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RESEARCH PAPER

The tomato (*Solanum lycopersicum* cv Micro-Tom) natural genetic variation *Rg1* and the DELLA mutant *procera* control the competence necessary to form adventitious roots and shoots

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Abstract

Despite the wide use of plant regeneration for biotechnological purposes, the signals that allow cells to become competent to assume different fates remain largely unknown. Here, it is demonstrated that the *Regeneration1* (*Rg1*) allele, a natural genetic variation from the tomato wild relative *Solanum peruvianum*, increases the capacity to form both roots and shoots *in vitro*; and that the gibberellin constitutive mutant *procera* (*pro*) presented the opposite phenotype, reducing organogenesis on either root-inducing medium (RIM) or shoot-inducing medium (SIM). Mutants showing alterations in the formation of specific organs *in vitro* were the auxin low-sensitivity *diageotropica* (*dgt*), the *lateral suppresser* (*ls*), and the *KNOX*-overexpressing *Mouse ears* (*Me*). *dgt* failed to form roots on RIM, *Me* increased shoot formation on SIM, and the high capacity for *in vitro* shoot formation of *ls* contrasted with its recalcitrance to form axillary meristems. Interestingly, *Rg1* rescued the *in vitro* organ formation capacity in *proRg1* and *dgtRg1* double mutants and the *ex vitro* low lateral shoot formation in *pro* and *ls*. Such epistatic interactions were also confirmed in gene expression and histological analyses conducted in the single and double mutants. Although *Me* phenocopied the high shoot formation of *Rg1* on SIM, it failed to increase rooting on RIM and to rescue the non-branching phenotype of *ls*. Taken together, these results suggest *REGENERATION1* and the *DELLA* mutant *PROCERA* as controlling a common competence to assume distinct cell fates, rather than the specific induction of adventitious roots or shoots, which is controlled by *DIAGEOTROPICA* and *MOUSE EARS*, respectively.

Key words: Cell fate, competence, determination, hormonal mutants, Micro-Tom, plant development, regeneration.

Introduction

A remarkable and intriguing aspect of plant development is the capacity to form new and adventitious organs during the whole life cycle. This capacity, which has both evolutionary (Fosket, 1994; Sugimoto *et al*., 2011) and ecological significance (Kauffman, 1991), confers the plasticity necessary for sessile organisms to face the changing environment. It also has a practical importance in agriculture, explored since the domestication of vegetatively propagated crops (Harlan,

Abbreviations: BA, benzyl adenine; CIM, callus-inducing medium; 2,4-D, 2,4-dichlorophenoxyacetic acid; GA, gibberellin; MT, Micro-Tom; NAA, naphthaleneacetic acid; PVC, polyvinyl chloride; RIM, root-inducing medium; SIM, shoot-inducing medium.

1992). Early studies in the field of mineral nutrition (White, 1934) and plant hormones (Miller *et al*., 1955) allowed the further sophistication of cloning practices (revised in Vasil, 2008), which were attempted to be extended to almost all agriculturally important crops as a prerequisite for modern genetic manipulations *in vitro*. Despite the importance of flexible plant development for agriculture, its *in vitro* manipulation remains largely empirical, and sometimes unsuccessful, since its molecular basis has only recently started to be unravelled (revised in Duclercq *et al*., 2011).

An important step in the process of formation of novel and ectopic organs is the acquisition of competence of a given cell or tissue to assume a new developmental fate. For instance, plant propagation by stem cuttings, although a rather simple horticultural technique, involves complex changes that culminate in the formation of cells with a new root identity at the base of the shoot cuttings. As proposed by Christianson and Warnick (1988) for *in vitro* propagation, the acquisition of competence precedes the phase of induction of different organs. Upon induction, a given cell or tissue become determined (committed) to form the induced organ, and this last developmental step can be interpreted as opposite to the initial non-committed state of competent cells or tissues (Wareing, 1982). Thanks to the seminal work of Skoog and Miller (1957), it is currently known that the induction of organs, such as roots and shoots, depends on the balance between the plant hormones auxin and cytokinin, rather than their absolute levels. This implies that any given exogenous or endogenous event that alters the levels of these two hormones, or the capacity to respond to them, will probably influence the capacity for organ formation. Thus, it is conceivable that some plants overproducing cytokinin become prone to form shoots (Estruch *et al*., 1991; Peres and Kerbauy, 1999; Catterou *et al*., 2002) and that enhanced auxin sensitivity usually increases root formation capacity (Visser *et al*., 1996; Lima *et al*., 2009). Additionally, it has been shown that the expression of genes controlling cytokinin response or shoot meristem identity, such as *ARABIDOPSIS RESPONSE REGULATOR5* (*ARR5*), *SHOOTMERISTEMLESS* (*STM*), and *WUSCHEL* (*WUS*), correlates with shoot induction, and these may also serve as markers for this event (Cary *et al*., 2002; Gallois *et al*., 2002; Che *et al*., 2006, 2007). However, a similar level of knowledge on the control of the phase of acquisition of competence is not yet available, despite its importance as a key step in organ regeneration (Christianson and Warnick, 1988).

Some transcription factors identified as important molecules in the shoot induction step in *Arabidopsis* (Cary *et al*., 2002; Gallois *et al*., 2002; Che *et al*., 2007) are homeoboxes (e.g. *STM* and *WUS*), whose homologues in maize and tomato were also proposed to regulate the switch from determinate to indeterminate cell fates in the control of developmental processes, such as leaf architecture (Sinha *et al*., 1993). Considering that adventitious organ formation is recognized to be dependent on the presence of indeterminate (non-committed) stem cells (Sugimoto *et al*., 2011), one may hypothesize that the acquisition of competence could be related to the action of homeobox genes, such as *STM* and *WUS*. However, contrary to the early hypothesis of a specific competence for each kind of organ to be formed (see Christianson and Warnick, 1985), there is recent evidence suggesting that the beginning of organogenesis follows a common and general pathway (Atta *et al*., 2009; Sugimoto *et al*., 2010). This pathway is then channelled toward the induction of roots or shoots under the influence of specific hormonal balances (Skoog and Miller, 1957). Thus, it may be that genes controlling competence should have an impact in the capacity to form both roots and shoots, which seems not to be the case for the shoot identity-associated homeobox genes studied thus far (Smith *et al*., 1997; Cary *et al*., 2002; Gallois *et al*., 2002; Che *et al*., 2007).

Moreover, it is generally assumed that the pre-incubation on auxin-rich 'callus-inducing medium' (CIM) helps the acquisition of competence to generate the respective organ in 'shoot-inducing medium' (SIM) or 'root-inducing medium' (RIM) (Christianson and Warnick, 1988; Valvekens *et al*., 1988). This assumption suggests auxin as a possible molecular player in the acquisition of competence, a knowledge that was also used in the identification of the genes *ENHANCER OF SHOOT REGENERATION 1* (*ESR1*), *ARR15*, *POLYGALCTURONASE INHIBITING PROTEIN 2* (*PGIP2*), and *WUS* as requiring CIM pre-incubation for up-regulation on SIM (Banno *et al*., 2001; Che *et al*., 2007). These findings indicate that genes and molecules controlling acquisition of competence may be upstream to the above-mentioned genes in a common cellular signalling pathway prior to the specification of the kind of organ to be formed. Direct approaches to find such genes would be the identification of genes expressed before the commitment to organogenesis (Santos *et al*., 2009) or the screening for genotypes (induced mutants and natural genetic variation) with higher or lower *in vitro* organ formation concomitantly on SIM or RIM. The identification of key genes/molecules controlling the acquisition of competence, at either cellular or organismal levels, is relevant to understand the molecular basis of the plant development plasticity.

In the present work, a collection of tomato (*Solanum lycopersicum* L.) mutants (www.esalq.usp.br/tomato) introgressed into the cv Micro-Tom (MT) genetic background (Scott and Harbaugh, 1989; Meissner *et al*., 1997) were used to look for genetic variation associated with *in vitro* regeneration capacity. The *Regeneration1* (*Rg1*) allele, a natural genetic variation originating from *S. peruvianum* (Koornneef *et al*., 1993), and the gibberellin (GA) constitutive mutant *procera* (*pro*), a loss-of-function in the DELLA-like protein (Bassel *et al*., 2008; Jasinski *et al*., 2008), were characterized as affecting the competence phase, since they alter both shoot and root *in vitro* formation and influence a series of apparently unrelated processes that are linked to the capacity to assume different cell fates.

Materials and methods

Plant material

Tomato (*S. lycopersicum* L.) cv. MT and the near-isogenic lines (NILs) (Table 1) harbouring the alleles *Rg1*, *pro*, and *diageotropica* (*dgt*) were obtained as described in previous studies (Pino *et al*., 2010; Carvalho

Table 1. Tomato genotypes in the cv Micro-Tom background used here

Genotype	Effect/gene function	Origin	Reference
diageotropica (dgt)	Low sensitivity to auxin. Defect in a cyclophilin biosynthesis gene (a putative auxin signal transduction component)	LA1529 cv unknown	Oh *et al*. (2006)
procera (pro)	Constitutive response to gibberellin. Contains a point mutation that convert the VHVID putative DNA-binding domain in the tomato *DELLA* gene to VHEID	LA0565 cv Condine Red	Bassel *et al*. (2008)
lateral suppresser (ls)	No initiation of lateral branches. Mutated in the VHIID domain of a gene from the *GRAS* family, which includes the *DELLA* gene	LA0329 hybrid	Schumacher *et al*. (1999)
Mouse ears (Me)	Highly dissected leaves. Overexpression of a tomato *KNOX* gene (*TKn2/LeT6*)	LA0715 cv unknown	Parnis *et al*. (1997)
DR5::GUS	Plants present enzymatic staining in sites where auxin accumulates. Synthetic auxin-responsive promoter fused to the reporter gene *uid* GUS (encoding a β-glucuronidase)	Micro-Tom	Martí *et al*. (2010)
Regeneration1 (Rg1)	High organ formation capacity in different explants, including roots. Unknown gene function	LA4136 hybrid	Koornneef *et al*. (1993)

et al., 2011). Similarly, the mutants *Mouse ears* (*Me*) and *lateral suppresser* (*ls*) were introgressed into MT as described for the *Rg1* allele (Pino *et al*., 2010), and *pro* and *dgt* mutants into MT (Carvalho *et al*., 2011). MT seeds carrying the *DR5::GUS* gene (Ulmasov *et al*., 1997) were kindly provided by Dr José Luiz Garcia-Martinez from Universidad Politécnica de Valencia, Spain. The crosses and phenotypical screening procedures used to obtain the double mutants *dgtRg1*, *proRg1*, *lsRg1 Mels*, and *Rg1DR5::GUS* were as described previously (Lima *et al*., 2009). All the genotypes used here are maintained in the tomato mutant collection of the Escola Superior de Agricultura 'Luiz de Queiroz' (ESALQ), Universidade de São Paulo (USP), Brazil (http://www.esalq.usp.br/tomato/).

Plant cultivation

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

Grafting and branch analyses

Grafting procedures were conducted as previously described (Peres *et al*., 2005) with some modifications. Briefly, 7-day-old seedlings cultivated in 150 ml pots were used as both scion and rootstock. Scions were prepared by cutting the stem with a razor blade below the second or third leaf from the apex into a wedge shape. Rootstocks were prepared by cutting transversely with a razor blade ~5 cm above soil level, followed by inserting the scion into a 'V'-shaped incision in the stock. A small peg was used to fasten the scion and rootstock together. Grafted plants were then covered with transparent polyethylene terephthalate (PET) bottles to provide a humid environment. After 1 week, the cover and peg were removed and the plants were maintained in the greenhouse as described above. The branching index, the ratio between the total length of lateral ramification and the main axis length (Morris *et al*., 2001), was estimated 33 d after grafting.

In vitro *culture*

Seeds were surface-sterilized by shaking in 100 ml of 30% (v/v) commercial bleach (2.7% sodium hypochloride) plus two drops of commercial detergent for 15 min, followed by three rinses with sterile water. The seeds were then germinated on media containing half-strength MS salts (Murashige and Skoog, 1962) and B5 vitamins (Gamborg *et al*., 1968); 15 g l^{-1} sucrose; and 6 g l^{-1} agar (Merck, Darmstadt, Germany). Medium pH was adjusted to 5.8 before autoclaving. Approximately 40 seeds were sown per flask containing 30 ml of medium. Cultures were sealed with polyvinyl chloride (PVC) and incubated at 25 ± 1 °C in the dark for 4 d, followed by 4 d or 8 d under a 16 h photoperiod provided by a 40 W cool white fluorescent tube (~45 μmol m^{-2} s^{-1} PAR). Cotyledons were then isolated from 8- or 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 SIM, composed of MS salts, B5 vitamins, 30 g l^{-1} sucrose, 6 g l^{-1} agar, and 5 μM 6-benzyl adenine (BA) (Sigma, St Louis, MO, USA), or RIM, which has the same composition as SIM, except that BA is replaced with 0.4 μM naphthalene acetic acid (NAA) (Sigma). The CIM has the same salt, sucrose, and vitamin composition as SIM and RIM, plus 0.5 μM BA and 1.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D). During explanting, a Petri dish containing potassium permanganate salts was kept inside the laminar flow hood to avoid ethylene accumulation, which can reduce tomato regeneration afterwards (Lima *et al*., 2009). Twenty cotyledonary explants were cultured per sterile polystyrene Petri dish (90 × 15 mm), with six plates per treatment. Plates were sealed with PVC and maintained under a 16 h photoperiod at 25 ± 1 °C for 3 weeks.

Histological analysis

For light microscopy analysis, five samples of cotyledons from 8-day-old seedlings grown *in vitro* or three samples of the fourth leaf of 33-day-old plants grown in the greenhouse were collected. Transverse sections (30–60 μm thick) of the base of the petiole were hand cut with a razor blade. Petiole sections were placed in a sodium hypochlorite solution (20%) for bleaching, and then washed with distilled water until total removal of sodium hypochlorite, as evidenced by the loss of the characteristic odour. Petiole sections were stained with 1% aqueous iodine green for 2 min, and then washed with distilled water to remove excess dye. Subsequently, the petiole sections were stained with Congo red for 30 s and then washed twice with distilled water. The petiole sections were then mounted on glass slides, adding a small amount of liquid glycerin gelatin (heated to 40 °C), before covering with a cover slip. Images from petiole sections were digitally captured using a Leica (Leica™, Wetzlar, Germany) DMLB microscope with a camera connected to a computer, and the

IM50 software (Leica™) was used for image analysis. Cotyledons were fixed in Karnovsky solution (Karnovsky, 1965) for 24 h at 8 °C. Dehydration was performed with a graded ethanol series 10–100%, followed by embedding in synthetic 2-hydroxyethylmethacrylate resin (Leica Historesin embedding kit™), according to the manufacturer's recommendations. Sections (5 µm) of cotyledons were obtained on a rotary microtome, and stained with 0.05% toluidine blue in phosphate buffer and citric acid pH 4.5 (Sakai, 1973). Slides were prepared with synthetic permanent resin (Entellan™). The images from cotyledon sections were obtained using a Zeiss Axiophot photomicroscope with a digital camera attached, and analysed by image acquisition software Axiovision 4.6 (Carl Zeiss™, Oberkochen, Germany).

DR5::GUS *reporter assay*

Histochemical *GUS* (β-glucuronidase) staining was performed in *DR5::GUS* transgenic MT and MT-*Rg1*, as described by Jefferson *et al*. (1987). Roots from 14-day-old seedlings or cotyledons from 8-day-old seedlings grown *in vitro* in basal MS medium were used. The cotyledons were incubated on RIM for 1 d before staining. Roots and cotyledons were incubated at 37 °C overnight in GUS staining solution [80 mM sodium phosphate buffer, pH 7.0; 8 mM EDTA; 0.4 mM potassium ferrocyanide; 0.05% Triton X-100; 0.8 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc); 20% methanol]. Following GUS staining, the reaction was stopped with 70% ethanol. The root tips and cotyledons were prepared and observed in a Trinocular Leica DM LB microscope. Representative phenotypes were photographed (Leica DC 300 F) at ×200 magnification.

RNA analysis and semi-quantitative RT-PCR

Briefly, total RNA was isolated from young leaves and shoot apexes using Trizol (Invitrogen). RNA was quantified in an Ultrospec 1100 pro spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA) and RNA integrity was examined by gel electrophoresis. A 1 µg aliquot of DNase I-treated RNA was used to perform cDNA synthesis using ImProm-II Reverse Transcriptase (Promega). Reverse transcription-PCRs (RT-PCRs) were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. Mature microRNA164 (miR164) was detected using stem–loop RT-PCR, a standard technique to quantify miRNAs accurately (Chen *et al*., 2005). Primers used for RT-PCR are described in Supplementary Table S1 available at *JXB* online.

Results

Characterization of Rg1*, a natural genetic variation improving both shoot and root formation* in vitro

Given that *Rg1* was originally characterized as improving shoot formation *in vitro* from root (Koornneef *et al*., 1993) and hypocotyl explants (Lima *et al*., 2004), whether it also improves root formation in adequate medium was tested here. Cotyledonary explants harbouring the *Rg1* allele not only formed significantly more shoots on SIM (Figs 1A, 2A), but also more roots on RIM (Figs 1B, 2B), when compared with explants from the NIL MT. Besides displaying more explants undergoing *de novo* shoot and root formation (Fig. 1), the number of both organs formed per explant also increased in MT-*Rg1* (Fig. 2). As previously reported by Koornneef *et al*. (1993), *Rg1* maintained the capacity for shoot regeneration in SIM after long-term pre-incubation on CIM, a capacity not present in most tomato cultivars (Koornneef *et al*., 1987, 1993), such as the cv MT used here (Fig. 1C).

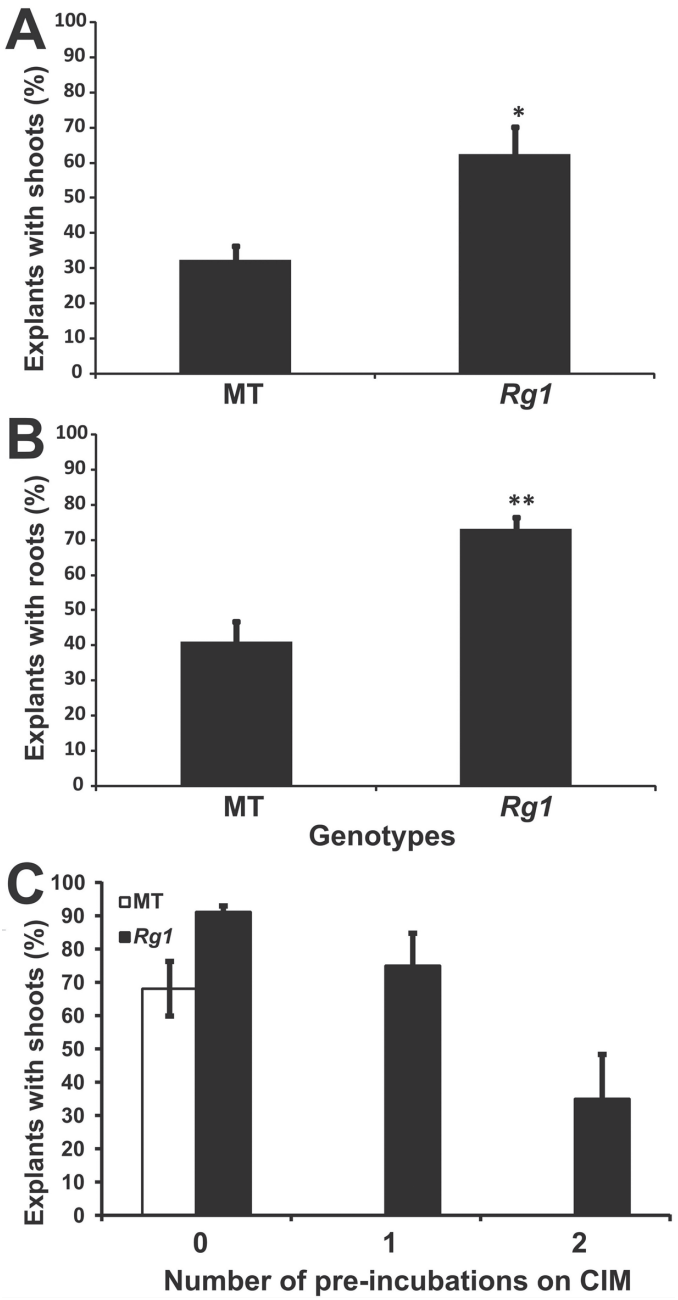

Fig. 1. *In vitro* regeneration ability of Micro-Tom (MT) and MT-*Rg1*. (A) Shoot formation in cotyledonary explants from 12-day-old (after *in vitro* sowing) seedlings cultivated on SIM (5.0 µM BA). (B) Root formation in cotyledonary explants from 12-day-old seedlings cultivated on RIM (0.4 µM NAA). (C) Shoot formation in callus explants cultivated on SIM. Cotyledonary explants from 8-day-old seedlings received no, one, or two pre-incubations of 21 d on CIM (0.5 µM BA+1.0 µM 2,4-D) before transfer to SIM. Error bars represent the mean ± SE, $n = 5$ Petri dishes each containing 20 cotyledons. *$P < 0.05$ and **$P < 0.01$, according to Student's *t*-test. The measurements were taken 10 d and 21 d after explant inoculation on RIM or SIM, respectively.

A phenotypic characterization of plants harbouring the *Rg1* allele was conducted comparing them with the NIL MT. The high *in vitro* shoot and root formation capacity of *Rg1* seemed to parallel an improved shoot (branching) and root

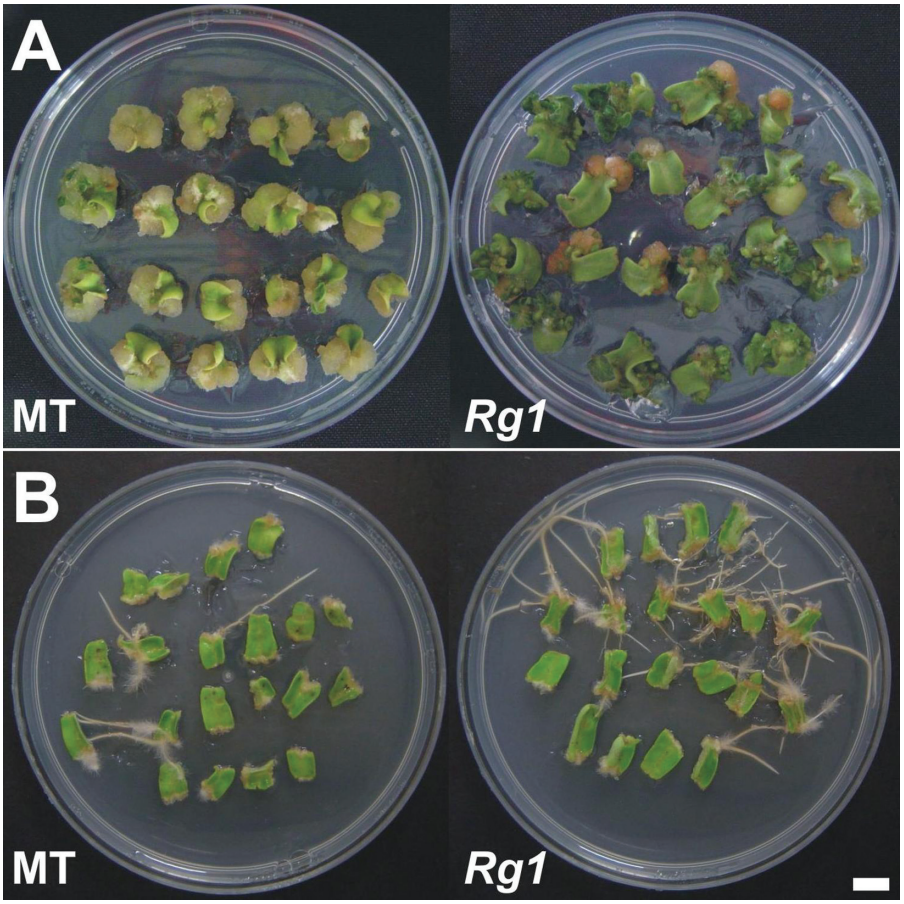

Fig. 2. *In vitro* regeneration of 8-day-old cotyledonary explants of MT (left) and MT-*Rg1* (right) cultivated on SIM (A) or RIM (B). Bar = 1 cm. (This figure is available in colour at *JXB* online.)

system in *Rg1* plants growing in the greenhouse (Fig. 3A, 3C). *Rg1* seedlings also tended to form supernumerary cotyledons (Fig. 3B), with an average number of seedlings forming three or bifurcated cotyledons, statistically different from MT (Table 2). It was also noticed that MT-*Rg1* plants presented thicker petiole bases (Fig. 3D) with a diameter significantly larger than that of MT (Table 2). Histological analysis of MT-*Rg1* petiole bases showed that they presented more parenchymatic cell layers, with smaller cells, when compared with MT (Fig. 3E). Although MT-*Rg1* tended to display increased fresh and dry mass of the roots and shoots, paralleling its capacity to form these organs *in vitro*, it did not differ statistically from MT (Table 2).

In vitro *shoot and root formation capacity of MT single mutants and MT-*Rg1 *double mutants*

Tomato mutants were searched based on their effects on branching, the main *ex vitro* phenotype presented by *Rg1*, and also based on their gene functions as indicative of a possible capacity to control cell fates (Table 1). Mutants (*dgt*, *pro*, *ls*, and *Me*) introgressed into the same MT genetic background were tested for their capacity to form organs in SIM or RIM. Double mutants were then produced, combining the selected mutants with MT-*Rg1*, and tested for their regeneration capacity (Fig. 4).

The *pro* mutant displayed a reduced capacity to form both roots and shoots *in vitro* (Fig. 4A, 4B), whereas *dgt* exhibited a reduced capacity to form roots (Fig. 4B). Although *Me* phenocopied the high shoot formation of *Rg1* on SIM, it failed to increase root formation in RIM (Fig. 4). It is noteworthy that *Rg1* rescued the low organ formation capacity of *pro* in the double mutant *proRg1* and the reduced root formation capacity of *dgt* in *dgtRg1*, appearing as epistatic to both mutations. On the other hand, the presence of *ls* in a double mutant with *Rg1* dramatically reduced the shoot formation capacity, while *ls* alone did not present a reduction in organ formation *in vitro*. This epistatic reduction in shoot formation presented by the double mutant *lsRg1* was not observed in *Mels*.

*Phenotypes of MT single mutants and MT-*Rg1 *double mutants*

Histological analysis of cotyledons from 8-day-old seedlings, which was the tissue used as explant in both RIM and SIM, was performed in both single and double mutants (Fig. 5). The *Rg1* cotyledons had more spongy parenchyma. This high proportion of spongy parenchyma was also present in *Me* cotyledons, which also seem to have increased layers of palisade parenchyma. On the other hand, a clear reduction in the number of spongy parenchyma layers was observed for *dgt* cotyledons. Although the reduced parenchyma proliferation

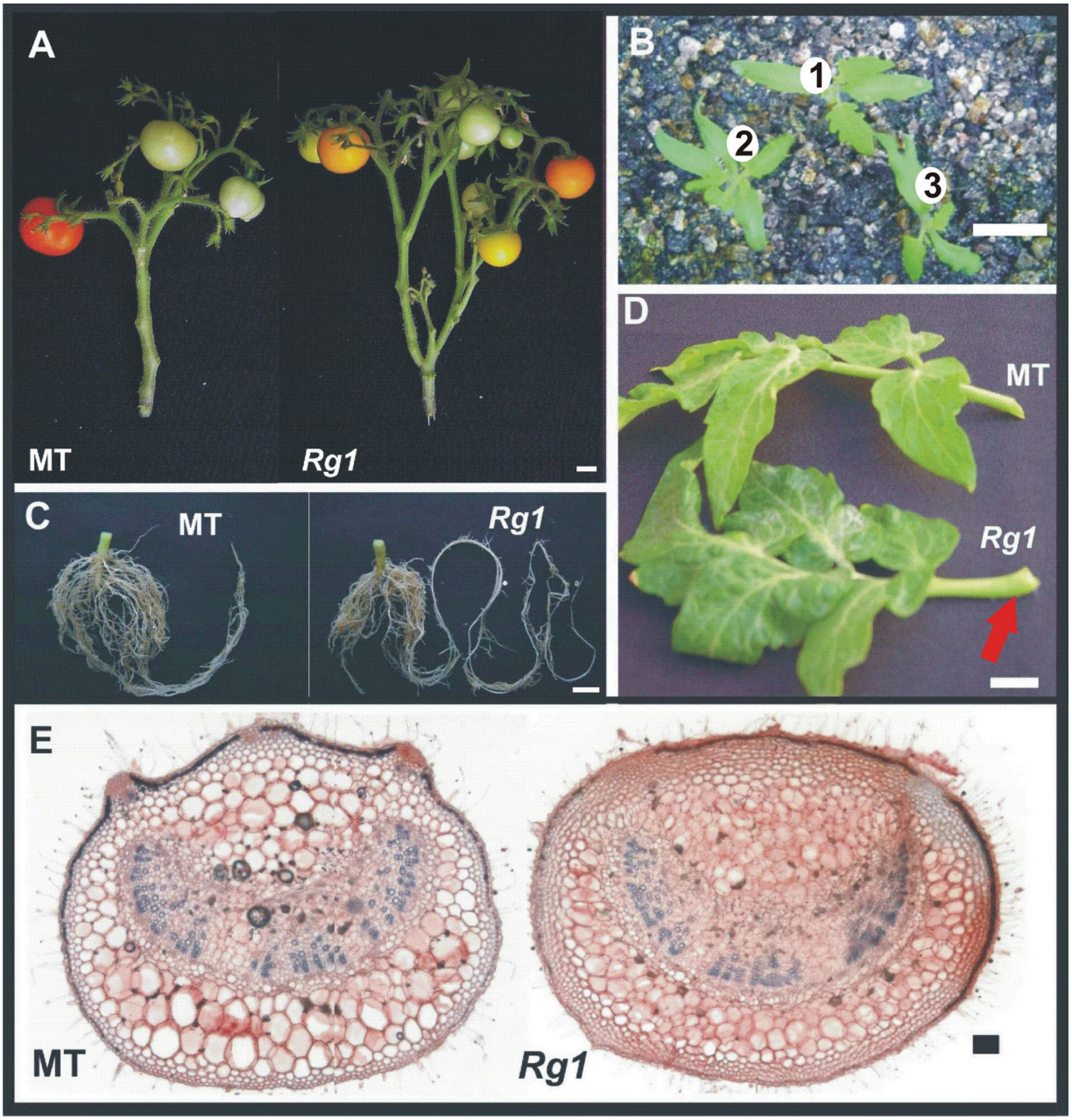

Fig. 3. The MT-*Rg1* phenotype. (A) Branching phenotype of MT-*Rg1*. (B) MT-*Rg1* seedlings showing two (1), three (2), and bifurcated cotyledons (3). (C) Root system of 85-day-old MT and MT-*Rg1* plants. (D) MT and MT-*Rg1* leaves. Note the thicker base of the MT-*Rg1* petiole (arrow). (E) Transversal section of an MT and MT-*Rg1* petiole. Bar = 1 cm (A–D) and 100 μm (E). (This figure is available in colour at *JXB* online.)

in *dgt* cotyledons was not reversed in the double mutant *dgtRg1*, *Rg1* increased the number of parenchyma cells in *pro* and *ls* double mutants. The increased number of parenchyma cells in the double mutant *lsRg1* was not observed in *Mels*, which indeed had fewer parenchyma cells than *Me* (Fig. 5).

Greenhouse-grown double mutant plants (Fig. 6) consistently presented phenotypes confirming the epistatic interactions observed *in vitro*, but also showing some additive *ex vitro* phenotypes. The double *dgtRg1* mutant showed increased root formation, as compared with the single mutant *dgt* (Fig. 6A), as well as a trend for a horizontal orientation in the first lateral roots (compare Fig. 6A with Fig. 3C). The *lsRg1* plants combined the absence of petals, a pleitropic effect of *ls* (Fig. 6B), with axillary bud formation (Fig. 6C) and vigorous branching of *Rg1* (Fig. 6D). Although the vigorous branching of *lsRg1* plants does not parallel the reduction of shoot formation observed *in vitro* for this genotype (Fig. 4A), these results confirm the epistatic interactions between *Rg1* and *ls*.

Although the epistasis of *Rg1* over the auxin low sensitivity mutant *dgt* might suggest that *Rg1* increased auxin sensitivity, it does not seem to be the case, as indicated by the activity of the synthetic auxin promoter *DR5* driving *GUS* expression in root tips (Fig. 6E). On the contrary, the staining of *Rg1* suggests less sensitivity to auxin, which is reinforced

Table 2. *Phenotypical data of MT and MT*-Rg1

Means followed by the same letters are not significantly different according to Student's *t*-test ($P < 0.05$). For cotyledons per seedlings, $n = 4$ trays, each containing 70 seedlings. For germination, $n = 3$ germinating boxes, each containing 50 seeds. For petiole diameter and organ weight, $n = 10$ plants. Shoot and root dry mass were determined on 60-day-old plants ($n = 10$) after drying in an oven at 60 °C.

Cotyledons per seedling (%)	MT	MT-Rg1
Two cotyledons	99.72 ± 0.28 a	90.45 ± 1.59 b
Three cotyledons	0.28 ± 0.28 b	3.71 ± 1.39 a
Bifurcated cotyledons	0.00 ± 0.00 b	5.84 ± 1.77 a
Germination (%)	97.33 ± 1.76 a	96.67 ± 1.76 a
Petiole diameter (mm)	3.54 ± 0.09 b	3.97 ± 0.06 a
Dry and fresh mass (g)		
Root fresh mass	2.81 ± 0.22 a	3.35 ± 0.25 a
Root dry mass	0.26 ± 0.02 a	0.28 ± 0.02 a
Shoot fresh mass	16.29 ± 1.17 a	16.81 ± 0.78 a
Shoot dry mass	1.64 ± 0.16 a	1.75 ± 0.08 a

by the GUS staining of *Rg1* cotyledons incubated on RIM (Supplementary Fig. S1 at *JXB* online). As for the double *proRg1* mutant, it was possible to observe a trend to revert the smooth leaflet margins of *pro* (Fig. 6F; Jasinski *et al*., 2008). However, it should be noted that *Rg1* plants do not display intensely dissected leaves (Fig. 3D), as the *knotted-like* homeobox mutant *Me* does (Fig. 6G).

The double *proRg1* mutant also showed that the presence of the *Rg1* allele was able to reverse the characteristic non-branching phenotype of *pro* plants (Bassel *et al*., 2008), as evidenced in the analysis of the branching index (Fig. 7A). As mentioned, the double *lsRg1* mutant proved that *Rg1* can rescue the lack of axillary meristem in *ls* (Fig. 6C), increasing the branching index (Fig. 7A). Conversely, the high branching phenotype of *Me* was reversed when combined with *ls* (Fig. 7A). While *Rg1* was able to improve the root system of *dgt* (Fig. 6A), its effect was less evident in shoots of the double mutant *dgtRg1*, as the plants showed significantly less branching than *Rg1*, but more branching than *dgt* (Fig. 7A).

One important question is whether the branching phenotype of *Rg1* is grafting transmissible. Reciprocal graftings between *Rg1* and MT were performed. A high branching phenotype was only observed when *Rg1* was the scion (Fig. 7B), suggesting that *Rg1* acts locally, probably modulating the capacity of the tissue to respond to the branching stimulus. This tissue-autonomous effect is consistent with the action of *Rg1* improving *in vitro* shoot and root formation in isolated explants (Fig. 1).

Gene expression analysis of different tomato mutants and their double mutants with Rg1

Semi-quantitative RT-PCR analysis was conducted of genes associated with meristem formation, as well as auxin and GA signalling in *Rg1* and double mutant plants. The aim of this analysis was to confirm, at the transcriptional level, the epistatic interactions observed in the *in vitro* and *ex vitro* phenotypes of the double mutants (Figs 4–7), as well as to gain more insight into the classes of genes that could be differentially regulated in *Rg1*.

One class of regulatory genes analysed was the tomato *Knotted-like* homeobox (*KNOX*) genes *Knotted1* (*TKn1*), *Knotted2* (*TKn2*), and *PETROSELINUM* (*PTS*). In tomato, which is a compound-leaf species, this class of genes is expressed in young leaves (Koltai and Bird, 2000; Kim *et al*., 2003), contrasting with the expression restricted to the shoot meristem in the simple-leaf species *Arabidopsis thaliana* (Lincoln *et al*., 1994; Barton, 2001). In general, *Rg1* presented a slight reduction in transcript accumulation of all *KNOX* genes analysed (*TKn1*, *TKn2*, and *PTS*; Fig. 8A). An increased transcript accumulation of *TKn1* in the *ls* mutant was noticeable, which was reverted in the double *lsRg1* mutant, thus resembling the epistasis presented by *Rg1* over *ls* in the analysis of branching phenotype (Figs 6, 67). The *Me* mutation is a gene fusion that causes the overexpression of *TKn2* (Chen *et al*., 1997; Parnis *et al*., 1997), and leaf tissues of *Me* plants consistently presented high transcript accumulation of this gene, when compared with the other genetic backgrounds, as expected (Fig. 8A).

For auxin-related genes, there was an increase in transcript accumulation of *SlPIN4* in the double *dgtRg1* mutant, when compared with *dgt* (Fig. 8B), suggesting that this gene, which is the most highly expressed PIN gene in tomato (Pattison and Catalá, 2012), may be one of the components of the epistatic interaction seen *in vitro* and *ex vitro* between *dgt* and *Rg1* (Figs 4–7). On the other hand, the low transcript accumulation of *IAA11* in *dgtRg1* when compared with *Rg1* and MT indicated an epistatic effect of *dgt*, which presented a low transcript accumulation of this gene, as previously reported (Nebenfuhr *et al*., 2000).

The same type of epistatic interaction at the transcript accumulation level was also observed for the GA pathway-associated gene *GAMyb-like1* (Fig. 8C). High *GAMyb-like1* transcript levels in leaf tissues of the GA-constitutive mutant *pro* (Fig. 8C) (see Bassel *et al*., 2008) was no longer observed in the double mutant *proRg1*, which correlated well with the epistasis of *Rg1* over *pro* observed *in vitro* and *ex vitro* (Figs 4–7). No obvious alterations in transcript accumulation of the miR164 and its target gene the NAC-domain *GOBLET* (*GOB*; Berger *et al*., 2009) were observed in *Rg1* vegetative apexes, when compared with MT (Fig. 8D).

Discussion

Organogenic capacity is not necessarily linked to alterations of hormonal status

In the present work, the characterization of the natural genetic variation *Rg1* was extended by demonstrating its additional capacity to improve root formation *in vitro*, besides shoot formation. This allele is also able to reverse the low levels of *in vitro* root and shoot formation in the *DELLA* mutant *pro*, as well as the low *in vitro* root formation of the cyclophilin mutant *dgt*. These results may suggest increased auxin and reduced GA signalling in *Rg1* to overcome the low auxin sensitivity of *dgt* and the constitutive GA signalling in *pro*, respectively. However, analysis of transcript accumulation from key

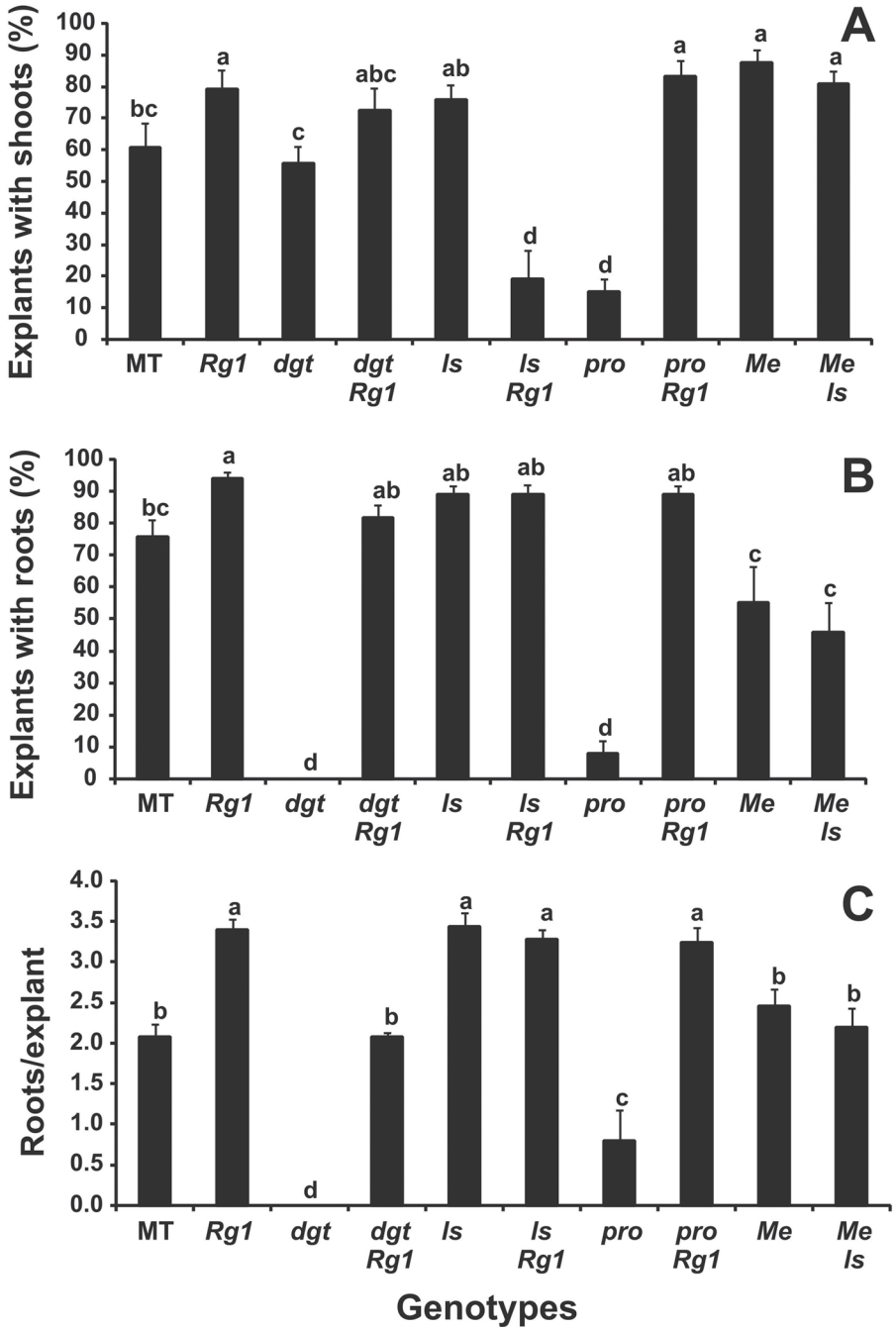

Fig. 4. Shoot (A) and root (B) regeneration *in vitro*, as well as the number of roots formed (C) in cotyledonary explants of single and double mutants. The explants were obtained from 8-day-old seedlings. SIM and RIM were used for shoot and root induction, respectively. Measurements were taken 21 d (A) and 8 d (B and C) after explant inoculation. Error bars represent the mean ±SE, $n = 6$ Petri dishes each containing 20 explants. Different letters indicate significant differences at $P \leq 0.01$ (unpaired Student's *t*-test). The mutants utilized are described in Table 1.

genes associated with the auxin signalling pathway (Fig. 8B), combined with the analysis of the activity of the synthetic *DR5* auxin response promoter (Fig. 6E; Supplementary Fig. S1 at *JXB* online), did not support an increase in auxin sensitivity in *Rg1*. Moreover, tomato mutants with increased auxin sensitivity would present a reduced complexity of leaf architecture (e.g. leaves with fewer leaflets and less dentated leaflets), as has been described for the *AUX/IAA9* tomato mutant *entire* (Wang *et al*., 2005; Zhang *et al*., 2007). This seems not to be the case for *Rg1* leaves (Fig. 3D), despite the fact that the RT-PCR analysis suggested that *Rg1* is expressed in leaves, since it affects the expression of a number of genes in young leaves (Fig. 8A–C). Similarly, a reduction in GA sensitivity in *Rg1* would result in plant dwarfism and low seed germination, as described for various GA-deficient tomato mutants at different intensities (Koornneef *et al*., 1990). Neither a reduction in seed germination (Table 2) nor dwarfism were observed in *Rg1* (Fig. 3A), which is consistent with the lack

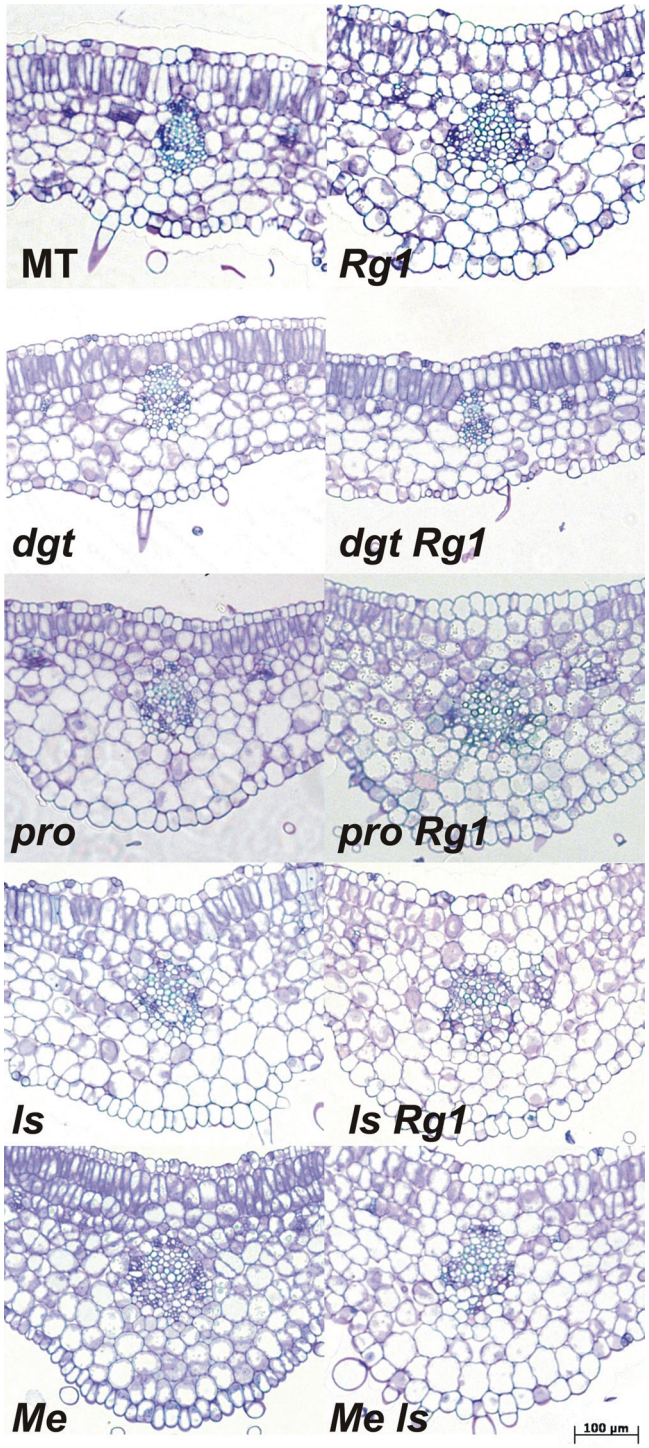

Fig. 5. Transversal sections of 8-day-old cotyledons of single and double mutants. Note the presence of the middle vein (vascular bundle) and part of the mesophyll constituted by palisade and spongy parenchyma in the upper (adaxial) and lower (abaxial) sides, respectively. Bar = 100 µm.

of alteration in levels of *GAMyb-like1* transcripts in MT-*Rg1* when compared with MT (Fig. 8C). Although the possibility that the *Rg1* locus affects distinct auxin- and GA-related pathways not tested here cannot be ruled out, *Rg1* is most probably not affecting hormonal homeostasis, but rather developmental processes also affected by the auxin and GA signalling pathway.

The relationship between in vitro *organogenic capacity and shoot branching*

A remarkable unexpected result was that the *Rg1* allele was able to reverse the absence of axillary meristems in the *ls* mutant, rescuing the non-branching phenotype. There is considerable evidence that the flux of auxin from lateral buds, which can be influenced by cytokinins and the novel hormone strigolactone, controls branching (Leyser, 2009). The role of GA in controlling branching is also evident (Fig. 7A; Bassel *et al*., 2008), although less considered in the current signalling pathway. Additionally, before bud outgrowth, the presence of pre-existing axillary meristems and lateral shoot primordia is required, and these are promoted by the GRAS protein LATERAL SUPPRESSER (LS). This predicts that the loss-of-function *ls* mutant is probably epistatic to most mutations affecting the main components controlling lateral bud outgrowth. In this context, *Rg1*, which is epistatic to *ls*, should be situated at least at the same genetic hierarchical level as *LS*, acting locally to promote the presence of cells capable of undergoing the formation of axillary meristem and bud primordium. This is consistent with the fact that *Rg1* is not graft transmissible (Fig. 7B), and that *Rg1* is also epistatic to the non-branching phenotype of the GA-constitutive mutant *pro*.

Competence as the capacity to improve different types of organs in vitro *and* ex vitro

The fact that *Rg1* can be epistatic to distinct and apparently unrelated non-branching mutants, together with its capacity to improve different types of organs *in vitro*, indicate thats *Rg1* is controlling a common requirement necessary for all these processes. This requirement would probably be the production of competent cells able to undergo further differentiation and to form roots and shoots *in vitro*, as well as axillary meristems *ex vitro*. The present results, together with both classical (Bonnet and Torrey, 1966) and recent studies (Atta *et al*., 2009; Sugimoto *et al*., 2010) suggesting a common initial developmental pathway for different organs, led to the reinterpretation of the Christianson and Warnick (1988) model, adding that the stage of 'acquisition of competence' is probably a general process, necessary for both shoot and root formation. This initial common competence would contrast with the stage of 'induction', which requires specific auxin-to-cytokinin balance (Skoog and Miller, 1957), leading to subsequent 'determination' to form shoots or roots, but not both organs. Based on these concepts, it is proposed here that *Rg1* and *pro* mutants are probably positive and negative regulators, respectively, of the stage of 'acquisition of competence', since they have a large influence on both root and shoot formation. On the other hand, the specific high shoot formation in *Me* (Fig. 4A) and the low root formation in *dgt* (Fig. 4B) suggest that these mutants are affecting the stage of 'induction'

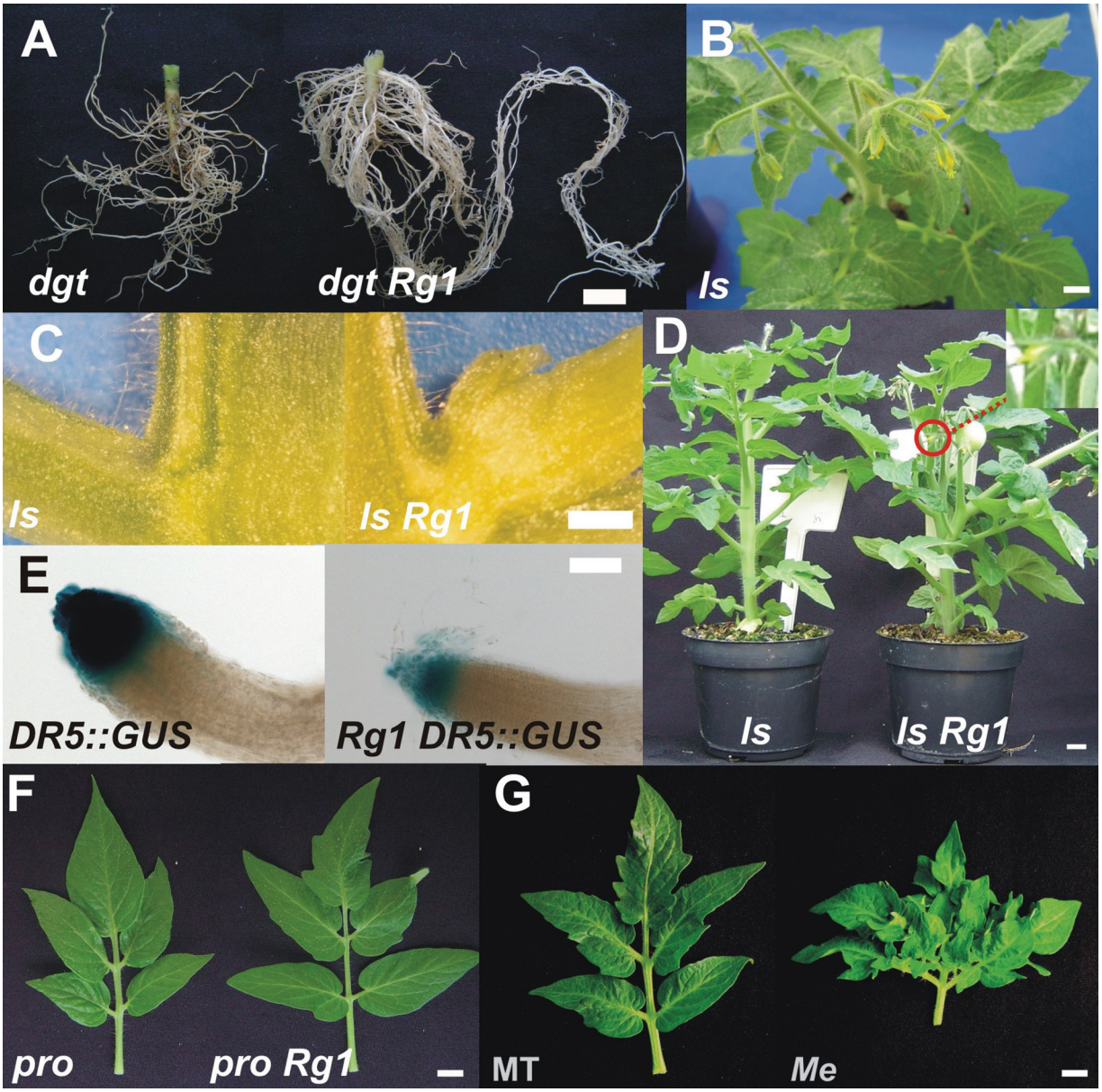

Fig. 6. Phenotype of mutants and double mutants. (A) Increased root formation and growth in the double mutant *dgtRg1*, as compared with *dgt*. (B) Absence of petals in *ls* flowers. (C) Lack of axillary bud formation in *ls* and the reversion of this phenotype in the double mutant *lsRg1*. (D) Branching formation in the double *lsRg1* mutant (right) and its absence in *ls* (left). Note that the double *lsRg1* mutant combines branch outgrowth with flowers lacking the corolla (inset). (E) GUS staining of *DR5::GUS* root tips in the MT (left) and the *Rg1* (right) background. (F) Increased lobing in leaflets of the double *proRg1* mutant (right), as compared with *pro* (left). (G) Aspect of an *Me* leaf showing high dissection. Bar = 1 cm (A, B, D, F, and G), 5 mm (C), and 1 mm (E). (This figure is available in colour at *JXB* online.)

of roots and shoots (Fig. 9). It is reasonable to assume that the low sensitivity of *dgt* to auxin (Kelly and Bradford, 1986), and the known effect of *KNOX* genes, such as *Me*, improving cytokinin levels (Hay *et al*., 2004), probably represent disturbances in the auxin-to-cytokinin balance, or the endogenous response to these hormones, disfavouring root induction in *dgt* and favouring shoot induction in *Me* (Fig. 9). Different from *Me* and *dgt*, *Rg1* seems to be controlling competence to form both shoots and roots, instead of the specific induction of these organs. If so, *Rg1* should not present conspicuous alterations in the auxin-to-cytokinin balance, which, otherwise, would improve the induction of one type of organ to the detriment of the other (Skoog and Miller, 1957; Li *et al*., 1992). This is supported by the evidence presented here that *Rg1* has no significant alterations in auxin, together with hormonal measurements made by Boiten *et al*. (2004), which showed that *Rg1* does not confer an increase in the endogenous levels of cytokinin. Moreover, it appears that the high competence of *Rg1* is able not only to revert the lack of competence of *pro*, consequently improving both root and shoot formation in the double mutant *proRg1*, but also to compensate the poor root induction in *dgt*, improving root formation in the double mutant *dgtRg1*.

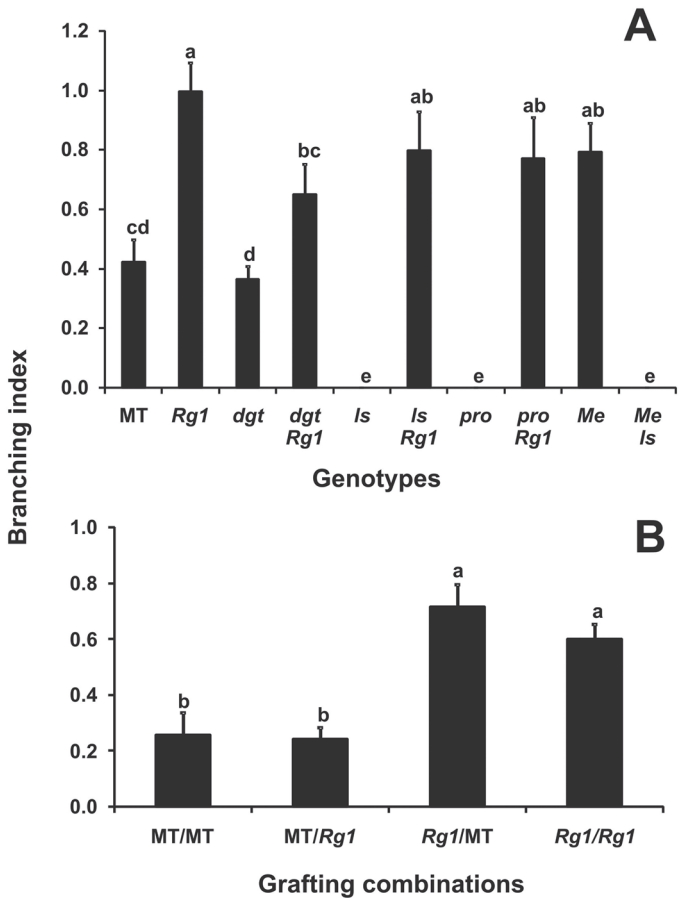

Fig. 7. Branching phenotype of MT-*Rg1* and double mutants. (A) Branching index of single and double mutants measured in 80-day-old plants. The mutants utilized are described in Table 1. (B) Branching index of reciprocal grafting measured in 50-day-old plants. Genotypes are expressed as scion/rootstock. Error bars represent the mean ±SE, $n = 7$ (A) or $n = 12$ (B) plants. Different letters indicate significant differences at $P \leq 0.01$ (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).

The more determined the less competent

One important corollary of the model proposed by Christianson and Warnick (1988) is that determination can also be interpreted as opposite to competence, since an explant highly committed ('determined') to a particular developmental pathway (Tran Thanh Van, 1973) will probably be more recalcitrant ('non-competent') to assume a different fate. These considerations may help further elucidation of the molecular basis of competence, since genes positively affecting this process, such as *Rg1*, may be arresting the specification of cell fates, or maintaining the population of indeterminate cells (stem cells) in a given explant (Sugimoto *et al.*, 2011). It is noteworthy that *Rg1* was initially selected by its capacity to maintain shoot regeneration after long-term *in vitro* callus culture, which is lost much more quickly for other tomato genotypes under the same circumstances (Koornneef *et al.*, 1987). This behaviour of *Rg1* was also confirmed here by comparing the shoot formation capacity of MT and MT-*Rg1* calli induced on CIM and then transferred to SIM (Fig. 1C). Similarly, in a previous study, it had been shown that the capacity for shoot formation in cotyledon explants of MT decreases proportionally with age, but it is maintained in the near isogenic *Rg1* line (see fig. 2B in Pino *et al.*, 2010). *Rg1* also presented an increased number of cells in transversal sections of petioles and cotyledons (Fig. 5). However, the higher regeneration capacity does not seem to be linked to this higher number of cells phenotype, since the double mutant *dgtRg1*, which presented enhanced regenerations of both roots and shoots, displays a reduced number of cells in the explants. Further, despite the fact that it is being considered here that *Me* is only affecting the phase of induction of shoots, but not the competence to form both roots and shoots, it presented an enhanced number of cells similar to that observed in *Rg1*.

The genetic basis of organogenic competence

Among the genes that maintain indeterminate cell fates are specific members of homeoboxes, such as the maize *KNOTTED1* (Sinha *et al.*, 1993). However, if *KNOTTED-like* genes in general can improve the capacity to assume different developmental fates, it would be expected that an improved root formation on RIM in the *TKn2* overexpresser mutant *Me* studied here would be obtained, which was not the case (Fig. 4B). Considering the model plant *Arabidopsis*, another important requisite of a gene controlling competence is probably the capacity to be expressed in roots, since these organs are the preferred source for explants (Valvekens *et al.*, 1988). Cultivated tomato does not exhibit the capacity to regenerate shoots from root explants (Koornneef *et al.*, 1993; Peres *et al.*, 2001), but this capacity is gained by the presence of the *Rg1* allele (Koornneef *et al.*, 1993; Lima *et al.*, 2004). When tomato cotyledons or true leaves are used as explants, an initial down-regulation of shoot-specific homeobox genes might be necessary. This may explain the observed reduction of transcript accumulation of *TKn1* and, to a lesser extent, *TKn2* in *Rg1* young leaves (Fig. 8A), which are tomato explants with the same regeneration capacity as cotyledons (Kut and Evans, 1982). Conversely, a surprising result was the observed high transcript accumulation of *TKn1* in the mutant *ls*. It is noteworthy that *ls* presented a high *in vitro* formation of shoots from cotyledon explants (Fig. 4A), which suggests that the developmental programme controlling *ex vitro* axillary meristem formation is distinct from that controlling *in vitro* adventitious meristem formation. Whether the homeobox *TKn1* is relevant for such developmental programmes remains to be determined. Curiously, in *Arabidopsis*, elevated expression of *KNAT1*, homologous to *TKn1* (Hay ande Tsiantis, 2010), alters leaf architecture (Chuck *et al.*, 1996), a phenotype not observed in the *ls* mutant.

In summary, the characterization of tomato mutants indicated that the acquisition of competence for organogenesis is a common pathway controlled by genes that are most probably upstream to those controlling the process of induction of specific organs. In the case of shoot induction in *Arabidopsis*,

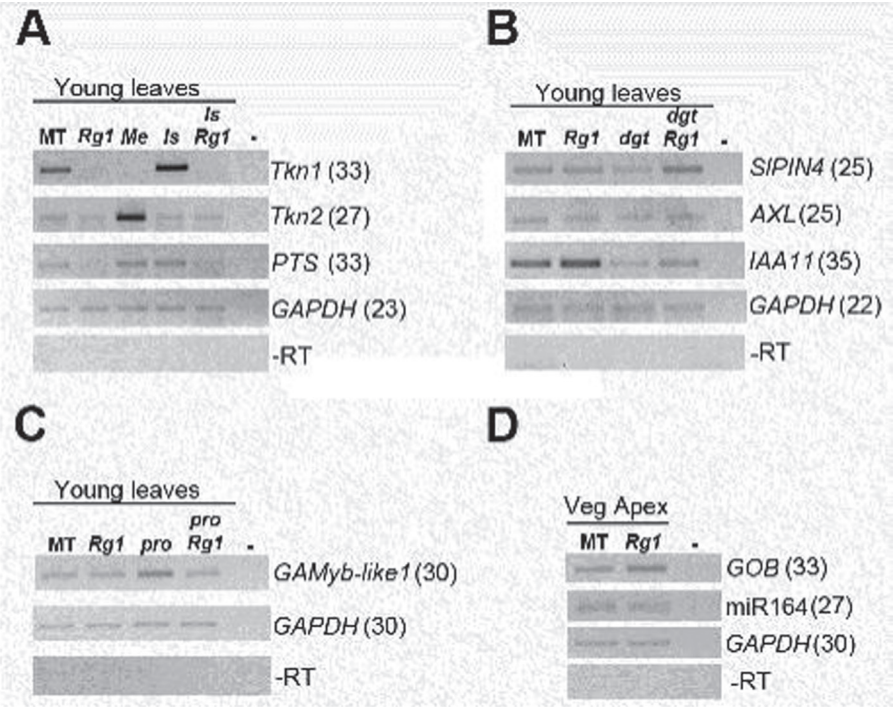

Fig. 8. Expression pattern of genes associated with meristem formation as well as auxin and gibberellin signalling in tomato cv Micro-Tom by semi-quantitative RT-PCR using GAPDH as the reference gene. (A) RT-PCR of tomato *Knotted1* (*TKn1*), tomato *Knotted2* (*TKn2*), and *PETROSELINUM* (*PTS*) in young leaves of the given genetic backgrounds: Micro-Tom, MT; *Rg1*; *Mouse ears*, *Me*; *lateral suppresser*, *ls*, and the *lsRg1* double mutant. (B) RT-PCR analysis of the auxin pathway-associated genes *S. lycopersicum PIN-FORMED4* (*SlPIN4*), *AXR1-like* (*AXL*), and *AUX/IAA11* (*IAA11*) in young leaves of MT, *Rg1*, *diageotropica* (*dgt*), and the *dgtRg1* double mutant. (C) RT-PCR analysis of the gibberellin pathway-associated gene *GAMyb-like1* in young leaves of MT, *Rg1*, *procera* (*pro*), and the *proRg1* double mutant. (D) Stem-loop RT-PCR analysis of the *microRNA164* (*miR164*) and its target the NAC-domain *GOBLET* (*GOB*) in MT and *Rg1* vegetative apexes. Tomato *GAPDH* was used as the reference gene. PCRs without reverse transcriptase (RT–) or without cDNA (–) are shown as negative controls. Numbers in parentheses represent PCR cycles for each amplicon. Two biological replicates were used for each genetic background with at least two technical PCR replicates.

there are indications that some of the downstream genes may be the *ENHANCER OF SHOOT REGENERATION 1* (*ESR1*), *ARR15*, *POLYGALCTURONASE INHIBITING PROTEIN 2* (*PGIP2*) (Banno *et al*., 2001; Che *et al*., 2007) and the homeobox genes *WUS* and *STM* (Cary *et al*., 2002; Gallois *et al*., 2002; Che *et al*., 2007), whose expression usually occurs after transfer to SIM. Currently, very few genes are known to act upstream of the aforementioned genes in the control of organ formation. However, a recent study (Motte *et al*., 2011) demonstrated that *CUP SHAPED COTYLEDON2* (*CUC2*), which is expressed upstream to the homeoboxes *WUS* and *STM* during shoot induction in cultured root explants (Gordon *et al*., 2007), marks the site of both shoot and lateral root primordium formation in *Arabidopsis*. It is noteworthy that the tomato mutant *Goblet-4d*, which has a gain of function in the *CUC2* gene (Berger *et al*., 2009), presents a high frequency of supernumerary cotyledons, a phenotype also shown by *Rg1* and other mutants affecting *in vitro* regeneration capacity (Chaudhury *et al*., 1993; Chandler, 2008). Therefore, it is possible that the expression of *CUC2* during acquisition of competence is necessary for further action of the *homeobox* genes in the determination of shoot meristems. The present data suggested that the action of the DELLA protein PROCERA is also required during acquisition of competence, which is consistent with previous studies in *Arabidopsis* (Ezura and Harberd, 1995) showing that reduced levels or sensitivity to GA, implying that the DELLA proteins are active as a growth repressor (Harberd *et al*., 2009), are associated with enhanced *in vitro* organ formation. It is interesting to note that PROCERA was also considered as modulating competence to respond to KNOX-dependent signals that direct leaflet formation (Jasinski *et al*., 2008). Besides *PROCERA* and the NAC transcription factor *CUC2*, there would be more genes controlling competence. One of those key genes is likely to be *RG1*, whose future cloning and identity will provide more insights into this developmental pathway, and the understanding of the intriguing capacity of plants to form organs during their life cycle.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. GUS staining of *DR5::GUS* cotyledons in MT (left) and *Rg1* genetic background.

Table S1. Primers used in the RT-PCR analysis.

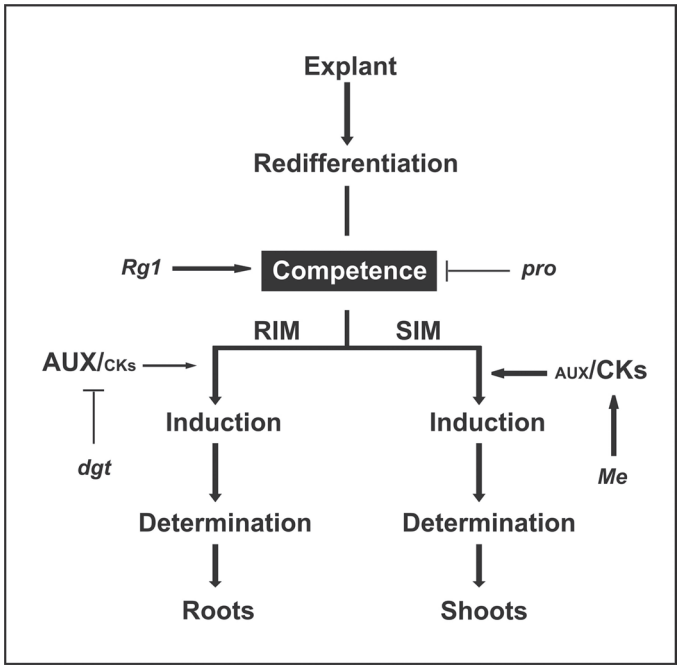

Fig. 9. A working hypothesis for the contribution of the mutations studied here (see Fig. 4) in the proposed phases controlling *in vitro* regeneration (Christianson and Warnick, 1988). The *pro* mutant and the *Rg1* allele have negative and positive effects, respectively, in the phase of acquisition of competence, which are reflected in their capacity to form both root and shoot. The *dgt* mutant probably affects the induction phase, since its low auxin sensitivity represents an altered response to the auxin-to-cytokinin balance (AUX/CKs) necessary for root induction. The known effect of KNOX genes (represented here by the *Me* mutant) increasing cytokinin (Hay *et al*., 2004) may cause an indirect effect, through alterations in the auxin-to-cytokinin balance or a direct effect on the shoot induction phase.

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