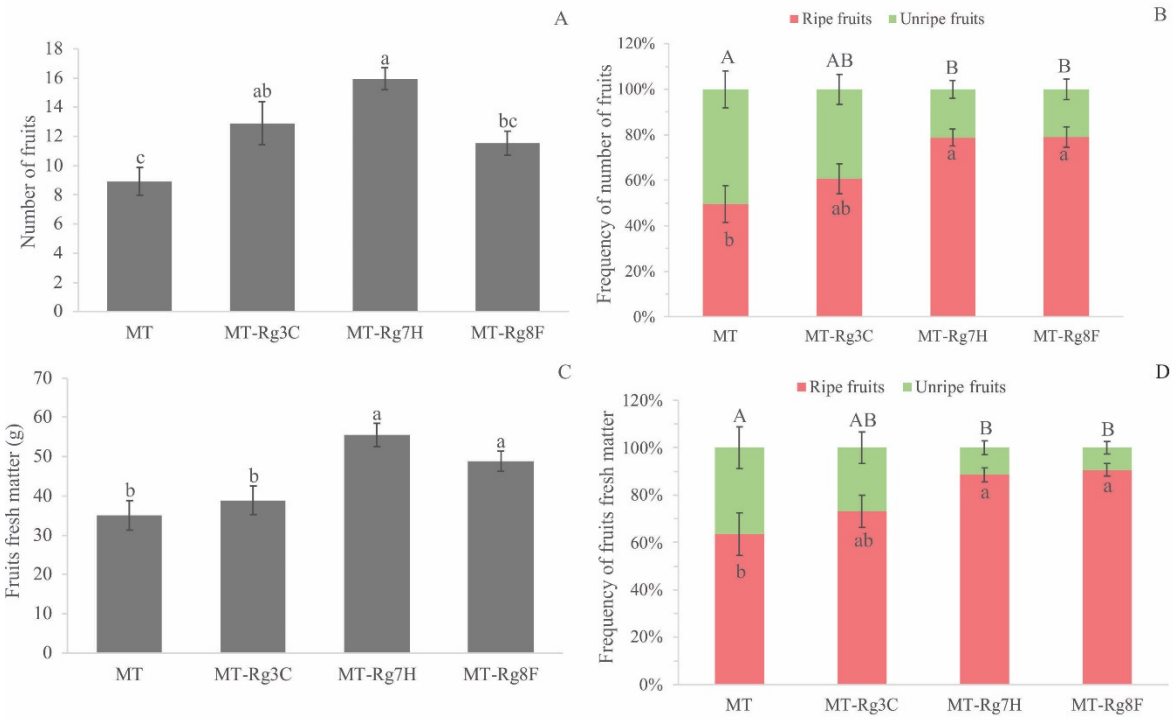

Figure 2.15 - Number and fresh matter of fruits in MT, MT-*Rg3C*, MT-*Rg7H* and MT-*Rg8F* harvested at 80 days after germination. (A) Total number of fruits. (B) Frequency of ripe and unripe fruits harvested per plant. (C) Fruit fresh matter. (D) Frequency of fresh matter of ripe and unripe fruits per plant. Error bars represent the mean±SE, n=15 plants. Different letters indicate significant differences at P ≤0.05 (unpaired Student's *t*-test)

MT-*Rg3C* fruit fresh matter, on the other hand, did not differ from MT, showing that despite the higher number of fruits produced by MT-*Rg3C*, the fruits were smaller than those of MT. Despite the differences in fruit number among the NILs, other fruit characteristics, such as Brix and seed number per fruit, did not differ among them (Figure 2.16).

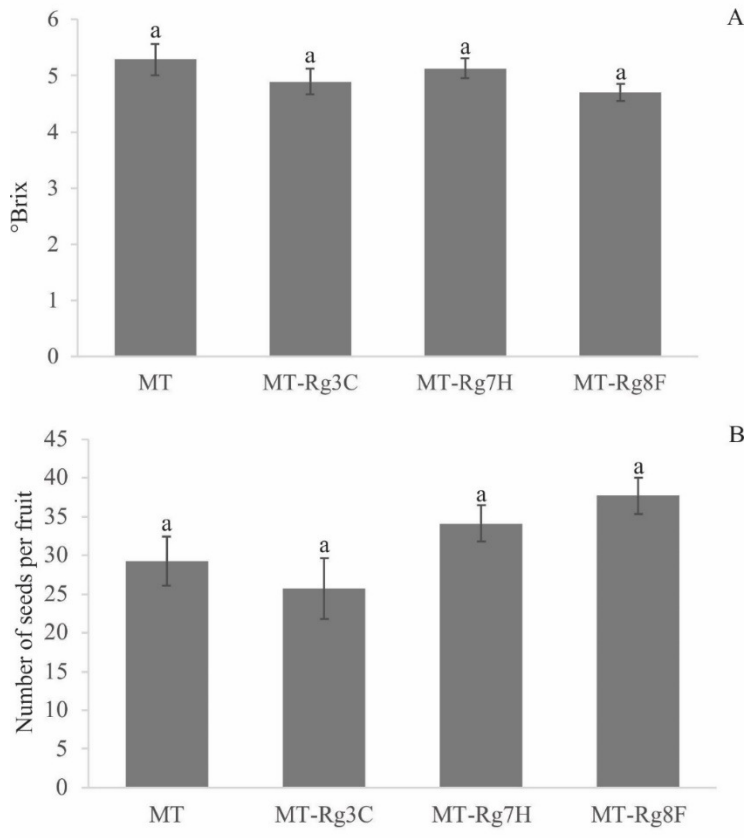

Figure 2.16 - Brix and number of seeds in MT, MT-*Rg3C*, MT-*Rg7H* and MT-*Rg8F* harvested at 80 days after germination. (A) Total soluble solids content in ripe fruits (Brix). (B) Number of seeds per fruit. Error bars represent the mean±SE, n=15. Each repetition consisted of the average of three fruits per plant. Different letters indicate significant differences at P ≤0.05 (unpaired Student's *t*-test).

2.4 Discussion

2.4.1 MT-*Rg3C*, MT-*Rg7H* and MT-*Rg8F* affect *in vitro* regeneration capacity through distinct pathways

In this work, after the introgression of the *S. pennellii* loci *Rg3C*, *Rg7H* and *Rg8F* into MT background, we confirmed the enhanced regeneration capacity they conferred. Both *in vitro* root and shoot regeneration were significantly higher in all three NILs than in the recurrent genotype, MT. These results confirm that observed by Arikita et al. (2013) for the M82 background.

Among the three NILs harboring loci from *S. pennellii*, MT-*Rg3C* was the one with the highest formation of roots on RIM. This result is not surprising since *Rg3C* is probably orthologous to the *Rg1* allele from *S. peruvianum* (KOORNNEEF et al., 1993; PERES et al., 2001), which was already characterized as harboring high *in vitro* formation of roots (LOMBARDI-CRESTANA et al., 2012). In MT-*Rg1*, the presence of the dominant *Rg1* allele

allows *in vitro* shoot formation to occur even in root explants (See Chapter 2), which is not observed in cultivated tomato genotypes but only in some wild species, including *S. peruvianum* (PERES et al., 2001), the original source of the *Rg1* allele. *Rg1* was mapped on chromosome three, near the *yellow flesh (r)* visual genetic marker, which corresponds to the chromoplast-specific gene, *PHYTOENE SYNTHASE* (KOORNNEEF et al., 1993). Indeed, the loss-of-function of *PHYTOENE SYNTHASE* in *S. peruvianum* imparts the yellow color to the fruits when introgressed into a *S. lycopersicum* background (FRAY; GRIERSON, 1993), which is also observed in MT-*Rg3C* homozygous plants. Taken together, the mapping and the enhanced *in vitro* organogenesis of MT-*Rg1* and MT-*Rg3C* strongly indicate that these NILs harbor two allelic versions of the same gene involved in organogenetic competence acquisition in *Solanum* spp.

Acquisition of competence is one of the key steps during shoot organogenesis, since cells must acquire the competence necessary to respond hormonal stimuli in order to proceed with, the following phases of organogenesis. In Arabidopsis, the acquisition of competence occurs during the incubation on Callus Induction Medium (CIM) for at least 2-3 days (CHE; LALL; HOWELL, 2007). In tomato, however, incubation on CIM is not necessary. MT explants acquire competence after 2-3 day incubation on SIM, a period remarkably similar to that of Arabidopsis. However, the time required for acquisition of competence in tomato genotypes with high regeneration capacity is known to be shorter, as already observed in MT-*Rg1*, which explants acquired competence for shoot organogenesis after just 1-2 days of incubation on SIM (AZEVEDO, 2012).

Among the NILs studied here, MT-*Rg3C* and MT-*Rg8F* presented a reduced time to start to acquire competence, as observed in MT-*Rg1*. This reduction may be one of the reasons for the enhanced organogenesis capacity observed in these NILs. Since acquisition of competence is a common pathway for both root and shoot formation, these results are consistent with the better performance of MT-*Rg3C* and MT-*Rg8F* on both RIM and SIM. Therefore, the genes coded by MT-*Rg3C* and MT-*Rg8F* are probably involved in the production of uncommitted cells able to assume different developmental fates.

On the other hand, it seems that the locus *Rg7H* is not affecting competence acquisition, since MT-*Rg7H* did not differ from MT regarding the time needed to acquire competence. Probably, this locus is enhancing organogenesis by affecting organogenesis induction, since this phase started 2 days earlier in this NIL than MT.

The organogenetic induction in MT-*Rg3C* occurred earlier than expected, shoot formation was induced after just three days on SIM, when in MT-*Rg1* it requires at least five days (AZEVEDO, 2012).

The molecular basis of *in vitro* organogenesis in plants, and especially shoot regeneration, has steadily been revealed in the last few years (MOTTE et al., 2014). However, the molecular basis of competence acquisition is much less understood. A candidate gene to control this phase in Arabidopsis is *CUP-SHAPED COTYLEDON 2* (*CUC2*). *CUC2* is a NAC transcription factor that marks the site of both shoot and lateral root primordia formation, indicating to be likely involved in cell competence (MOTTE et al., 2011). In tomato, the *CUC2* homolog is *GOBLET* (*GOB*; Solyc07g062840). *GOB* is expressed in narrow stripes at the leaf margins, flanking the distal side of the future leaflet primordia, and between the boundaries of the shoot apical meristem and leaf primordia (BERGER et al., 2009). A large increase of *GOB* expression in MT-*Rg3C* explants in comparison to MT was observed after six days of incubation on SIM. This suggests that in MT-*Rg3C* the *Rg3C* and *GOB* may be acting on the same pathway enhancing the acquisition of competence. Other features that reinforce this hypothesis is the enhanced branching and presence of bifurcated and triple cotyledons in MT-*Rg3C* plants (not shown) and *Rg1* (LOMBARDI-CRESTANA et al. 2012). These phenotypes are also observed in the tomato mutant, *Gob-4d*, which express high levels of *GOB* mRNA (BERGER et al., 2009).

Studies using Arabidopsis led to the identification of genes involved in *in vitro* regeneration. The genes *SHOOTMERISTEMLESS* (*STM*), *WUSCHEL* (*WUS*), *CLAVATA* (*CLV*) and *ARABIDOPSIS RESPONSE REGULATORs* (*ARRs*) are markers of *in vitro* shoot induction, in addition to their participation in shoot meristem identity (ATTA et al., 2009; CARY; CHE; HOWELL, 2002; CHE; LALL; HOWELL, 2007; CHE et al., 2006; GALLOIS et al., 2002).

WUS expression marks the onset of shoot meristem development. Amongst all genotypes studied, *WUS* expression was significantly higher on day 1 in MT-*Rg7H* explants, while its expression in the other NILs did not differ from that of MT. This may indicate that meristem formation in *MT-Rg7H* started earlier than in the other genotypes, which is consistent with the early shoot induction observed in this genotype.

LeT6 in tomato is orthologous to *STM* in Arabidopsis (BHARATHAN et al., 1999). Tomato leaves constitutively expressing *LeT6* developed ectopic meristems (JANSSEN; LUND; SINHA, 1998), which suggests that this gene may be involved in *in vitro* shoot regeneration in both species. High expression of *Let6* was observed at 0, 2 and 21 days of

incubation in MT-*Rg7H* explants. The highest expression on day 2 corroborates the idea that the induction in this NIL started earlier than in the other genotypes. In Arabidopsis, *STM* expression coincides with the moment when the explants can be transferred from SIM to a basal, hormone-free medium without loss of regeneration capacity, thus marking the timing of shoot determination (ZHAO; FISHER; AUER, 2002).

Auxin is essential for *in vitro* shoot regeneration. In Arabidopsis, the division of pericycle cells, which will form organogenic callus, is driven by a local auxin maximum (CHE; LALL; HOWELL, 2007). Auxin-dependent gene expression is generally mediated by AUXIN RESPONSE FACTORS (ARFs) (ULMASOV et al., 1997; ULMASOV; HAGEN; GUILFOYLE, 1999). Among ARFs, ARF5/MONOPTEROS is required for root organogenesis (SMET et al., 2010) in order to increase the frequency of *de novo* shoot formation and overcome the organogenic recalcitrance of tissues in Arabidopsis (CKURSHUMOVA et al., 2014).

As observed for *WUS* and *Let6*, *ARF5* expression was markedly different in MT-*Rg7H* when compared to MT explants. *ARF* expression was considerably high in MT-*Rg7H* explants on day 2, one day after the highest *WUS* expression and coinciding with the highest *Let6* expression in this NIL. Interestingly, *ARF5* expression in MT-*Rg7H* explants on day 6 was lower than MT, which coincides with the lowest *WUS* expression on that day for this NIL. Some studies have suggested an overlapping role of *ARF5* and *WUS* in locally modulating cytokinin signaling (ZHAO; FISHER; AUER, 2002).

The analysis of gene expression of key factors controlling the regeneration program showed that, for the three genotypes studied, the expression pattern was not equivalent, and it as especially distinct for MT-*Rg7H*. This indicates that the *S. pennellii* alleles on the different chromosomal fragments introgressed in these NILs may be affecting the regeneration capacity through distinct pathways. Further studies involving double mutant analyses combining the three alleles as well as the tomato mutants already available for *Let6* and *GOBLET* will shed more light on this subject.

2.4.2 Adventitious *in vitro* shoot development and *ex vitro* axillary bud formation are induced by different genetic pathways

Many genes related to *ex vitro* meristem formation probably are also involved in *in vitro* shoot regeneration (MOTTE et al., 2014). Some genotypes that have enhanced shoot

regeneration, such as MT-*Rg1*, also show pronounced branching (LOMBARDI-CRESTANA et al., 2012), suggesting a genetic connection between the development of these traits.

Our branching analysis of the NILs reported here showed that enhanced shoot regeneration is not necessarily associated to increased branching. In MT-*Rg3C* plants, branching was higher than MT, but the same was not true for MT-*Rg7H* and MT-*Rg8F* plants. The dissociation between these traits indicates that *in vitro* adventitious shoot formation and *ex vitro* axillary bud formation/outgrowth is induced by different genetic pathways.

Besides increased branching, other traits only observed only in MT-*Rg3C* were short length of the main shoot and a reduced number of leaves up to the first inflorescence. Interestingly, such features are usually observed in early-flowering tomato genotypes (VICENTE et al., 2015). This suggests that the *Rg3C* locus may be affecting growth habit in addition to *in vitro* regeneration and branching.

On the other hand, MT-*Rg7H* and MT-*Rg8F* did not differ from the recurrent control genotype, MT. However, MT-*Rg8F* plants showed a slight but statistically significant difference in leaf morphology, with the development of more lobes than MT. Many genes affecting leaf morphology are also related to the shoot meristem formation, such as *KNOX 1* (HAKE et al., 2004) and *GOB* (BERGER et al., 2009). During our molecular analysis, we observed that MT-*Rg8F* cotyledons showed higher *GOB* expression before the SIM treatment when compared to MT or the other NILs. Thus, it is likely that the naturally higher expression of *GOB* in leaves may be related to the enhanced lobes formation observed in MT-*Rg8F*.

The phenotypic characteristics of MT-*Rg7H* and MT-*Rg8F* with no enhanced branching (Figure 2.12), high fruit productivity and Brix (Figure 2.15-16) open the possibility to breed tomato varieties and hybrids with improved capacity of *in vitro* regeneration and transgenic plant production without the undesired trait of enhanced side shoots (suckers) and other yield penalties.

2.4.3 Implications of high organogenesis capacity for tomato adaptation and domestication

The observation that many wild species of tomato regenerate *in vitro* at higher rates than the domesticated species leads to the intriguing question about the possible adaptive value of this trait in natural environments. It is well established that the post-embryonic development of plants, *i.e*. their continuous organ formation throughout the life cycle, enables them to better explore the environment and to cope with stresses in a sessile lifestyle

(FOSKET, 1994). Thus, it is reasonable to assume that alleles improving adventitious shoot formation will help in the survival after fortuitous episodes, such as fire (KAUFFMAN, 1991) or herbivory (MEIJDEN; WIJN; VERKAAR, 1988). As for the ability to form adventitious roots, besides its evident importance to cope with flooding (MANO et al.; 2005) and to improve nutrient allocation (OCHOA et al., 2006), alleles enhancing adventitious organ development could be especially useful in non-climbing vine plants like tomato. The ability to form adventitious roots along the stem close to the soil (Figure 2.17) allows for superficial nutrient mining, as well as for restarting a new rooting system to replace one attacked by pathogens (GRIEVE, 1941). It is noteworthy that, different from its original environment, the tomato was domesticated as a climbing (stalked) vine, besides being later bred for determinate growth (STEVENS; RICK, 1986). Therefore, it is tempting to speculate that the high organogenic capacity might have evolved as an ability useful in the natural environment that became futile during domestication, and that is now sought after for modern biotechnology.

Figure 2.17 - *Ex vitro* adventitious root formation is observed in tomato growing untamed as a non-climbing vine. In modern cultivation systems, the plant usually grows as a stalked vine, which refrains the development of adventitious roots

2.5 Conclusions

S. pennellii ILs 3-2, 8-3 and 7-1 were introgressed into the Micro-Tom (MT) background after six backcrossings and selection for enhanced *in vitro* shoot and root regeneration;

The time required on SIM for induction of shoot organogenesis on basal medium MT-occurred respectively one and two days earlier for *Rg7H* and MT-*Rg3C* compared to MT. MT-*Rg8F* did not differ from MT regarding induction time.

The time required for acquisition of competence occurred one day earlier in MT-*Rg3C* and MT-*Rg8F* compared to MT. MT-*Rg7H* did not differ from MT regarding competence acquisition period.

The locus *Rg3C* affected *in vitro* regeneration by decreasing the time required do acquire competence and to start the induction phase. In MT-*Rg8F*, the quicker competence acquisition is probably the single factor responsible for its highest *in vitro* regeneration rate among all genotypes studied. The locus *Rg7H* does not seem to be involved in the acquisition of competence since the timing of this first phase did not differ from MT. Thus, this locus probably enhances *in vitro* shoot regeneration by decreasing the time necessary to start the induction phase.

The expression patterns of key genes related to shoot regeneration in the three NILs under study were dissimilar, suggesting that the loci studied affect regeneration capacity through distinct pathways.

The enhanced branching of MT-*Rg3C* is so evident that shoot formation could be observed even in the cotyledonary axil. On the other hand, MT-*Rg7H* and MT-*Rg8F* branching did not differ from MT, which is key evidence that enhanced *in vitro* shoot formation is not necessarily related to enhanced branching.

In addition to higher branching, *Rg3C* also affected growth habit since both height and leaf number were smaller than MT. MT-*Rg8F* did not differ from MT in other traits except number of lobes in leaves.

Rg7H did not differ from MT on plant growth traits but it produced more fruits that ripened more uniformly than MT. This may be a good genotype for plant transformation protocols, and efforts should focus to characterize the exact gene within the recombined region that confers the enhanced shoot regeneration.

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3 THE TOMATO *REGENERATION 1* GENE HAS GAIN-OF-FUNCTION ALLELES IN THE GREEN-FRUITED WILD RELATED SPECIES *Solanum peruvianum* AND *S. pennellii* ENHANCING BOTH ROOT AND SHOOT FORMATION *IN VITRO* AND HIGH BRANCHING PHENOTYPE *EX VITRO*

Abstract

The molecular basis of plant *in vitro* regeneration is not fully understood yet, despite its evident importance for several biotechnological applications. The *Rg1* allele from the tomato wild specie *Solanum peruvianum* is known for its effect enhancing both root and shoot formation *in vitro*. *Rg1* probably acts in the phase of acquisition of competence for organ formation, which is a common step for both root and shoot regeneration. The locus for this allele was initially mapped on the chromosome 3, between the genes *BETA-CAROTENE HYDROXYLASE* (*CrtR-b* Solyc03g007960*)* and *PHYTOENE SYNTHASE* (*PSY1* Solyc03g031860) a segment harboring 301 genes. Later, analysis using new molecular markers reduced the number of candidate genes to 136. Studies involving introgression lines, developed from segments of *S. pennellii* introgressed and mapped into the tomato (*S. lycopersicum*) cultivar M82, identified the allele *Rg3C*, which has a similar effect and chromosomic location of *Rg1*. This information raised the hypothesis that *Rg1* and *Rg3C* are alleles of the same gene, which probably lost its function in cultivated tomato before domestication. Here, we present a comparative analysis of two Near-Isogenic Lines (NILs) in the cultivar Micro-Tom (MT) harboring the *S. peruvianum* (MT-*Rg1*) or the *S. pennellii* (MT-*Rg3C*) alleles. The main characteristics of MT-*Rg1* were also observed in MT-*Rg3C*. Both root and shoot regeneration capacities were very similar comparing the NILs, although they differ in the lack of the capacity of shoot formation from roots explants in the MT-*Rg3C*. Both NILs also coincide in having a branching phenotype *ex vitro*. We determined that this effect is, at least in part, due to the linkage drag with a strong allele (*SP3D*) of the *SINGLE FLOWER TRUSS* gene, which is known to be present in both *S. pennellii* and *S. peruvianum*. Thus, the analysis of a NIL harboring the *SP3D* allele showed that it is more branched than MT, although not affecting *in vitro* regeneration capacity. *Rg1* was previously shown to be epistatic to the *lateral suppressor* (*ls*) mutant, an effect that could not be attributed to *SP3D*, as evidenced here by the analysis of the double mutant *SP3D ls*. This suggests that the genetic identity of *Rg1/Rg3C*, which we further narrowed to only 27 candidate genes, also affect branching by itself and probably belongs to a novel pathway that interacts with the LS gene and other members of the GRAS superfamily.

Keywords: Branching; Competence; Organogenesis; *Solanum lycopersicum*

3.1 Introduction

Both plants and animals are capable, throughout their lives, to restore damages caused by injury, disease or predators attack using regeneration process. However, this regenerative ability is more remarkable in plants than in animals, since plants, which have a sessile nature, are more susceptible to all kinds of physical damages (PULIANMACKAL et al., 2014). Plant cells own an unconventional plasticity, which allows an initial cell fate to be completely

changed during post-embryonic development (BERG et al., 1995). The regeneration process in plants can occur in several ways, from the regeneration of a single tissue, after an injury, to the regeneration of a complete organism from a tissue or somatic cell (SUGIMOTO; GORDON; MEYEROWITZ, 2011). Thus, a hundred years old tree are still able to regenerate new organs year after year, demonstrating the incredible plant development capacity (AICHINGER et al., 2012).

Initially, the plant continuous organogenic capacity has been explored with agricultural proposes for the propagation of selected varieties. After, researches working with plant tissue culture extended this ability to *in vitro* culture, enabling the development of a set of biotechnological techniques, from the production of virus free seedlings to plant genetic transformation (RAMGAREEB et al., 2010; SUSSEX, 2008).

The possibility to direct plant development using plant hormones has been raised the interest of scientists for a long time. The use of plant hormones, added to the culture medium with optimal minerals, organic nutrients (sugar) and vitamins composition, enables the formation of shoots or roots *in vitro* (CARY; CHE; HOWELL, 2002). Skoog and Miller (1957) classical studies showed the influence of plant hormones, and mainly their interaction, in *in vitro* adventitious organ formation. They observed the auxin/cytokinin balance directing *in vitro* organogenesis. A high auxin/cytokinin ration enables roots formation, while a low auxin/cytokinin ratio enables shoot formation. The intermediate balance between these two hormones leads to the formation of cell masses called callus.

Acquisition of competence to assume new developmental fates is, among the events that are involved in new organs formation, the most important step. According to Christianson and Warnick (1985), the organogenesis process is composed of the following steps. The first one is the acquisition of competence, when the cells acquire the competence necessary to respond to the hormonal stimulus in the next phase. In the second step, the competent cells are induced to form the correspondent organ in response to an auxin/cytokinin balance, as proposed by Skoog and Miller (1957). After induction, in the third step, the cells become committed to follow a developmental fate. Finally, in the last step, the structure of the new organs begins to develop, regardless the hormonal medium composition.

Probably, the explants naturally own the capacity to form adventitious roots and shoots through organogenic processes. However, it is likely that there is a genetic or developmental blockage that prevents some explants to acquire the competence necessary to respond to the inductive signals (AUER et al., 1999, CHRISTIANSON; WARNICK, 1985, GILISSEN et al., 1996). For this reason, many works related to *in vitro* organogenesis are

focused in overcoming this blockage, allowing the explants to acquire de competence to induce the organogenesis (CARY; CHE; HOWELL, 2002).

A gene involved in organogenic process in Arabidopsis is *CUP SHAPED COTYLEDON2 (CUC2)*, which is a NAC transcription factor expressed previously to the homeoboxes genes *WUSCHEL* (*WUS*) e *SHOOT MERISTEMLESS* (*STM*) (GORDON et al., 2007). This gene is a marker of the places where roots or shoots will be formed *in vitro* in Arabidopsis. For this reason, this is a candidate gene to control the acquisition of competence phase. In tomato the homologous to these gene is *GOBLET* (BERGER et al., 2009), which has both loss-of-function and gain-of-function mutations *gob3* and *Gob-4*, respectively. However, recent studies in our lab showed that *gob3* and *Gob-4* has little impact in the capacity to form both shoots and roots *in vitro* (AZEVEDO, 2016). Recently, it was demonstrated the role of *PLETHORA* (*PTL*) genes, which are AP2 transcription factors, in the *in vitro* regeneration process in Arabidopsis. These genes, beside act in the competence establishment by activating root stem cells regulators, also act in the regulation of *CUC* genes during the regeneration process (KAREEM et al., 2015). Despite the recent progress in understanding the molecular mechanisms involved in the regeneration process, new studies will provide for the discover of other genes controlling the acquisition of competence, since this event is not yet completely understood.

The genes and molecules involved in the control of acquisition of competence are believed to act, in the signaling pathway, before the genes specifying the organ that will be formed. One of the ways to identify such genes is the characterization of those expressed in a previous step to the commitment to organogenesis (SANTOS et al., 2009), or alternatively the search for genotypes (induced mutants or natural genetic variation) with high or low organ formation in both Shoot-Inducing Medium (SIM) and Root-Inducing Medium (RIM) (ARIKITA et al., 2013; LOMBARDI-CRESTANA et al., 2012). The identification of such genes/molecules involved in the regeneration regulation would be an important step to understand the molecular events involved in the organogenic process, especially in the phase of acquisition of competence (KOORNNEEF et al., 1993; LOMBARDI-CRESTANA et al., 2012).

Tomato (*Solanum lycopersicum* L.) is an excellent model to study natural genetic variations controlling in vitro regeneration capacity, since some wild species related to it have a high organogenic capacity. *Solanum peruvianum* is one of these wild species. Its high *in vitro* shoot formation capacity was associated mainly with two dominant alleles called *REGENERATION 1* (*Rg1*) and *REGENERATION 2* (*Rg2*) (KOORNNEEF et al., 1987).

Alone, the allele *Rg1* is enough to induce the formation of shoots in root explants, a capacity not present in cultivated tomato (PERES et al., 2001). The *Rg1* allele was mapped on the chromosome three (KOORNNEEF et al., 1993), between the genes *BETA-CAROTENE HYDROXYLASE* (*CrtR-b*) (GALPAZ et al., 2006) and *PHYTOENE SYNTHASE* (*PSY1*) (BARTLEY et al., 1991; FRAY; GRIERSON, 1993). *S. peruvianum* harbors the recessive allele *yellow flesh* (*r*) of the gene *PSY1*, which represents a loss of function that gives rise to a yellow fruit when introgressed in *S. lycopersicum* background (KOORNNEEF et al., 1987).

The presence of the allele *r* in green fruit species allowed its use as morphological marker for the introgression of the allele *Rg1* into the cultivated tomato. Using this approach, *Rg1* allele was introgressed in the cultivar Micro-Tom (MT) (LOMBARDI-CRESTANA et al., 2012), which is considered a genetic model due to its small size and short life cycle (MEISSNER et al., 1997; CAMPOS et al., 2010). The introgression produced a genotype (MT-*Rg1*) with high regeneration capacity and dwarf size proposed to be a base for genetic transformation of the MT model (PINO et al., 2010).

MT plants harboring the *Rg1* allele, besides the expected enhanced capacity to form shoots *in vitro*, also enhance the number of roots formed when cultivated in the appropriate medium (LOMBARDI-CRESTANA et al., 2012). *Rg1* allele is also capable to revert the low formation of shoots and roots *in vitro* in mutants with low rate of regeneration, such as the *procera* mutant. This mutant has a gibberellin constitutive response through the loss of function of a DELLA protein belonging to the GRAS superfamily of transcription factors (JASINSKI et al., 2008). The double mutant *proRg1* had a higher number of both roots and shoots *in vitro*, which in normal conditions is very low in the *procera* mutant. The reversion of the low organs formation of the *procera* mutant in the double mutant *proRg1* shows the occurrence of epistasis between these two mutations (LOMBARDI-CRESTANA et al., 2012), which indicates that these two genes may be in signal transduction pathways that converge at some point. A further an interesting phenotype of MT-*Rg1* is an enhanced branching phenotype which was epistatic to the classical *lateral suppresser* mutant (*ls*) (LOMBARDI-CRESTANA et al., 2012). Since *ls* also belongs to the GRAS superfamily, this reinforce the hypothesis that *Rg1* is somewhat related to this signal transduction pathway.

New analysis using molecular markers decreased the size of the initial chromosomal segment proposed by Koornneef (1993) for the localization of *Rg1*. In none of the markers used it was observed polymorphism between MT-*Rg1* e MT, neither in a segregating population resulted by the cross of these two genotypes. However, for the same markers it was observed polymorphisms between MT and *S. peruvianum*. According to this new

mapping, *Rg1* is located between the gene *CrtR-b* and the molecular marker P5 (Solyc03g025320) decreasing the number of candidate genes to *Rg1* from 301 to 136 (AZEVEDO, 2012).

It was postulated that most green-fruited wild tomato related species with high organogenic capacity, and harboring the *r* allele, probably also has the *Rg1* allele (PERES et al., 2001). Hence, *S. pennellii* LA716, a green-fruited species, presented a high *in vitro* organogenesis capacity (ARIKITA et al., 2013). In addition, previous results have suggested the presence of other loci controlling *in vitro* regeneration in tomato (FARIA et al., 2002; KOORNNEEF et al., 1993). Using a collection of 50 Introgression Lines (ILs), developed by Eshed and Zamir (1994), each one containing a small segment of a *S. pennellii* 'LA716' chromosome, introgressed and mapped in the cultivar M82, six ILs with high regeneration capacity (*Rg3C*, *Rg7H*, *Rg8F*, *Rg9DE*, *Rg10F*, *Rg6A*) were identified. These ILs were partially introgressed in MT, and their *in vitro* regeneration capacity was tested. Of the six alleles introgressed, four showed high capacity to form both roots and shoots *in vitro* (*Rg3C, Rg7H, Rg8F, Rg10F*), indicating that these alleles are probably related to the acquisition of competence phase, according to the concepts proposed by Christianson and Warnick (1988). The other two alleles (*Rg9DE*, *Rg6A*) enhance only the formation of shoots *in vitro*, and are probably affecting the organogenesis induction phase (ARIKITA et al., 2013).

The locus *Rg3C* is located in the chromosome 3 in the bin 3C (Figure 1) and, as *Rg1*, the allele from *S. pennellii* enhance regeneration capacity. The location of the gene conferring high regeneration capacity in the bin 3C was possible due to the fact that IL 3-2 has high regeneration capacity, while IL 3-3 does not have such characteristic. Thus, all the genes in *S. pennellii* segment shared by ILs 3-2 and 3-3 (Figure 1) can be discarded as candidate genes for controlling acquisition of competence capacity (ARIKITA et al., 2013).

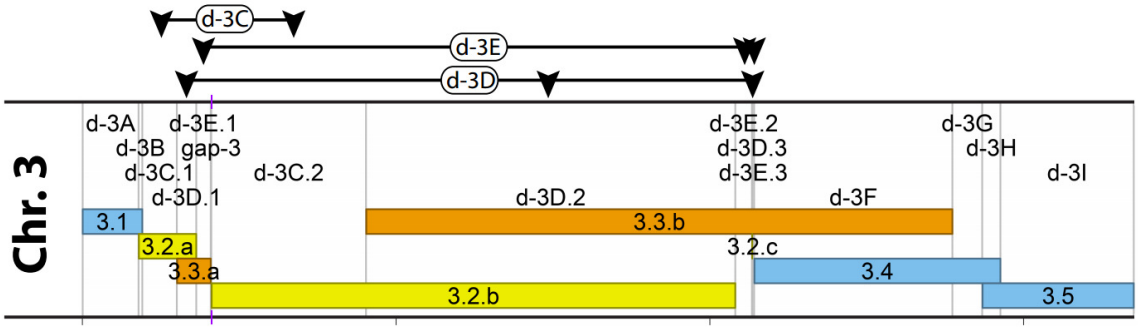

Figure 2.1 – Map showing the architecture of ILs based on precisely defined introgression boundaries determined from the sequenced tomato genome and next-generation sequencing data. IL size is proportional to the number of annotated genes harbored in each introgression. Bins, or intervals defined by unique combinations of IL overlap, are indicated by capital letters from A to I. Note that ILs can be non-contiguous (indicated by orange or yellow) as well as bins (indicated above graphs with arrowheads and lines). Figure taken from Chitwood et al. (2013) (Supplemental figure 4)

Since the *Rg3C* segment is located on chromosome 3, it is believed that this is the *S. pennellii* allele for the gene *RG1* (ARIKITA et al., 2013). Probably, *Rg1* and *Rg3C* are gain-of-function alleles of a same gene, coming from the wild species *S. peruvianum* and *S. pennellii*, that lost their function in *S. lycopersicum* after domestication. However, until today no comparative analysis between *Rg1* and *Rg3C* have been done. Herein we created the Near-Isogenic Line (NIL) MT-*Rg3C* and presented a comparative analysis of MT-*Rg1*, MT-*Rg3C* and the control MT, contributing to the assertion that *Rg1* and *Rg3C* are two alleles of the same gene. Based on different sources of *in silico* data for *S. pennellii* LA716 and cultivated tomato, we propose a list of 27 genes candidates to *Rg1/Rg3C* and analyzed their expression pattern in different organs and genotypes. We also analyze the impact of these two alleles in different plant phenotypes with special emphasis on their effect on shoot branching.

3.2 Material and Methods

3.2.1 Plant material

Tomato (*S. lycopersicum* L.) cv. MT and the NILs harboring the alleles *Rg1*, *Rg3C*, *ls* and *SP3D* were used in the present study. The MT-*Rg1* genotype was described previously (LOMBARDI-CRESTANA et al., 2012) and the MT-*Rg3C*, whose initial introgression into MT background was presented in Arikita et al. (2013), had its introgression finished here using the same procedure described previously (PINO et al., 2010; Chapter 1). The MT-*SP3D* was introgressed in a parallel work that will published elsewhere.

The crossing to obtain the double mutant MT-*SP3D ls* was done by conventional methods with plants holding either *SP3D* or *ls* allele, already introgressed into the MT genetic background. F1 plants were allowed to self-pollinate to further produce homozygous F2 plants. The screening of F2 plants for the presence of the recessive *ls* mutation was done by the observation of the flowers, since homozygous *ls* flowers fail to produce petals.

The plants homozygous for the *ls* allele were then screened for the allele *SP3D*. For the screening of the dominant *SP3D* allele we used a SCAR marker (Table 3.1), which allows the identification of homozygous and heterozygous plants.

Table 3.1 - *SP3D* primers sequence of SCAR marker

Name	Sequence	Product size
SP3D FW	GCCATAAGTATTTCCCCATTC	MT - 880bp
SP3D RV	CAAGTCACTAATTCAAATAAATTGAG	*S. pen*. - 970bp

3.2.2 Growth conditions

Plants were grown in a greenhouse at the Laboratory of Hormonal Control of Plant Development, ESALQ-USP (Piracicaba, SP, Brazil). The growth conditions were mean temperature of 28 ºC, 11.5 h/13 h (winter/summer) photoperiod, 250–350 mol photons m^{-2} s^{-1} PAR irradiance, attained by a reflecting mesh (Aluminet, Polysack Indústrias Ltda, Leme, Brazil), and automatic irrigation to field capacity four times a day. Seeds were germinated in 350 mL pots with a 1:1 mixture of commercial potting mix Basaplant® (Base Agro, Artur Nogueira, SP, Brazil) and expanded vermiculite supplemented with 1 g L^{-1} 10:10:10 NPK and 4 g L^{-1} dolomite limestone ($MgCO_3$ + $CaCO_3$). Upon appearance of the first true leaf, seedlings of each genotype were transplanted to pots containing the soil mix described above, except for NPK supplementation, which was increased to 8 g L^{-1}.

3.2.3 *In vitro* culture

Seeds from *S. lycopersicum* cv. Micro-Tom and the NILs MT-*Rg1,* MT-*Rg3C* and MT-*SP3D* were surface-sterilized by shaking in 20 mL 30% (v/v) commercial bleach (2.7% sodium hypochlorite) 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 (MURASHIGE; SKOOG, 1962), half strength B5 vitamins (GAMBORG; MILLER;

OJIMA, 1968), 30 g L^{-1} sucrose, and 2.3 g L^{-1} phytagel. 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 days, followed by 8 days under 16-h photoperiod.

Cotyledons were then isolated from 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 medium composed by MS salts with B5 vitamins, 30 g L^{-1} sucrose, 2.3 g L^{-1} fitagel, and 5 µM benzylaminopurine (BAP) for Shoot-Inducing Medium (SIM), or 0.4 µM naphthalene acetic acid (NAA) for Root-Inducing Medium (RIM). Twelve cotyledonary explants were cultured per Petri dish (90 × 15 mm), with 10 plates per treatment. Plates were sealed with PVC and maintained under 16 h photoperiod at 55 µmol PAR $m^{-2}s^{-1}$ and 25 ± 1°C. After 9 days was evaluated the number of roots per explant in RIM, and after 21 days the percentage of explants with shoots in SIM.

To the analyses of regeneration from roots, five-millimeter root tip segments were placed onto Petri dishes containing MS salts with B5 vitamins, 30 g L^{-1} sucrose, 2.3 g L^{-1} fitagel, and 5 µM zeatin (ZEA). Twelve root explants were cultured per Petri dish (90 × 15 mm), with 5 plates per treatment. Plates were sealed with PVC and maintained under 16 h photoperiod at 55 µmol PAR $m^{-2}s^{-1}$ and 25 ± 1°C. After 35 days was evaluated the percentage of explants with shoots or secondary roots.

3.2.5 Phenotypic characterization

Phenotypic characterization was performed 50 days after germination (dag). Branching index, height of the plant on the primary shoot and number of leaves up to the first inflorescence were measured.

The branching index represents the sum of the length of lateral branches divided by the main stem length (MORRIS et al., 2001).

In the individual axis branching analysis, each line represents a leaf axis and each column, an individual plant. Grey squares represent an empty axis, white squares the presence of one or two leaves, light green squares the presence a side shoot and dark green squares the presence of a well-developed side shoot (more than 10 cm). C1 and C2 represent cotyledonary leaves (Analysis adapted from BUSCH et al., 2011).

3.3 Results

On the hypothesis that *Rg1* and *Rg3C* are gain-of-function alleles of a same gene, coming from the wild species *S. peruvianum* and *S. pennellii*, respectively, we decided to perform a comparison of the NILs MT-*Rg1* and MT-*Rg3C*, evaluating features that are known to be characteristic of *MT-Rg1* (LOMBARDI-CRESTANA et al., 2012).

We evaluated the capacity for root and shoot formation *in vitro* from cotyledonary explants, since this is the trait of interest in both NILs. In both MT-*Rg1* and MT-*Rg3C* the number of roots per explant was significantly higher than in MT, and very close from each other (Figure 3.2).

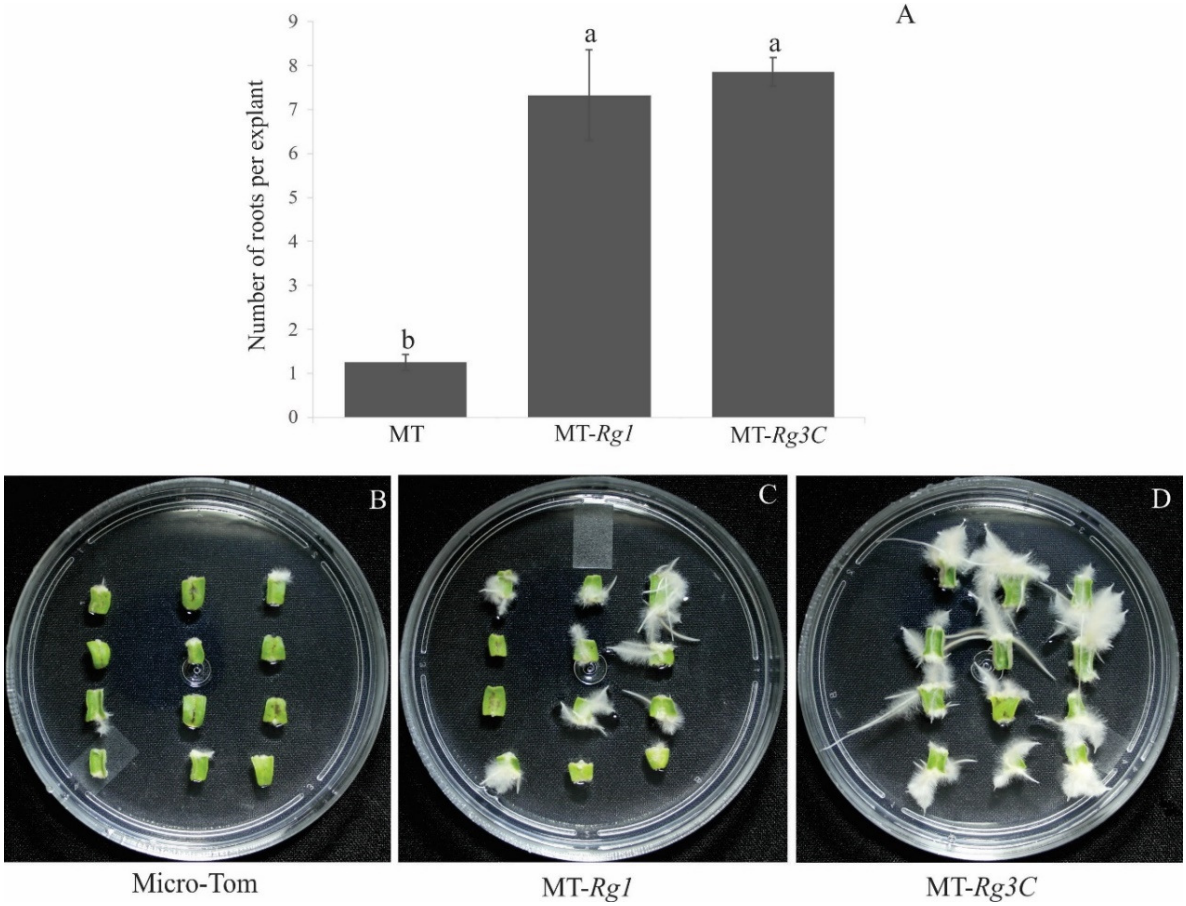

Figure 3.2 - *In vitro* root regeneration capacity of the genotypes Micro-Tom (MT), MT-*Rg1* and MT-*Rg3C* (A) 12-day-old seedling cotyledon explants were cultivated during 9 days on MS media with 0.4 µM NAA. (B-D) Aspect of root regeneration from selected genotypes. The bars depicted with the same letter are not significantly different ($p>0.01$) according to the unpaired Student's t-test (n = 10 Petri dishes, each containing 12 cotyledon explants)

Similar results were observed regarding the regeneration of shoots. Both MT-*Rg1* and MT-*Rg3C* had a higher regeneration rates than had MT. Moreover, the percentage of explants with shoots in both genotypes were higher than 85% (Figure 3.2).

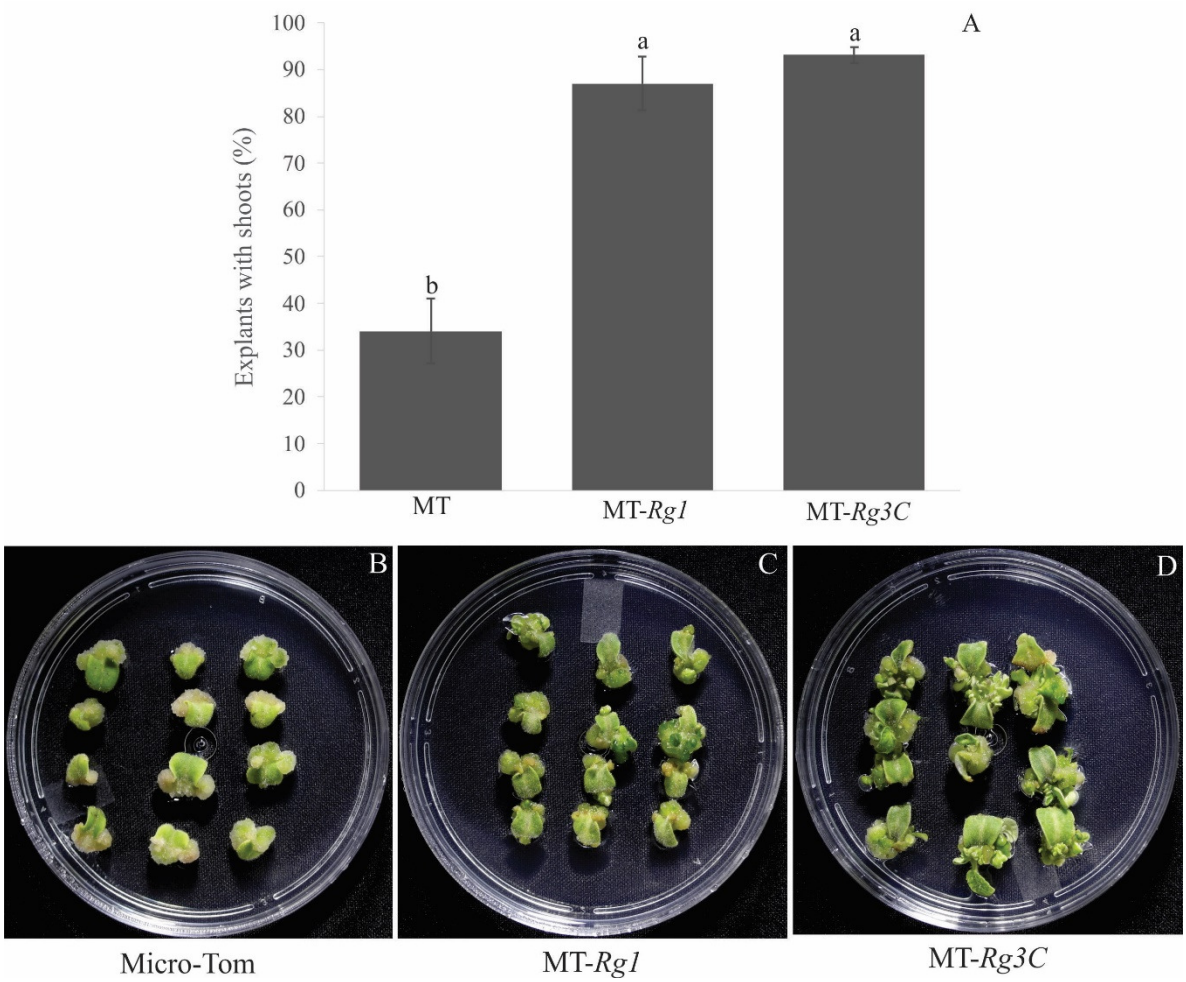

Figure 3.3 - *In vitro* shoot regeneration capacity of the genotypes Micro-Tom (MT), MT-*Rg1* and MT-*Rg3C*. (A) 12 day-old seedling cotyledon explants were cultivated during 21 days on MS media with 5.0 µM BAP. (B-D) Aspect of shoot regeneration from selected genotypes. The bars depicted with the same letter are not significantly different ($p>0.01$) according to the unpaired Student's t-test (n = 10 Petri dishes, each containing 12 cotyledon explants)

A remarkable feature of MT-*Rg1* is the capacity to regenerate shoot from roots explants (KOORNNEEF et al., 1993). So, this was the next feature evaluate in the NILs. Although 17.3% of the MT-*Rg1* root explants regenerated shoots (Table 3.2), in none of the MT-*Rg3C* root explants were observed the regeneration of shoots, the same was observed in the control MT (Figure 3.4).

Table 3.2 - *In vitro* shoot and root regeneration from roots of the genotypes Micro-Tom (MT), MT-*Rg1* and MT-*Rg3C*. Means followed by the same letters are not significantly different according to Student's t-test ($P < 0.01$). (n = 5 Petri dishes, each containing 10 root explants)

Genotype	Explants with shoots (%)	Explants with roots (%)
MT	0 b	7.3 a
MT-*Rg1*	17.3 a	3.3 a
MT-*Rg3C*	0 b	2.2 a

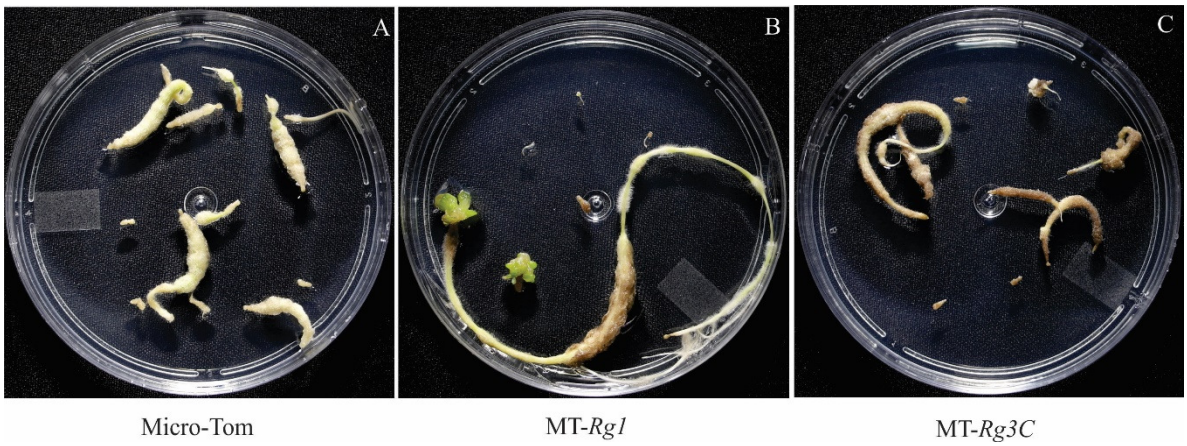

Figure 3.4 - *In vitro* regeneration from roots of the genotypes Micro-Tom (A), MT-*Rg1* (B) and MT-*Rg3C* (C) and. 12-day-old seedling were used as explant source. Roots were cultivated during 35 days on MS media with 5.0 µM Zeatin

We also evaluate the formation of roots in the explants, although, it was not observed difference neither in MT-*Rg1* in MT-*Rg3C* compared to MT.

Regarding phenotypic characteristic, branching in MT-*Rg1* is very pronounced, so we evaluate that feature in MT-*Rg3C*. The branching index of MT-*Rg1* and MT-*Rg3C* were very close and considerably higher than in MT (Figure 3.5).

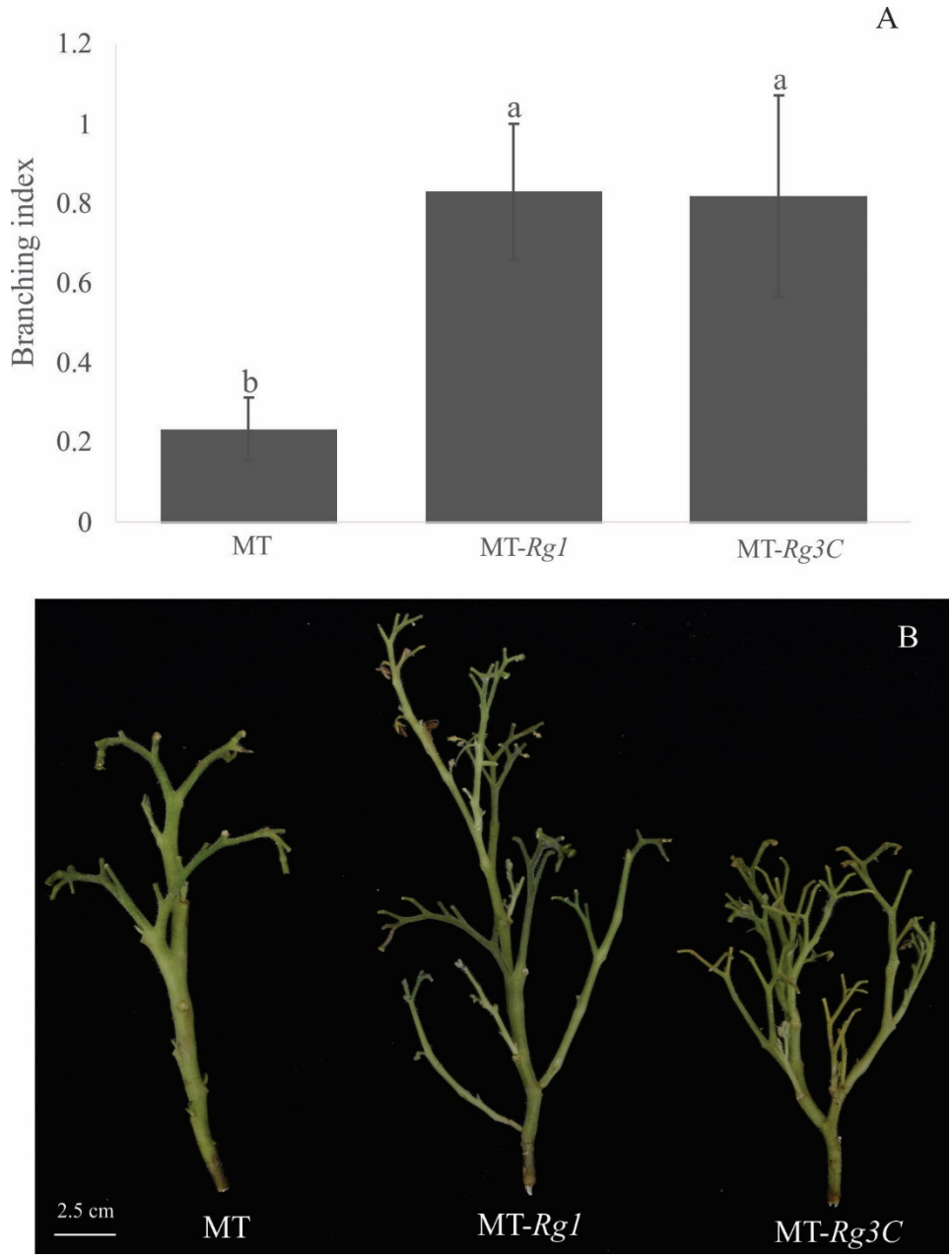

Figure 3.5 - Branching phenotype of MT, MT-*Rg1* and MT-*Rg3C* (A) Branching index of 60 days-old plants. Error bars represent the mean ±SE, n = 12 plants. Different letters indicate significant differences at P ≤0.01 (Scott-Knott test). The branching index represents the sum of the length of lateral branches divided by the main stem length (Morris et al., 2001) (B) Branching in MT, MT-*Rg1* and MT-*Rg3C* 90 days-old plants

Furthermore, in both genotype we observed the presence of shoots even in the cotyledonary axil (Figure S3B and C).

Although no difference in height was observed between MT-*Rg1* plants and MT, this feature was very noticeable in MT-*Rg3C* plants that was significantly shorter than MT and MT-*Rg1* (Figure 3.6A).

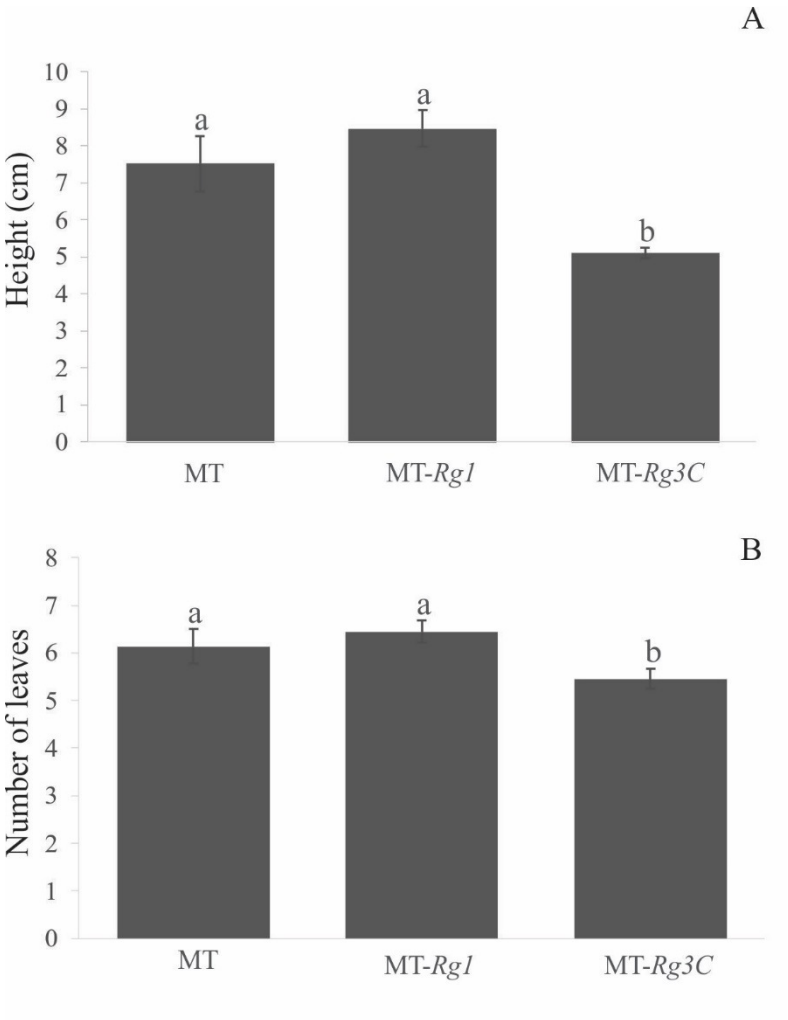

Figure 3.6 - Plant height and number of leaves up to the first inflorescence of MT, MT-*Rg1* and MT-*Rg3C* (A). Plant height until the first inflorescence measured in 60-day-old plants. (B) Number of leaves until the first inflorescence measured in 60-day-old plants. Error bars represent the mean ±SE, n = 12. Different letters indicate significant differences at P ≤0.05 (unpaired Student's t-test)

Wondering the reason why MT-*Rg3C* plants were shorter than MT and MT-*Rg1*, we evaluate the number of leaves up to the first inflorescence in these genotypes. The MT-*Rg3C* had fewer leaves than MT and MT-*Rg1* (Figure 3.6B), implying that its short shoot (Figure 3.6A) is probably due to a precocious flower induction.

Since the genetic map of IL 3-2, from which the locus *Rg3C* was introgressed into the MT background, is known, we searched for genes in the whole IL3-2 segment that would be affecting the number of leaves and/or height in MT-*Rg3C*. The *S. pennellii´*s allele *SP3D* is located in the IL 3-2, in the bin 3D (Figure 7). The *SP3D* is a strong allele of the gene *SINGLE FLOWER TRUSS* (*SFT*), which is considered the long-sought florigenic signal that induces flowering (LIFSCHITZ; ESHED, 2006). Hence, the introgression of *SP3D* into indeterminate tomato cultivars will reduce the sympodial index from 3 to 2, a phenotype leading to more compact plants with commercial interest to save apace and increase

productivity (US PATENT APPLICATION, 2010). Thus, it is likely that MT-*Rg3C* also harbors the *SP3D* allele, which is reducing the number of leaves up to the first inflorescence (Figure 3.6B). In order to test this, a SCAR marker was used and it was confirmed that MT-*Rg3C* still harbors the allele *SP3D* from *S. pennellii* (Figure S4). Since *SP3D* is not in the same bin as *Rg3C*, it was probably inheriting as a linkage drag, due to the lack of recombination of the region near to the centromere, during the introgression of *Rg3C* from IL3-2 into the MT background.

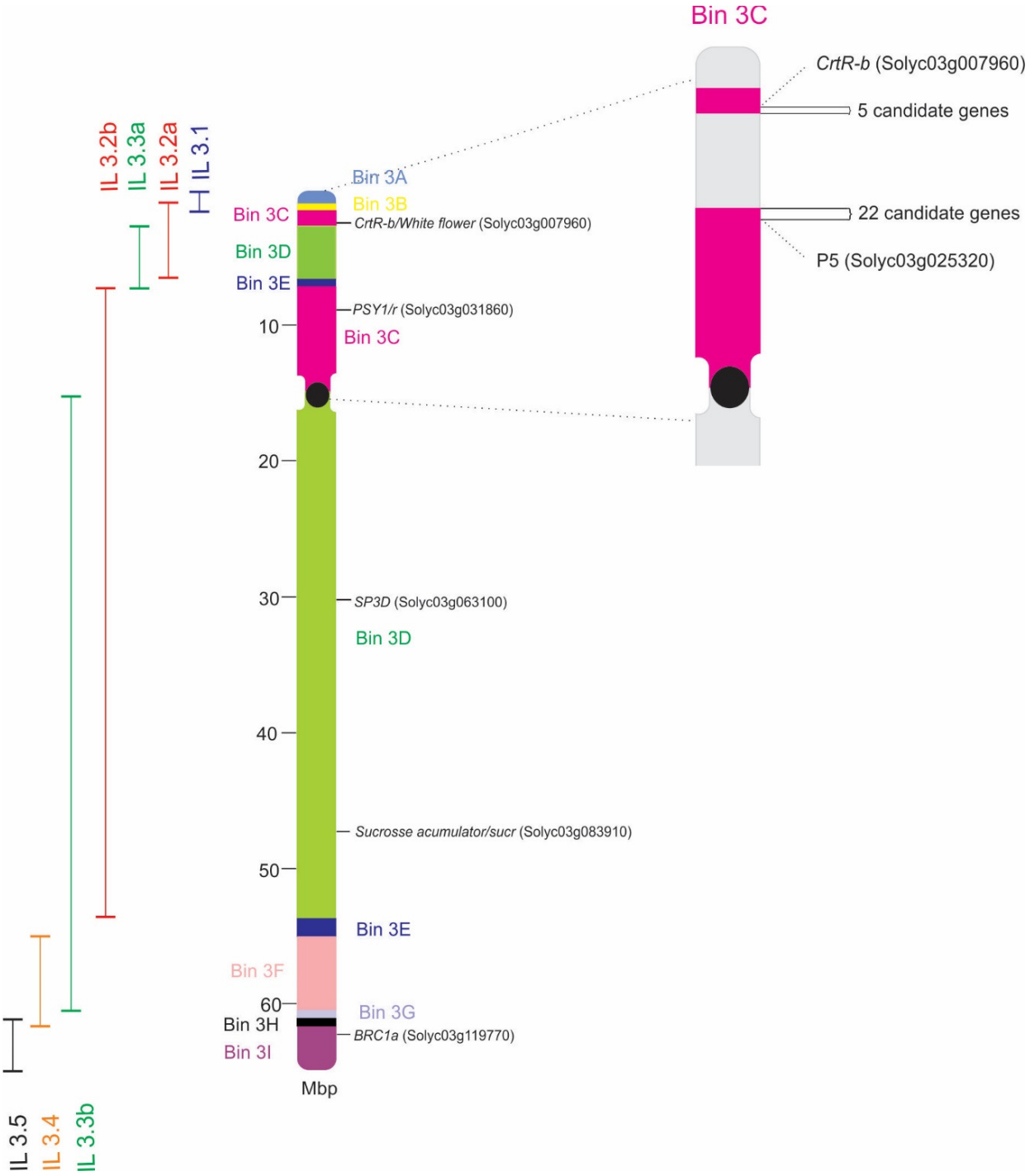

Figure 3.7 - Schematic figure of tomato chromosome 3 indicating the introgression lines, bins and main genes presents in the introgression lines. Bin 3C (pink) represents the segment from *S. pennellii* introgressed into MT background in the NIL MT-*Rg3C*. In the detail the *S. pennellii* segment present in the bin 3C. This bin is formed by the segments presents in the IL 3-2 and not in the IL 3-3. This detail also shows *CrtR-b* (Solyc03g007960) and the molecular marker P5 (Solyc03g025320) location. The fact that *Rg1/Rg3* is located in the bin 3C (pink segments) and between *CrtR-b* and P5, which is interrupted by a segment of bins 3D and 3E, decrease the list of candidate genes to 27 (5 + 22). Image designed from the data presented in Chitwood et al., 2013 and Tomato genome consortium, 2012

We also observed that the NIL MT-*SP3D* have an increased branching phenotype, which rose the possibility that *SP3D*, and not *Rg3C*, is responsible for the increased branching in the NIL MT-*Rg3C*. Since we know that the gene *Rg1* was able to revert the non-branching phenotype of MT-*ls* in the double mutant *Rg1 ls* (LOMBARDI-CRESTANA et al., 2012), we also analyzed the double mutant *SP3D ls* to see if *SP3D* would be capable to revert the non-branching phenotype of MT-*ls*.

The branching index of the double MT-*SP3D ls* was similar to that of MT-*ls* and do not followed the high branch index of the MT-*SP3D*, implying that that *SP3D* was not capable to rescue the non-branching phenotype of MT-*ls* in the double mutant (Figure 3.8).

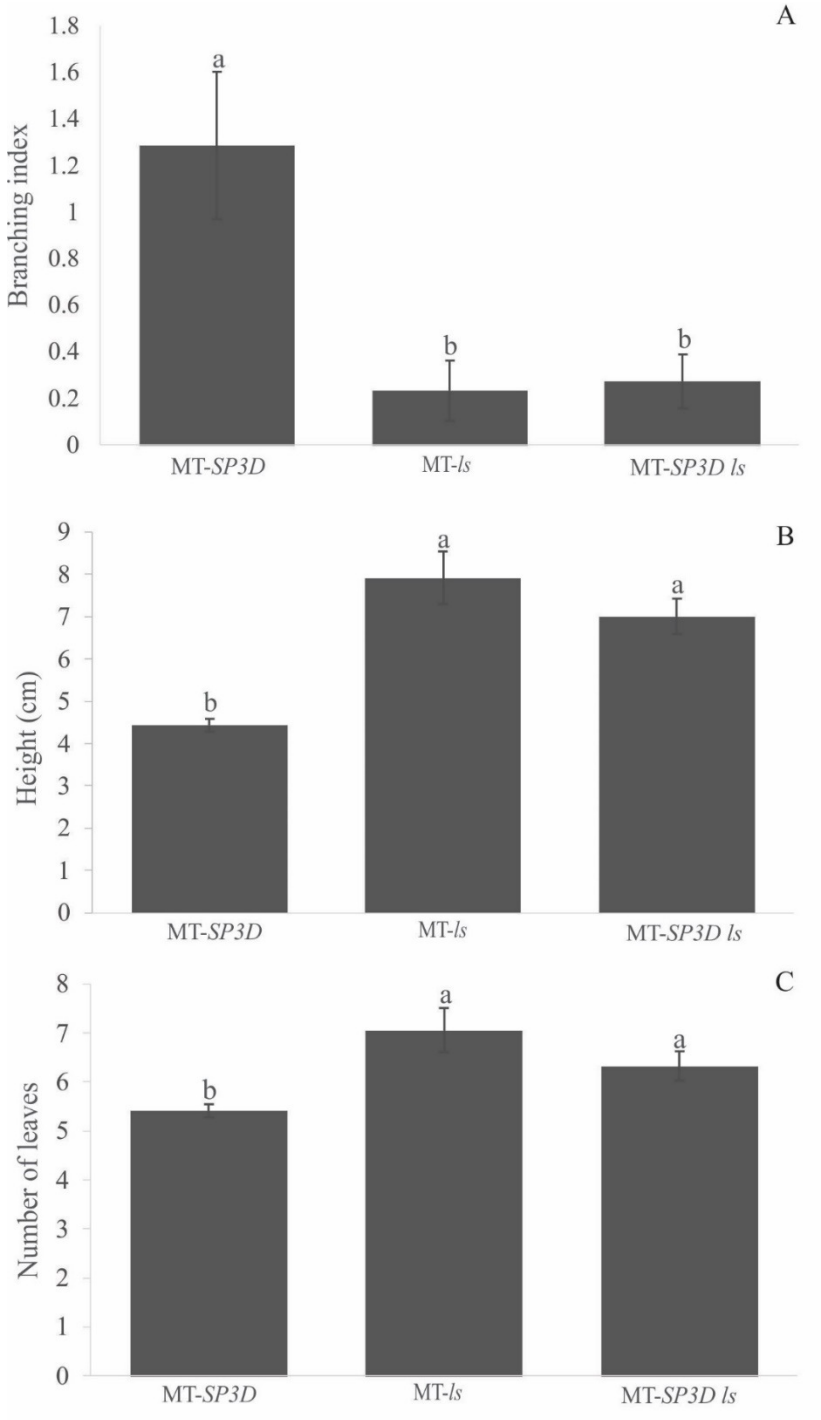

Figure 3.8 - Branch Index plant height and number of leaves up to the first inflorescence of MT-*SP3D*, MT-*ls* and the double mutant MT-*SP3D ls* (A) Branching index of 60 days-old plants. The branching index represents the sum of the length of lateral branches divided by the main stem length (Morris et al., 2001) (B) Plant height until the first inflorescence measured in 60-day-old plants. (C) Number of leaves until the first inflorescence measured in 60-day-old plants. Error bars represent the mean ±SE, n = 12. Different letters indicate significant differences at P ≤0.05 (unpaired Student's t-test)

Although the double mutant MT-*SP3D ls* was a little shorter and had a fewer leaves up to the first inflorescence than MT-*ls*, this difference was not significantly different. MT-*SP3D* was the NIL with the lower height and number of leaves up to the first inflorescence, consistent with its nature as a strong allele of the florigenic signal *SFT*.

3.4 Discussion

3.4.1 MT-*Rg1* and MT-*Rg3C* are different alleles from the same gene

The discovery that the wild specie S. *pennellii* harbors, in the chromosome 3, an allele that enhance both shoot and root *in vitro* regeneration, launched the idea that this would be the allele from *S. pennellii* corresponding to the allele *Rg1* of *S. peruvianum* (ARIKITA et al., 2013). *Rg1* is an allele already known to enhance *in vitro* organogenesis (LOMBARDI-CRESTANA et al., 2012). Although, so far, no comparative analyses had been made between the NILs MT-*Rg1* and MT-*Rg3C*. In this work, we presented some evidences that strongly indicate that MT-*Rg1* and MT-*Rg3C* are two alleles of the same gene. We also propose a list of candidate genes crossing the mapping information of *Rg1* and *Rg3C*.

Rg1`s high organogenic capacity was initially characterized by Koornneef et al. (1987), who localized the allele in the chromosome 3, between the mutations *white flower* (*wf*) and *yellow flesh* (*r*) (KOORNNEEF et al., 1993). The most remarkable characteristics conferred by this allele are *in vitro* regeneration, even from root explants, and increased branching *ex vitro* (KOORNNEEF et al., 1993; LIMA et al., 2004; LOMBARDI-CRESTANA et al., 2012).

After evaluate both root and shoot regeneration in the NILs MT-*Rg1* and MT-*Rg3C*, we confirmed that their regeneration rate are very close. Previous results reinforce this affirmation, the analysis of MT-*Rg3C* acquisition of competence defined that this NIL acquire competence sooner than MT (Chapter 1), what was also observed in MT-*Rg1* (AZEVEDO, 2012). Probably, both alleles enhance organogenesis by decreasing the time required to acquire competence for the organogenic process.

Although MT-*Rg1* and MT-*Rg3C* organogenesis from cotyledonary explants were very similar, the same was not observed for regeneration from root explants. As expected, MT-*Rg1* was capable to regenerate shoots from roots, but in MT-*Rg3C* explants no shoots regenerated.

This result, however, doesn`t discard the hypothesis that *Rg3C* and *Rg1* are alleles of a same gene. There is a possibility that *Rg3C* allele is weaker than *Rg1* and the experimental conditions was not favorable to MT-*Rg3C* regeneration from roots. Other additional assays testing regeneration from roots in this NILs, and even in IL 3-2 in M82 background, would be informative, and help to elucidate definitely whether the *Rg3C* has the capacity to regenerate shoots from roots.

The enhanced branching, another remarkable characteristic of MT-*Rg1*, was very similar between the two NILs, what can be observed in the branching index (Figure 3.5A) and in the image of the stems without leaves or flowers (Figure 3.5B). Although the branching index was not statistically different between MT-*Rg1* and MT-*Rg3C,* the image passes an idea that MT-*Rg3C* branching is even higher than in MT-*Rg1*. Although, this may be an impression caused by the lower height observed in MT-*Rg3C* plants (Figure 3.5B).

3.4.2 *SP3D* is not responsible for MT-*Rg3C* branching

Since we observed differences in height in the NILs, we decided to evaluate the height up to the first inflorescence. As we suspected during branching evaluations, MT-*Rg3C* plants are shorter than MT-*Rg1* and MT (Figure 3.6A). Among the causes for differences in height among genotypes are the length of the internode, or the number of internodes.

Although we didn`t evaluate the internode length, we observed that the number of leaves, and consequently the number of internodes, up to the first inflorescence are lower in MT-*Rg3C* plants than in MT and MT-*Rg1* (Figure 3.6B). The reduced height and number of leaves up to the first inflorescence are usually characteristics observed in tomato genotypes with early flowering (VICENTE et al., 2015).

As we do not observe this characteristic in MT-*Rg1* plants, we wondered if another gene present in the locus *Rg3C* would be affecting the height and the number of leaves up to the first inflorescence in this NIL. Since the genetic map of Introgression Lines in the chromosome 3 is known (Figure 3.7), we searched, in the segment 3-2, for genes that would affect this characteristic.

The promotor of the gene *SP3D*, from *S. pennellii*, is known to reduce the plant sympodial index from 3 to 2 when introgressed in *S. lycopersicum* background, reducing also the number of leaves up to the first inflorescence (US PATENT APPLICATION, 2010). This gene is located in the chromosome 3, in the bin 3D. Although *Rg3C* and *SP3D* are located in different bins, both are in the IL 3-2. Thus, it is likely that during the introgression of *Rg3C*

into MT background, although we screened the plants for the high regeneration capacity, the 3-2 segment containing *SP3D* was also introgressed together with *Rg3C*. We confirmed this hypothesis by using CAPS markers.

Since we already knew that probably *SP3D* is the responsible for MT-*Rg3C* short height, we wondered if this gene would be also the responsible for the increased branching in MT-*Rg3C* plants. To elucidate this question, we evaluate the branching index in MT-*SP3D* plants. We also add to the analysis the mutant *lateral suppressor* (MT-*ls*) and the double mutant MT-*ls SP3D*.

The cells in the axil of leaf primordia in the *ls* mutant fail to retain their meristematic character leading to the absence of side shoots. Axillary meristems in this mutant are only initiated during the reproductive development in the two leaf axils preceding the inflorescence (MALAYER; GUARD, 1964).

The *Rg1* allele was capable of rescue the branching phenotype of *ls* in the double mutant MT-*Rg1 ls*, although the regeneration of shoots in this double mutant was extremely low, even lower than in the mutant MT-*ls* (LOMBARDI-CRESTANA et al., 2012). Based on this, we decided to use the double mutant MT-*SP3D ls* in the analysis to evaluate the possibility of epistasis between *SP3D* and *ls*.

The branching index in the double mutant wasn`t different from MT-*ls*, what indicates that *SP3D* was not capable of revert the absence of branching caused by the *ls* allele, and there is no epistasis between *ls* and *SP3D*. The analyses of the presence of shoots in the individual axils also shows that in MT-*SP3D ls* the only axil that produces shoots are the last two before the inflorescence, as observed for MT-*ls* (Figure S3).

Probably *SP3D* is affecting MT-*Rg3C* flowering and consequently the height and number of leaves up to the first inflorescence, but is not responsible for the branching phenotype. The branching in MT-*Rg3C* and MT-*Rg1* may be caused by the same gene that enhance *in vitro* regeneration, or alternatively by another gene present in the segment 3-2. In MT-*SP3D* the branching may not be caused by the *Rg3C* allele, since it is not present in this genotype that was produced from the introgression of the IL 3-3. This was also confirmed by the regeneration rate of this NIL in both RIM and SIM medium that was extremely low (Data not presented). This reinforce the possibility that another gene than *SP3D* and *Rg3C* are the cause of the branching phenotype. Another possibility is that in the MT-*SP3D* plants the early flowering, are the cause of the branching, releasing the lateral shoots from the auxin dominance and letting the lateral meristems to develop (MÜLLER; LEYSER, 2011)

The search for recombinants, coming from the NIL MT-*Rg3C*, that harbor the allele *Rg3C* and not the allele *SP3D* from *S. pennellii*, may clarify the questions regarding the branching in these two genotypes.

Although some differences were observed between MT-*Rg1* and MT-*Rg3C*, the main characteristics evaluate, that was the high regeneration rate and the increased branching, was very close in these NILs. That fact, together with the mapping information of these two NILs, strongly indicate that they are two alleles of the same gene.

Considering this fact, we decided to analyze together the mapping information already available for *Rg1* and *Rg3C*.

The allele *Rg1* was mapped in the chromosome 3, between the mutations *white flower* (*wf*) and *yellow flesh* (*r*) (KOORNNEEF et al., 1993). This mutations code for the genes *BETA-CAROTENE HYDROXYLASE* (*CrtR-b*) (GALPAZ et al., 2006) and *PHYTOENE SYNTHASE* (*PSY1*) (BARTLEY et al., 1991; FRAY; GRIERSON, 1993), respectively. The segment between *CrtR-b* and *PSY1* comprises 301 genes (Figure 3.7).

New analysis using MT-*Rg1* and molecular markers diminished the segment proposed by Koornneef et al. (1993) for the *Rg1* localization. Analyzing a segregating population, resulting from crossing the genotypes MT and MT-*Rg1*, Azevedo (2012) didn`t observe polymorphism for any of the markers used, neither in the segregating population, nor between MT and MT-*Rg1*. Although, using the same markers, it was observed polymorphism between MT and *S. peruvianum*. According to this new mapping, *Rg1* is localized between the genes *CrtR-b* (*Solyc03g007960*) and the molecular marker P5 (*Solyc03g025320*) (Figure 3.7), diminishing the number of candidate genes to 136 (AZEVEDO, 2012).

MT-*Rg3C* are localized in the bin 3C, since the IL 3-2 has a high organogenic capacity, while the IL 3-3 doesn't own this feature (ARIKITA et al., 2013). The bin 3C has 601 genes (Figure 3.7).

The ILs developed by Eshed and Zamir (1994) were recently remapped by Chitwood et al. (2013). After the new mapping it was identified gaps in some ILs and non-contiguous segments. In the chromosome 3, it was observed an interruption in the bin 3C, where *Rg3C* are localized (Figure 3.7).

From *Rg1* mapping data and the updated ILs mapping data, published by Chitwood et al. (2013), we were able to reduce the possible location area of *Rg1/Rg3C* to 27 (5+22) candidate genes (Figure 3.7). The 27 genes are presented in the Appendices (Table S1 and S2).

3.4.3 A first look at the candidate genes

The segment proposed here to harbor *Rg1*/*Rg3C*, by the crossing of *Rg3C* and *Rg1* mapping information, is short. However, since these alleles are natural genetic variations, the number of SNPs among *S. pennellii*, *S. peruvianum* and *S. lycopersicum* (MT) in that segment are extremely high. So, the simple search for SNPs, that could be the cause of the increased regeneration in MT-*Rg1* and MT-*Rg3C* compared to MT, is not possible.

On the other hand, some *in silico* analysis using public databases and the search for function of the genes in the list may give us some tips of the most likely candidates to *Rg1*/*Rg3C*. Unfortunately, many genes in the list are unknown proteins, which may difficult this kind of analysis.

Many of the genes in the list code for proteins with known function in other organisms. UDP-N-acetylmuramate-L-alanine ligase (Solyc03g007970), for example, is an enzyme widely studied in bacteria due to its participation in peptidoglycan biosynthesis (SMITH, 2006). Although, to the best of our knowledge, the function of this enzyme in plants wasn`t already described.

Septum site-determining protein MinD (Solyc03g007980) is required in bacteria, together with the MinC and MinE proteins, for proper placement of the septum in cell division (BOER; CROSSLEY; ROTHFIELD, 1989). In Arabidopsis, MinD have been shown to be involved in the positioning of the division site in chloroplasts. Transgenic plants with reduced expression of *MinD* had asymmetric plastid division, leading to an abnormally heterogeneous distribution of plastid sizes in leaf cells, although, their aspects of growth and development didn`t differ from the wild type (COLLETTI et al., 2000). As expected this gene in *S. lycopersicum* has an enhanced expression in leaves compared to roots (Table S2).

Some of the genes also have known involvement in several kinds of cancer. Since in cancers the body's cell begins to divide without stopping (NATIONAL CANCER INSTITUTE), similar to what happens during plant regeneration, these may be considered potential candidates to *Rg1/Rg3C*.

PPPDE peptidase domain-containing protein 1 (Solyc03g008010) belongs to the PPPDE superfamily, this family are involved in the ubiquitination signaling system. Studies relates the presence of this protein with cancers, since it exhibits decreased expression of varying degrees in certain tumors (HE et al., 2013).

The gene *Retinoblastoma-binding protein 6* (*RBBP6*) (Solyc03g025260), to the best of our knowledge, is not yet characterized in plants. Although, this gene is deeply studied in

human science due to its role in several cancers (DLAMINI et al., 2016; MOELA; CHOENE; MOTADI, 2014; MOTADI; BHOOLA, DLAMINI, 2011). In humans, *RBBP6* codes for 3 protein products (GAO; SCOTT, 2002; GAO; WITTE; SCOTT, 2002; SAKAI et al., 1995), and interacts with the tumor suppressors proteins, Retinoblastoma (Rb binding domain) (WITTE; SCOTT, 1997) and p56 (p56 binding domain) (SIMONS et al., 1997). Besides Rb and p55 domain, human *RBBP6* also has a DWNN (Domain With No Name), a CCHC zinc finger and a RING finger domain (PUGH et al., 2006). All the *RBBP6* homologues identified in other organisms at the moment include the DWNN, a CCHC zinc finger and a RING finger domain and are found as single copy genes (PUGH et al., 2006). Although *RBBP6* function is not yet characterized in plants, it is known that plants *RBBP6* lacks both Retinoblastoma and p53 binding domain (PUGH et al., 2006). However, both DWNN and RING finger domain demonstrated also to be involved in cancer diseases. DWNN is a ubiquitin-like domain that are involved in cell cycle regulation (MBITA et al., 2012), and the RING finger domain shows to interact to the protein YB-1 (Y-box binding protein 1), a transcription factor with important role in tumorigenesis, leading to its ubiquitination and degradation by the proteasome (CHIBI et al., 2008).

Other candidate gene is a GRAS family transcription factor (*GRAS10*) (Solyc03g025170). These are plant specific proteins that play diverse roles in root and shoot development and gibberellic acid signaling (HIRSCH; OLDROYD, 2009). Besides that, mutants *procera* and *ls*, both belonging to GRAS family, interacted with MT-*Rg1*, suggesting a link between the GRAS family and *Rg1* (LOMBARDI-CRESTANA et al., 2012). Also, in previous analysis, the gene *GRAS10* was highly expressed in MT-*Rg1* cotyledons cultivated for 1 day in MS supplemented with NAA auxin (AZEVEDO, 2012). The *GRAS10* homologous gene in Arabidopsis is *SCL8* (AZEVEDO, 2012). Little is known about these genes function in both Arabidopsis and tomato. In Arabidopsis, it is known that *SCL8* shows enhanced expression in root tissues (PYSH et al., 1999), and are target of the trichome formation transcription factors GL3 and GL1, suggesting a need for SCL8 function at early stages during trichome initiation (MOROHASHI; GROTEWOLD, 2009). In *S. lycopersicum* this gene has higher expression in roots than in leaves (Table S2). Due to the known role of this family of transcription factor in root and shoot development, its expression analysis in MT-*Rg1*, as well as the interaction of *procera* and *ls* with *Rg1*, this GRAS transcription factor is a strong candidate to be *Rg1/Rg3C*.

In the list of genes candidates there is also a *RNA-binding protein* (*RBP*) (Solyc03g025280). These proteins in eukaryotes have crucial roles in all aspects of post-

transcriptional gene regulation. They act by regulating pre-mRNA splicing, polyadenylation, RNA stability and export, and influence chromatin modification (LORKOVIĆ, 2009). The *Arabidopsis thaliana* genome encodes for more than 200 different RBPs (LORKOVIĆ; BARTA, 2002), and these genes have important roles in developmental process and plant adaptation to various environmental conditions (AMBROSONE et al., 2011, LORKOVIĆ, 2009; RAI et al., 2016; ZHANG et al., 2015). Although we have not found any work relating RBPs with plant regeneration, this gene may not be discard as a candidate gene to *Rg1/Rg3C*, since RBPs develop a wide range of functions in plant development.

Two genes in the list codes for proteins involved in transport across membranes. Twin-arginine translocation (Tat) protein (Solyc03g025300) is one of the membrane proteins that compose the Tat translocation pathway (CLINE; ETTINGER; THEG, 1992; MOULD; ROBINSON, 1991). This is a transport pathway found in plants, bacteria and archaea. In plants, the Tat pathway is located in the thylakoid membrane of the chloroplast (ALBINIAK; BAGLIERI; ROBINSON, 2012), being essential for plant photosynthesis (PALMER; BERKS, 2012). Although we don't want to completely discard this gene as *Rg1/Rg3C* candidate, it doesn't seem to be a good candidate to control plant regeneration.

A cluster formed by seven genes (Solyc03g025190, Solyc03g025200, Solyc03g025210, Solyc03g025220, Solyc03g025230, Solyc03g025240, Solyc03g025250) belonging to the Multi Antimicrobial Extrusion (MATE) family is located in the middle of the segment. Multidrug resistance protein mdtk is member of the MATE family, and are found in bacteria, archaea and eukaryotes. MATE transporters in plant species appears to be largely involved in the detoxification of endogenous secondary metabolites (GOMEZ et al., 2009) and xenobiotics (DIENER; GAXIOLA; FINK, 2001). Besides that, they also function in the synthesis of natural products like proanthocyanidins (ZHO; DIXON, 2009). Interestingly, one of the Multidrug resistance proteins (Solyc03g025240) has the expression very lower in *S. lycopersicum* than in *S. pennellii* and in IL 3-2 (Table S1). This data makes this gene a likely candidate to be *Rg1/Rg3C*.

The candidate gene *Hydroxyproline-rich glycoprotein* (*HRGP*) (Solyc03g025290) codes to the major protein component of plant cell walls (CHEN; VARNER, 1985). In Arabidopsis, a HRGP gene, *ROOT-SHOOT-HYPOCOTYL-DEFECTIVE* (*RSH*) is essential for the correct positioning of the cell plate during cytokinesis of cells in developing embryo (HALL; CANNON, 2002). In maize, the mRNA of *HRGP* accumulates in young organs rich in dividing cells, but it decreased in mature tissues (BALUŠKA et al., 2003). HRGP seems also to be essential for development, germination and regeneration of banana somatic

embryos (XU et al., 2011). However, in tomato, to the best of our knowledge, these proteins are mostly related to pathogen and herbivores defense (BENHAMOU et al., 1991; PEARCE; RYAN, 2003).

We also have as candidate a rRNA 2'-O-methyltransferase fibrilarin (Solyc03g025270), that is a basic nucleolar protein involved in rRNA processing and has the ability to methylate both RNAs and proteins (MULLER et al., 2014; PIH et al., 2000).

Besides these genes with known function, the Genomic DNA chromosome 5 BAC (Solyc03g008000) clone F6N7 is more expressed in fruits than in the other organs (Data not presented), suggesting that this gene probably develop a role in fruits, and probably is not related to the regeneration process.

Also, a gene coding to an unknown protein (Solyc03g007990) is twice more expressed in *S. lycopersicum*, than in *S. pennellii* and in the IL 3-2, suggesting that this gene should be more investigated in future studies.

Although some of the proteins code by the genes in the list don`t seem to be related to plant regeneration, we have to be careful in discard any of them, since most doesn`t have been deeply studied in plants and may have functions not yet described. Therefore, further studies involving *in silico* and expression analyses will be important to narrow the candidate list to the most likely genes to be *Rg1/Rg3C*.

3.5 Conclusions

The evidences presented in this work strongly indicate that *Rg1* and *Rg3C* are alleles of a same gene controlling enhanced regeneration capacity.

Some phenotypic differences observed between MT-*Rg1* and MT-*Rg3C*, such as height and number of leaves up to the first inflorescence, are caused by the allele *SP3D* introgressed into MT-*Rg3C* together with *Rg3C*.

SP3D was not capable to rescue the lack of branching in the *ls* mutant, as reveled by the double mutant MT-*SP3D ls*. This indicates that the enhanced branching in MT-*Rg3C* are not caused by *SP3D*.

By integrating *Rg1* and *Rg3C* mapping information we diminished to 27 the list of candidate genes to be *Rg1/Rg3C*.

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APPENDIXES

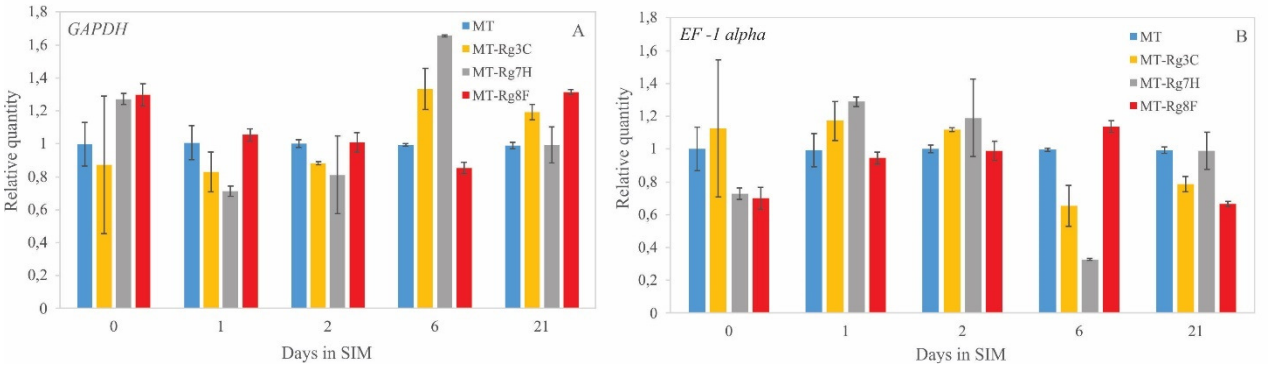

Figure S1 - Relative expression of housekeeping genes used for normalization of gene expression during *in vitro* shoot regeneration process (Days 0, 1, 2, 6 and 21 in SIM) in *S. pennellii*, MT, MT-*Rg3C*, MT-*Rg7H* and MT-*Rg8F*. (A) Relative expression of *GAPDH*. (B) Relative expression of *EF-1 alpha*

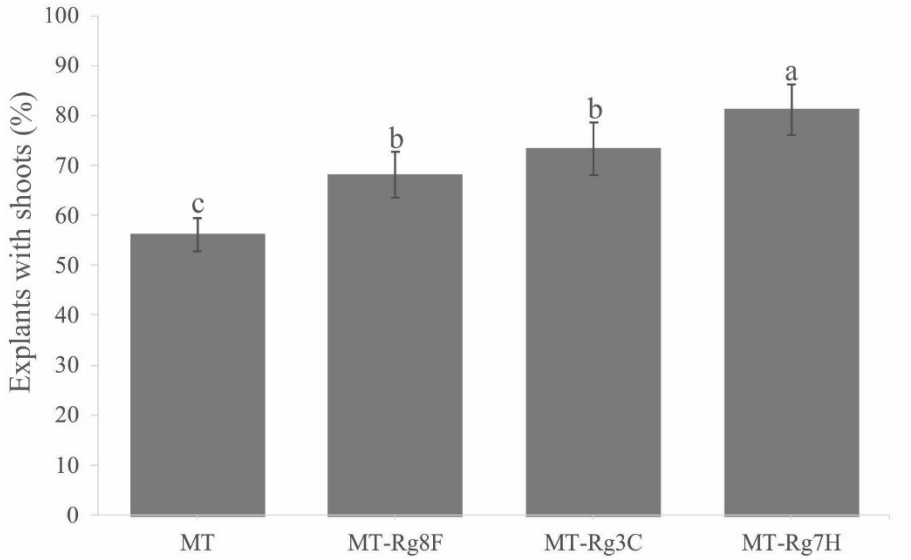

Figure S2 **-** *In vitro* shoot regeneration assay used to collect the samples for RT-PCR analysis. In the graphic, the regeneration rate of cotyledons of *S. lycopersicum* cv. MT, and lines MT-*Rg8F*, MT-*Rg3C* and MT-*Rg7H* after 21 days on SIM (shoot inducing medium). The bars depicted with the same letter are not significantly different (p>0.05) according to Scott Knott's Test

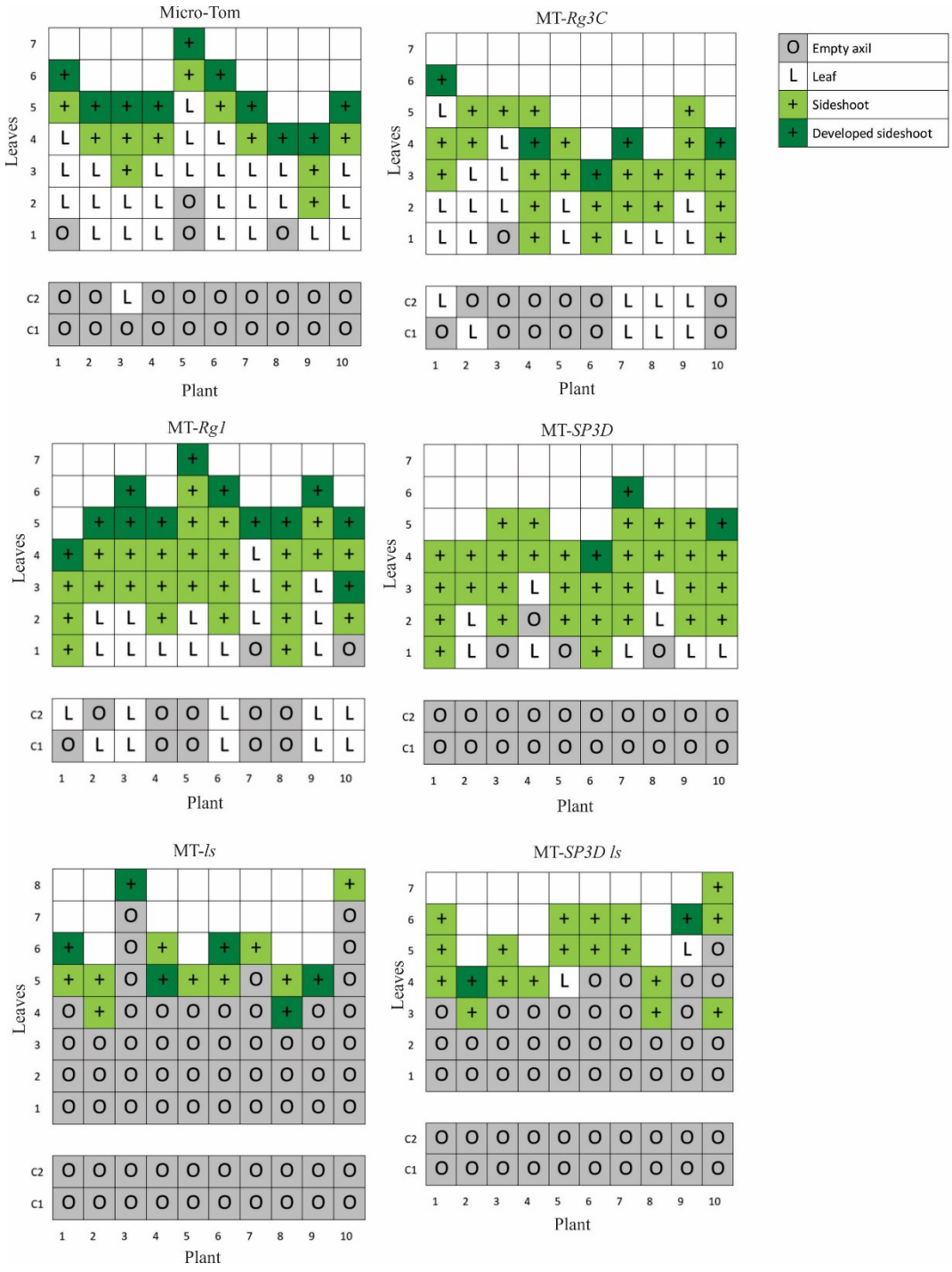

Figure S3 - Branching phenotype of MT, MT-*Rg3C,* MT-*Rg1*, MT-*SP3D*, MT-*ls*, MT-*SP3D ls*. Individual axis branching representation. Each line represents a leaf axis and each column an individual plant. Grey represents an empty axis, white the presence of one or two leaves, light green the presence a sideshoot and dark green the presence of a well-developed sideshoot. C1 and C2 represent the cotyledonary leaves (Figure adapted from BUSCH et al., 2011)

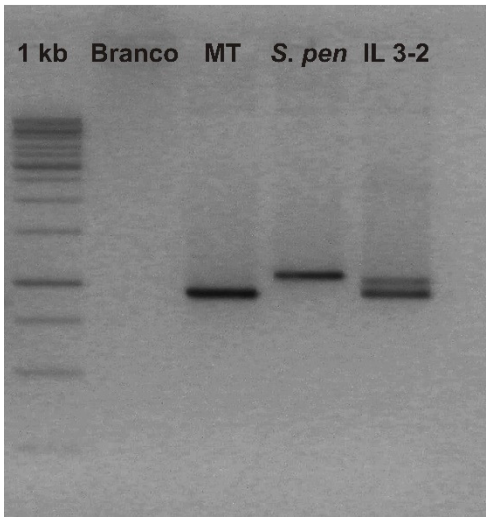

Figure S4 - PCR analysis using SCAR markers for the gene *SP3D*. According to the figure, MT-*Rg3C* still harbors the gene *SP3D* from *S. pennellii*

Table S1 - Candidate genes to *Rg1*/*Rg3C* and RNA-seq data available in Tomato eFP Browser. Data collected from Chitwood et al. (2013) and represent RNA-seq derived data from 8 days old seedling (cotyledons and mature leaves were excluded, and the remaining tissues above the hypocotyl midpoint were pooled). Available in: http://bar.utoronto.ca/efp_tomato/cgibin/efpWeb.cgi?dataSource=ILs_Leaf_Chitwood_et_al.

	Solyc Number	Description	IL 3-2	*S. pennellii*	*S. lycopersicum*
1	Solyc03g007970	UDP-N-acetylmuramate-alanine ligase	2.78	3.51	3.56
2	Solyc03g007980	Septum site-determining protein MinD	41.74	35.76	30.89
3	Solyc03g007990	Unknown Protein	10.14	9.24	20.79
4	Solyc03g008000	Genomic DNA chromosome 5 BAC clone F6N7	35.89	30.43	42.24
5	Solyc03g008010	PPPDE peptidase domain-containing protein 1	64.65	54.03	73.01
6	Solyc03g025100	Unknown Protein	0.0	0.0	0.0
7	Solyc03g025110	Unknown Protein	0.0	0.0	0.0
8	Solyc03g025120	Unknown Protein	0.0	0.0	0.0
9	Solyc03g025130	Unknown Protein	0.0	0.0	0.0
10	Solyc03g025140	Unknown Protein	0.0	0.0	0.0
11	Solyc03g025150	Unknown Protein	10.67	13.56	12.39
12	Solyc03g025160	Unknown Protein	69.59	62.05	57.91
13	Solyc03g025170	GRAS family transcription factor	12.14	9.61	9.71
14	Solyc03g025180	Unknown Protein	2.79	3.7	3.88
15	Solyc03g025190	Multidrug resistance protein mdtK	71.96	74.74	65.26
16	Solyc03g025200	Multidrug resistance protein mdtK	4.82	5.63	6.22
17	Solyc03g025210	Mate efflux family protein	0.0	0.0	0.0
18	Solyc03g025220	Multidrug resistance protein mdtK	1.32	0.84	1.35
19	Solyc03g025230	Multidrug resistance protein mdtK	34.86	31.25	40.13
20	Solyc03g025240	Multidrug resistance protein mdtK	2.65	2.66	0.1
21	Solyc03g025250	Multidrug resistance protein mdtK	0.0	0.0	0.0
22	Solyc03g025260	Retinoblastoma-binding protein	0.0	0.0	0.0
23	Solyc03g025270	rRNA 2'-O-methyltransferase fibrillarin	361.2	383.3	332.5
24	Solyc03g025280	RNA-binding protein	14.84	20.23	17.34
25	Solyc03g025290	Hydroxyproline-rich glycoprotein family protein	7.16	9.79	6.61
26	Solyc03g025300	Unknown Protein	0.0	0.0	0.0
27	Solyc03g025310	Sec-independent protein translocase tatA/E homolog	57.07	57.77	67.74

Table S2 - Candidate genes to *Rg1*/*Rg3C* and RNA-seq data available in Tom Express (Micro Tom Whole root, lateral root, root tip, whole leaf, whole flower and whole fruit). The tissues are indicated in the table and represent RNA-seq row sequence data available in public database (SRA/EMBL-EBI-ENA). Available in http://gbf.toulouse.inra.fr/tomexpress/www/welcomeTomExpress.php

	Solyc Number	Description	Micro-Tom							
			DMSO	CK	AUX	DMSO	CK	AUX	DMSO	CK
			Whole Root			Lateral Root			Whole Leaf	
1	Solyc03g007970	UDP-N-acetylmuramate-alanine ligase	0.012	0.036	0.037	0.007	0.018	0.020	0.003	0.005
2	Solyc03g007980	Septum site-determining protein MinD	0.065	0.078	0.067	0.063	0.066	0.069	0.104	0.118
3	Solyc03g007990	Unknown Protein	0.907	0.783	0.731	0.805	0.827	0.707	15.38	10.41
4	Solyc03g008000	Genomic DNA chromosome 5 BAC clone F6N7	0.708	0.608	0.486	0.610	0.628	0.532	0.844	0.664
5	Solyc03g008010	PPPDE peptidase domain-containing protein 1	0.401	0.617	0.742	0.494	0.646	0.610	0.246	0.214
6	Solyc03g025100	Unknown Protein	0.005	0.011	-	0.006	0.009	-	0.009	0.002
7	Solyc03g025110	Unknown Protein	0.027	0.020	0.021	0.023	0.021	0.017	0.004	0.001
8	Solyc03g025120	Unknown Protein	-	0.002	0.002	-	-	0.004	-	0.001
9	Solyc03g025130	Unknown Protein	0.002	-	0.006	0.002	0.012	0.003	0.001	0.002
10	Solyc03g025140	Unknown Protein	0.018	0.036	0.016	0.015	0.030	0.030	0.012	0.015
11	Solyc03g025150	Unknown Protein	0.122	0.135	0.167	0.126	0.144	0.173	0.137	0.132
12	Solyc03g025160	Unknown Protein	0.097	0.071	0.104	0.111	0.075	0.097	0.065	0.044
13	Solyc03g025170	GRAS family transcription factor	0.203	0.199	0.170	0.153	0.171	0.180	0.068	0.037
14	Solyc03g025180	Unknown Protein	0.020	0.020	0.020	0.020	0.025	0.023	0.017	0.012
15	Solyc03g025190	Multidrug resistance protein mdtK	0.002	0.005	0.002	0.001	0.001	-	0.039	0.013
16	Solyc03g025200	Multidrug resistance protein mdtK	0.053	0.090	0.068	0.078	0.106	0.071	0.021	0.021
17	Solyc03g025210	Mate efflux family protein	0.029	0.027	0.031	0.034	0.022	0.026	0.006	0.002
18	Solyc03g025220	Multidrug resistance protein mdtK	0.022	0.023	0.043	0.031	0.036	0.044	0.006	0.008
19	Solyc03g025230	Multidrug resistance protein mdtK	0.084	0.078	0.074	0.071	0.062	0.058	0.047	0.050
20	Solyc03g025240	Multidrug resistance protein mdtK	0.008	0.002	0.001	0.006	0.001	0.002	-	-
21	Solyc03g025250	Multidrug resistance protein mdtK	0.001	0.000	0.001	0.001	0.001	0.002	0.005	0.003
22	Solyc03g025260	Retinoblastoma-binding protein	0.002	0.003	0.004	0.004	0.003	0.002	0.003	0.002
23	Solyc03g025270	rRNA 2'-O-methyltransferase fibrillarin	0.817	0.768	13.27	0.926	0.709	1.512	0.243	0.582
24	Solyc03g025280	RNA-binding protein	0.131	0.132	0.142	0.140	0.131	0.151	0.123	0.141
25	Solyc03g025290	Hydroxyproline-rich glycoprotein family protein	0.099	0.125	0.084	0.099	0.123	0.074	0.278	0.172
26	Solyc03g025300	Unknown Protein	-	-	-	-	-	-	0.013	0.007
27	Solyc03g025310	Sec-independent protein translocase tatA/E homolog	0.076	0.081	0.076	0.077	0.074	0.086	0.150	0.196