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

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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.

2.5 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).

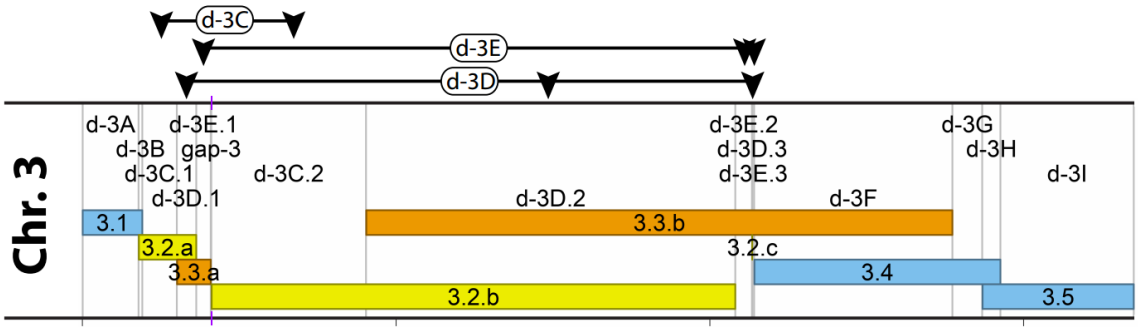

Figure 2.1 – Map showing the architecture of ILs based on precisely defined introgression boundaries determined from the sequenced tomato genome and next-generation sequencing data. IL size is proportional to the number of annotated genes harbored in each introgression. Bins, or intervals defined by 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.

3.5 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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