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Research Article

Approaching the genetic dissection of indirect adventitious organogenesis process in tomato explants

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ABSTRACT

The screening of 862 T-DNA lines was carried out to approach the genetic dissection of indirect adventitious organogenesis in tomato. Several mutants defective in different phases of adventitious organogenesis, namely callus growth (*tdc-1*), bud differentiation (*tdb-1*, *-2*, *-3*) and shoot-bud development (*tds-1*) were identified and characterized. The alteration of the *TDC-1* gene blocked callus proliferation depending on the composition of growth regulators in the culture medium. Calli from *tds-1* explants differentiated buds but did not develop normal shoots. Histological analysis showed that their abnormal development is due to failure in the organization of normal adventitious shoot meristems. Interestingly, *tdc-1* and *tds-1* mutant plants were indistinguishable from WT ones, indicating that the respective altered genes play specific roles in cell proliferation from explant cut zones (*TDC-1* gene) or in the organization of adventitious shoot meristems (*TDS-1* gene). Unlike the previous, plants of the three mutants defective in the differentiation of adventitious shoot-buds (*tdb-1*, *-2*, *-3*) showed multiple changes in vegetative and reproductive traits. Cosegregation analyses revealed the existence of an association between the phenotype of the *tdb-3* mutant and a T-DNA insert, which led to the discovery that the *SlMAPKKK17* gene is involved in the shoot-bud differentiation process.

1. Introduction

The ability to induce morphogenesis *in vitro* is the basis of all applications arising from plant tissue culture techniques in micropropagation and genetic improvement. For this reason, gaining new insights into how explant cells acquire competence and which genes determine the morphogenetic response *in vitro* is essential from both a basic and applied point of view.

The underlying causes determining the acquisition of competence in explant cells have been discussed over the years. In the traditional view, cells were thought to dedifferentiate and acquire competence when they divide to form a callus [1], so that *de novo* organogenesis would represent a reprogramming process from this partially undifferentiated state. Thus, based on the temporal requirement of explants for a specific balance of phytohormones, Christianson and Warnick [2–4] divided the organogenetic process into three phases. In the first, cells experience a dedifferentiation process and acquire competence to respond to hormone signals, which results in the formation of a mass of undifferentiated cells (*i.e.* a callus). In the second the competent cells are committed or determined for specific organ development by inductive signals according to the hormonal composition in the culture medium. During the third phase, the morphogenesis proceeds independently of the exogenously supplied phytohormones. Recent findings in *Arabidopsis thaliana* changed the perception in the acquisition of competence for callus growth and organogenesis *in vitro*. By fusing the coding regions of reporters (GUS, GFP) to promoters of selected developmental genes, or to

Abbreviations: IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, α-Naphthaleneacetic acid; K, Kinetin (6-furfurylaminopurine); Z, trans-zeatin; BAP, 6-benzylaminopurine; TDZ, Thidiazuron; MS, Murashige and Skoog (1962) mineral solution.

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promoters of genes encoding indicators of the auxin response or transport, cytokinin response or synthesis, or mitotic activity, Atta et al. [5] showed that regenerated shoots from root and hypocotyl explants of *Arabidopsis* originated directly or indirectly from pericycle or pericycle-like cells adjacent to xylem poles. In addition, shoot regeneration appeared to be partly similar to the formation of lateral root meristems. Sugimoto et al. [6] subsequently demonstrated that the initial process of callus formation resembles the tip of a root meristem, not only in roots but also in explants derived from aerial organs such as cotyledons and petals. Also, by analyzing the expression pattern of a pericycle specific marker they found the signal to be enriched around the midvein and vasculature of aerial organs such as cotyledon and leaf. Taken together, these results indicate that pre-existing pericycle-like cells in *Arabidopsis* explants may behave like stem cells as they show an intrinsic capacity for proliferation and regeneration. Despite the significance of these findings some questions need to be clarified. Assuming that in eudicotyledonous species other than *Arabidopsis* pericycle-like cells are also present in aerial organs, we still need to know if differences in the number and distribution of this particular cell type can explain the variability of the morphogenetic response of different species, or genotypes and explants of a given species. Also, it remains unclear how pericycle-like cells, or some other cell types which may behave like stem cells, perceive and respond to hormone signals.

As far as the genetic basis is concerned, it is well known that morphogenetic potential *in vitro* is highly dependent on the genotype [7, 8]. Unfortunately, many relevant genotypes are recalcitrant or display a low morphogenetic response, thus limiting the use of tissue culture techniques for breeding purposes. The habitual solution is to carry out a trial and error-based approach in order to search for an explant with competent cells, adjust the auxin to cytokinin ratio [9] as well as other growth regulators and several components of the culture medium, and discover an adequate combination of cultural conditions. An alternative approach would consist of transferring the genes determining high morphogenetic response into elite genotypes which despite their agronomic interest show a low response *in vitro*. This would not be an easy task if the morphogenetic response were controlled by many genes with minor effects (*i.e.* a polygenic trait). However, if it were controlled by only a few genes with major effects (*i.e.* an oligogenic trait), the transfer of those genes could be addressed through conventional backcrossing or, provided that molecular markers were previously identified, through marker-assisted backcrossing programs (MABC). In this respect accumulating evidence points toward the notion that, from a genetic point view, morphogenesis *in vitro* may be considered an oligogenic trait since, irrespective of the great number of intervening genes, it is possible to detect a few genes with major effects [10–13].

Regarding adventitious organogenesis in *Solanum* species, a genetic analysis of several cell culture traits in the progeny of a cross between a low responsive cultivar from tomato (*Solanum lycopersicum*) and a high responsive accession of the wild related species *Solanum peruvianum* showed that regeneration capacity from explants was apparently controlled by two dominant genes [7,14]. Competence for leaf disc regeneration was also studied in the parental, F_1, and F_2 generations of selected genotypes of *Solanum phureja*. Leaf regeneration data could be explained by a two-gene model with recessive alleles at each locus required for the highest response, a dominant allele at either of the loci resulting in a marginal response, and dominant alleles at both loci resulting in no response [15]. Using a similar experimental design, the genetic control of *in vitro* shoot regeneration from leaf explants in *Solanum chacoense* was analyzed [16]. A hypothesis involving three genes was formulated to explain the variability in the organogenetic response. This model implies that homozygous recessive alleles at any two out of three *loci* are required for the highest response, the presence of homozygous recessive alleles at any one of the three *loci* produces an intermediate response, and a dominant allele at all the three *loci* results in non-responsiveness [16]. Overall results from genetic analysis show that the ability to regenerate plants through adventitious organogenesis mainly depends on a few major genes controlling the trait. Such a relatively simple genetic control suggests that the genes controlling *in vitro* plant regeneration could be easily transferred into economically important cultivars with recalcitrance or low response. In the case of the tomato, the transference of genes controlling organogenetic response has been mainly approached through interspecific hybridization with wild related species. Thus, following a backcrossing program with a *Solanum peruvianum* accession, a tomato genotype (MsK) readily accessible to genetic manipulation through *Agrobacterium* leaf disc transformation and direct gene transfer in protoplast-derived calli was selected [7,14]. Subsequently, after six rounds of backcrossing, the genes conferring high organogenetic competence from the MsK genotype were transferred to the rapid life cycle plant Micro-Tom [17]. Introgression of regeneration capability in tomato has also been achieved using as donors selected accessions of *Solanum pimpinellifolium* [18] and *S. pennellii* [19].

The identification of the sequence of genes determining high morphogenetic response would open new perspectives in basic and applied research. From a practical point of view, once identified, they could be efficiently transferred and expressed in elite genotypes in order to enhance their morphogenetic response. Alternatively, their proper or neighboring sequences could be used as markers in MABC programs. From a basic point of view, the knowledge of the sequence of those genes would shed light on the nature of the molecular changes underlying the differences in the morphogenetic response conferred by different alleles of a given gene or their respective orthologs in related or unrelated species. Furthermore, by using the new gene-editing techniques, such as CRISPR-Cas9 [20,21], it would be possible to gain significant insights into the effects of specific changes in the sequence of those genes.

As regards tomato and related wild species, studies with a set of markers defining all 12 tomato chromosomes allowed to map a dominant *S. peruvianurn* allele at a locus (named *Rg-1*) near the middle of chromosome 3, determining efficient shoot regeneration on root explants in combination with dominant alleles at one or two other loci of either the wild or cultivated species [11]. A second major dominant gene from *Solanum chilense*, which was designated as *Rg-2*, has also been located on chromosome 3 [10]. More recently, the analysis of two mapping populations derived from a tomato cultivar with a low regeneration ability and a high regeneration accession of *Solanum pennellii* (PE-47), allowed the identification of six QTLs on chromosomes 1, 3, 4, 7 and 8. In particular, a putative allele of *Rg-2* (named *Rg-3*) was detected in a QTL on chromosome 3 [13]. Despite these interesting results, the sequences of the *Rg-1*, *Rg2* and *Rg-3* genes are still to be determined and therefore it remains unclear whether they represent different loci or different alleles of the same gene. On the other hand, based on its expression pattern, it has been proposed that *LESK1*, a gene encoding a putative serine/threonine kinase could be used as marker of the competence phase in the regeneration process from hypocotyl and cotyledon explants of tomato [22,23]. *LESK1* was also positioned on chromosome 3 but it is not located in the *Rg-3* QTL [13]. It remains to be determined whether *LESK1* corresponds to *Rg-1*.

We are using a mutant based-approach to address the genetic dissection of adventitious organogenesis in the tomato as, in our opinion, the finding of mutants impaired in explant regeneration may be the most adequate way for the identification of key genes involved in this process. Following the screening of a collection of T-DNA lines generated with an enhancer trap, five tomato mutants have been found with alterations at different steps of the organogenetic process, namely cell proliferation, bud differentiation, and shoot-bud development. Furthermore, taking advantage of the co-segregation between a T-DNA insert and the phenotype of mutant *tdb-3*, we discovered that Sl*MAPKKK17* gene plays an important role in adventitious shoot-bud differentiation in tomato explants.

2. Material and methods

2.1. Screening of T-DNA lines and genetic analysis of tomato mutants defective in adventitious organogenesis

A collection of tomato T-DNA lines was generated in our laboratory by *Agrobacterium*-mediated transformation following the protocol previously developed [24,25]. The enhancer trap vector used for transformation was pD991 (kindly supplied by Dr Thomas Jack; Department of Biological Sciences, Dartmouth College, USA), which has been previously described [26]. This vector contains the selection marker gene *neomycin phosphotransferase II* (*nptII*) at the 3′ end of the T-DNA, controlled by the 5′ mannopine synthase (*mas*) promoter and the 3′ *mas* terminator. The scrutiny of tomato (cv. Moneymaker) T-DNA lines was performed in T0 progenies (*i.e.* T1 plants). Seeds were surface sterilized by immersion in 12.5 % commercial bleach (a sodium hypochlorite solution equivalent to 50 g L^{-1} of active chlorine) for 20 min followed by three rinses with sterile distilled water, and sown aseptically on seed germination medium (SGM) consisting of MS salt solution [27] supplemented with 1 % sucrose and 0.8 % Agar 'Bacteriológico Europeo' (Pronadisa) in 20 × 195 mm test tubes. Cotyledons from 7-day-old seedlings were cut off and portions of 2 mm width along the proximal and distal edges were removed before they were placed in 9 cm Petri dishes with the abaxial side towards the culture medium. The tomato shoot inducing medium (TSIM) consisted of MB3 basal medium [12] supplemented with 225 μM indole-3-acetic acid (IAA), 19 μM 6-furfurylaminopurine (kinetin, K) and 4,5 μM trans-zeatin (Z). The pH was adjusted to 5.7 before autoclaving at 121 °C for 25 min. Growth regulators were added after sterilization of the culture medium. Explants were incubated in a growth chamber at 25 ± 1 °C under a photoperiod of 16 h light/8 h dark and a photon fluence rate of 90 μmol $m^{-2}s^{-1}$ (Grolux, Sylvania, fluorescent tubes). Upon detecting a putative mutant two to three additional experiments with T0 progenies were performed to confirm the reproducibility of the phenotype. Eight mutants impaired in different phases of the indirect organogenesis process were detected and five were selected for further characterization (Table 1). The explants of T-DNA line *2225-etmm* failed to give rise to a callus on TSIM culture medium and the mutant was named *tomato defective in callus proliferation 1* (*tdc-1*). Cotyledon segments of T-DNA lines *1920-etmm*, *1524-etmm* and *1801-etmm* formed a callus but did not differentiate adventitious buds; these mutants were named *tomato defective in bud differentiation 1, 2, 3* (*tdb-1*, *-2*, *-3*). The explants of T-DNA line *1311-etmm* formed calli and differentiated buds but were not able to develop normal shoots; the mutant was named *tomato defective in shoot development 1* (*tds-1*). The inheritance pattern of each mutant was studied by incubating sixty or more cotyledon explants of T1 plants on TSIM medium. Similarly, the number of T-DNA inserts with a functional *nptII* gene was estimated by sowing explants on TSIM medium supplemented with 100 mg l^{-1} kanamycin [25].

Table 1
Tomato mutants defective in different phases of adventitious organogenesis.

Mutant *1	Organogenic response*2			Phenotype *in vitro* *3	Phenotype *in vivo* *4
	Callus*2	Buds*2	Shoots*2		
tdc-1 (*2225-etmm*)	–	–	–	WT	WT except fruit size and seeds
tdb-1 (*1920-etmm*)	±	–	–	Lower development	Lower development
tdb-2 (*1524-etmm*)	±	–	–	SAM alteration Lower development (root)	Slow growth and further collapse
tdb-3 (*1801-etmm*)	+	–	–	WT except leaves	Several alterations in late development
tds-1 (*1311-etmm*)	+	+	Abnormal	WT	WT

*1: Name of mutants: tomato defective in callus proliferation 1 (tdc-1); tomato defective in bud differentiation 1, 2, 3 (tdb-1, -2, -3); tomato defective in shoot development 1 (tds-1). The codes of the T-DNA lines from which the mutants were identified are indicated in brackets (et = enhancer trapping; MM: cv. Moneymaker).
*2: Callus growth; bud differentiation; shoot-buds development.
*3: Phenotype of axenic plants growing on MB3 basal culture medium.
*4: Phenotype of greenhouse-grown plants.

2.2. Characterization of tomato mutants defective in adventitious organogenesis

2.2.1. Cultural and morphogenetic response in vitro

Upon detecting a tomato mutant defective in different phases of indirect organogenesis several kinds of explants and culture media were tried. Cotyledon explants from seven-day-old T1 seedlings were prepared as described above and 6−7 mm length hypocotyl segments were cut off and sown being placed on the culture medium. Besides, 1 cm wide leaf explants were obtained from axenic plants grown for 25 days on MB3 basal medium and sown with the abaxial side towards the culture medium. In order to check whether the incapacity of mutants for callus proliferation, bud differentiation or shoot development was due to a deficiency in the concentration of growth regulators in the TSIM culture medium, we assayed several modifications of TSIM by doubling the concentration of auxin (45 μM IAA), each cytokinin (37 μM K or 9 μM Z), both cytokinins (37 μM K plus 9 μM Z), and of all the growth regulators (45 μM IAA, 37 μM K, 9 μM Z). Moreover, for some mutants, other synthetic auxins (*e.g.* α-naphtalenacetic acid, 2,4-diclorophenoxiacetic acid) as well as an adenine-type cytokinin (6- benzylaminoapurine) were assayed.

2.2.2. Root development in vitro

Primary and lateral root development was studied in seedlings grown on SGM medium for 25–30 days. To study adventitious rooting shoot apexes were subcultured in MB3 basal medium and incubated for 30 days. In addition, cotyledon, hypocotyl and leaf segments were sown on MB3 medium supplemented with 225 μM IAA (ERM medium) to study adventitious root formation from different kinds of explants.

The nomenclature and composition of the culture media used in this work (Sections 2.2.1 and 2.2.2) are shown in Supplementary Table 1.

2.2.3. Vegetative and reproductive development in vivo

Vegetative and reproductive development in T0 progenies (*i.e.* T1 plants) was assessed by comparing the phenotype of the mutant plants with that of WT sister plants from the same progeny. In the case of fertile mutants, T1 progenies were also obtained and the phenotype of T2 mutant (homozygous) plants was compared with that of Moneymaker plants. All evaluations *in planta* were conducted in a controlled greenhouse environment, under the following conditions: long-day photoperiod (16 h of natural light supplemented with Osram lamps Powerstar HQI-BT, 400 W), temperature fixed at 24 °C during the day and 18 °C at night, and automatic fertigation. Plants were daily irrigated with Hoagland's nutrient solution [28] in 6 L pots with a mixture of peat: vermiculite (1:1 v/v). In order to check whether mutations affecting callus growth, shoot-bud differentiation or adventitious shoot development also affected vegetative traits, parameters related to shoot apical meristem development, leaf size and shape, number and length of phytomers of the initial segment (before the first inflorescence) and that of the successive sympodial segments up to the seventh inflorescence, were all evaluated. As regards reproductive development, the architecture of inflorescence and that of the four flower whorls (sepals, petals, stamens and carpel) as well as fruit size and shape, skin color and seed

number were analyzed.

2.3. Histology techniques

Plant tissue was fixed in FAE (50 % [v/v] ethanol, 5 % [v/v] formaldehyde, 10 % [v/v] acetic acid) and stored in 70 % [v/v] ethanol. Subsequently, tissues were dehydrated in 100 % [v/v] ethanol and embedded in paraffin (Paraplast Plus) blocks using plastic containers. Sections (8 μm thick) of material were cut with a Leica RM2025 microtome. The sections were stained with toluidine blue: 2–5 min in 0,05 % (w/v) toluidine blue and rinsed with water. The samples were observed with a Leica MZZ16 F light microscope (Leica Microsystems, Wetzlar, Germany).

2.4. Co-segregation analysis through the study of the association between the mutant phenotype and the expression of the nptII marker gene

As described by Jáquez-Gutierrez et al. [29], the comparison of the genetic models corresponding to the existence, or not, of co-segregation between the mutant phenotype and a T-DNA insert with a functional *nptII* gene indicate that, in the second case, the probability associated to the appearance of a mutant plant (M) sensitive to kanamycin (kanS) is equal to 1/16. Therefore, if 47 plants are analyzed and no M-kanS plant is detected, the existence of cosegregation may be accepted with a probability of 95 % (or 99 % if 72 plants are assessed).

2.5. GUS histochemical assays

The enhancer trap acts as a dominant element, whose expression pattern can be detected in hemizygous state [26]. GUS histochemical assays were carried out as described previously [30]. The samples of different tissues of T0 plants were collected and placed in GUS staining solution [100 mM sodium phosphate at pH 7.0, 0.1 % Triton X-100, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mg/mL X-Gluc, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 20 % methanol] and incubated at 37 °C for 20–24 h. Subsequently, the GUS-stained tissues were washed with 70 % ethanol for chlorophyll removal and examined under a zoom stereomicroscope (MZFLIII, Leica). Three replicates of each sample were analyzed.

2.6. Cloning of T-DNA flanking sequences and PCR genotyping

Genomic DNA of *tdb-3* mutant was extracted from young leaves by using the DNAzol® Reagent kit (Invitrogen Life Technologies, San Diego, CA, USA) and quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The sequences flanking T-DNA insertion sites were isolated by a modified anchor-PCR according to the protocol previously described [31]. PCR products were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer's instructions. The cloned sequences were compared with SGN Database (http://solgenomics.net/tools/blast/) to assign the T-DNA insertion site on the tomato genome. Co-segregation of the T-DNA insertion site with the *tdb-3* phenotype in the T2 progeny was checked by PCR using (i): the specific genomic forward and reverse primers (1801_genot_F/_R) to amplify the WT allele (without T-DNA insertion); and (ii) one specific genomic primer (1801_genot_R) and the specific T-DNA right border primer (ARB-3) to amplify the mutant allele (carrying the T-DNA insertion). The sequences of genotyping primers used are listed in Supplementary Table 2.

2.7. Generation of RNAi lines

An interference RNA (RNAi) approach was performed to down-regulate candidate genes following the protocol previously described [32]. Briefly, to generate the RNAi *SlMAPKKK17* construct, a 250-bp fragment of *SlMAPKKK17* cDNA was cloned in sense and antisense orientation into the vector pKannibal [33], which was digested with *Not*I, and the resulting fragment was cloned into the binary vector pART27 [34] following the method previously described [35]. Likewise, a 211-bp fragment of the *SlPYL6* cDNA was used to generate the RNAi *SlPYL6* construct. The sequences of primers used to generate the silencing constructs are shown in Supplementary Table 2. RNAi constructs were electroporated into *Agrobacterium tumefaciens* and *Agrobacterium*-mediated transformation of Moneymaker tomato cotyledons was performed following the protocol previously described [24]. Flow cytometry was used to evaluate the ploidy level in transgenic plants according to the protocol described in reference [30] and diploid transgenic lines were selected for further phenotypic and expression analyses.

2.8. qRT-PCR gene expression analysis

Total RNA was extracted with TRIZOL (Invitrogen Life Technologies, San Diego, CA) according to the manufacturer's instructions. Contaminating DNA was removed using the DNA-freeTM kit (Ambion, Austin, TX, USA). RNA quantity and quality were estimated by spectrophotometer analysis using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and checked by gel electrophoresis. The qRT-PCR analysis was performed as described previously [36]. Briefly, the first-strand cDNA was synthesized from 500 ng of total RNA using M-MuLVreverse transcriptase (Fermentas Life Sciences, Hanover, MD, USA). qRT-PCR reactions were performed using gene-specific primers (Supplementary Table 2) and the SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA) on the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The qRT-PCR analysis was performed using three biological and two technical replicates. The tomato *Ubiquitine3* (*Solyc01g056940*) gene was used as an internal control and the quantification of gene expression was performed using the ΔΔCt calculation method [37].

3. Results

3.1. Characterization of the indirect organogenesis process in wild-type cotyledon explants

In the wild type (cv Moneymaker) a color change on both explant sides due to anthocyanin synthesis was seen after two days of incubation, a change that was much more intense in the abaxial surface of the cotyledon segment. The first rounds of cell division in response to the wound reaction were observed at three days in the proximal zone of the explant, *i.e.* the cutting zone nearest the shoot apical meristem. The formation of a disorganized callus generally extended six-seven days more. During the process of callus formation, the explant expanded laterally, along the cotyledon midrib direction, and the disorganized callus growing in the proximal part of the explant progressively invaded the cotyledon midvein. The emergence of green organized growth areas usually began at ten days in the proximal zone, the differentiation of adventitious buds with a defined structure and a bright green color occurred at fourteen-fifteen days, the development of shoot-buds at sixteen-nineteen days, and the development of shoots extended from twenty to twenty-five days (Fig. 1a).

3.2. Screening of tomato T-DNA lines and genetic analysis of mutants impaired in different phases of adventitious organogenesis

To gain new insights into the control of indirect organogenesis in the tomato, we screened for mutants defective in different phases of the process. The scrutiny of the tomato T-DNA lines was carried out by incubating cotyledon segments from seven-day-old seedlings on TSIM culture medium (see Supplementary Table 1). After the evaluation of 862 T_1 progenies, five mutants impaired in different aspects of the

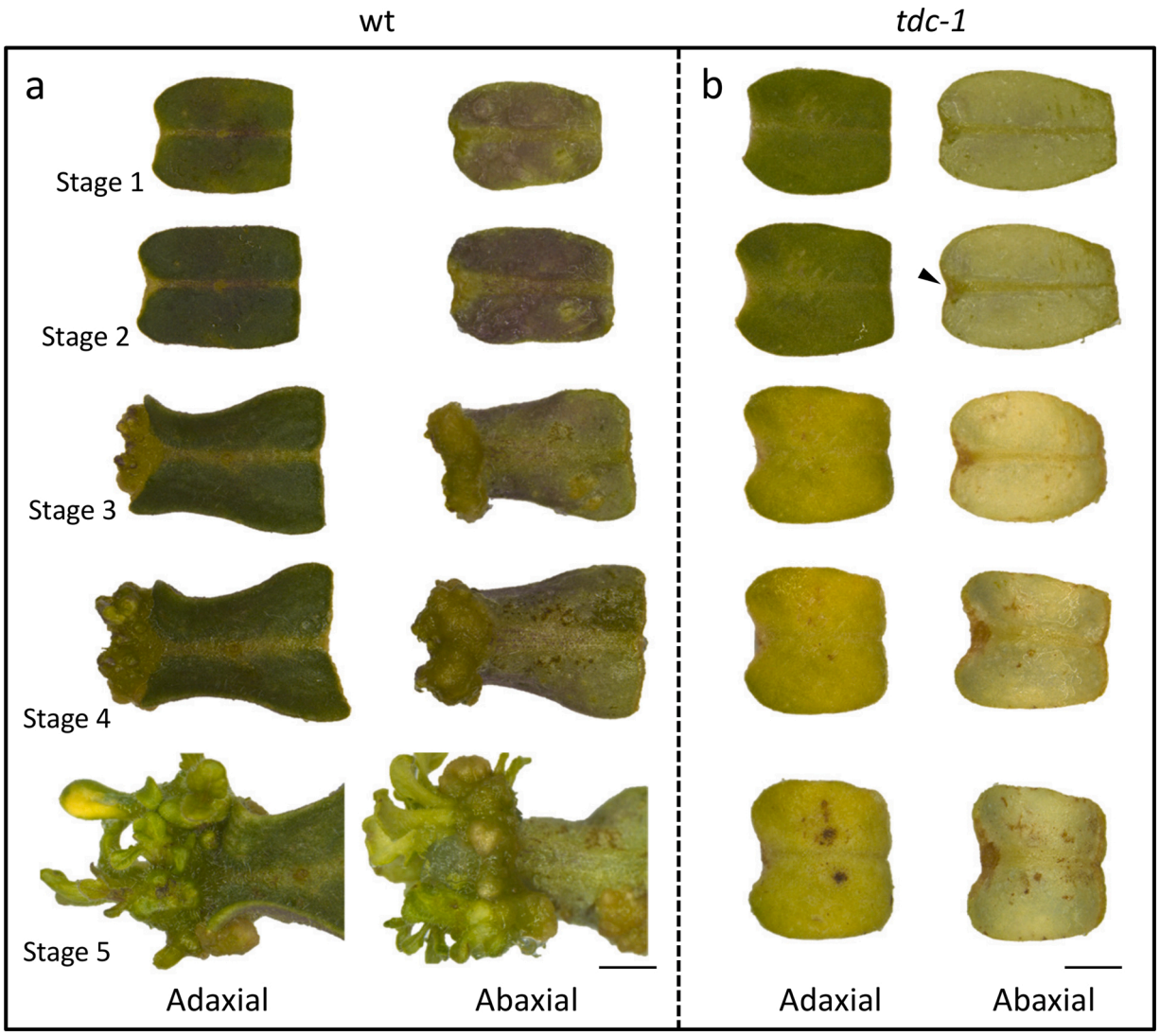

Fig. 1. Evolution of the wild-type and *tdc-1* cotyledon explants cultivated on TSIM culture medium. (a) Evolution of the WT cotyledon explants. In stage 1 (2.0 ± 0.0 days) a color change was observed on both sides of the explant due to the synthesis of anthocyanins. In stage 2 (3.7 ± 0.3 days) the first rounds of cell division in response to the wound reaction were seen in the cutting area closest to the shoot apical meristem. In stage 3 (12.7 ± 0.7 days) began the differentiation of green organized growth areas in the proximal zone of the explant. In stage 4 (15.0 ± 0.6 days) the differentiation of the adventitious shoot-buds with a defined structure and a bright green color occurred. In stage 5 (23.3 ± 1.5 days) shoot development was clearly visible. (b) Evolution of the *tdc-1* cotyledon explants. In stage 1 there were almost no symptoms of anthocyanin accumulation in *tdc-1* explants. In stage 2 wound reaction symptoms were seen in the proximal zone of *tdc-1* explants (black arrow) but, in contrast to the WT explants, cell proliferation stopped. In stage 3, the *tdc-1* explants began to acquire a yellowish color in the adaxial side and a whitish color in the abaxial one. In stages 4 and 5, necrotic areas appeared on the surface of *tdc-1* explants. To characterize the developmental stages of explants three experiments were performed. The values indicate the mean and the standard error of the number of days. Scale bars: 0,5 cm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

indirect organogenesis process were selected for further characterization (Table 1). The explants of one mutant failed to give rise to a callus on TSIM culture medium (*tomato defective in callus proliferation*; *tdc-1*), those of another three formed calli but did not differentiate adventitious buds (*tomato defective in bud differentiation*; *tdb-1*, *-2*, *-3*), and explants of the fifth mutant formed calli and differentiated buds but were not able to develop normal shoots (*tomato defective in shoot development*; *tds-1*). Genetic analysis showed the recessive nature of all the mutants impaired in callus proliferation or morphogenetic response (Supplementary Table 3). The coincidence between observed and expected data was excellent except for the *tdc-1* mutant. In this case, there was a lower number of mutant seedlings with respect to the expected segregation, which might be due to a certain degree of sublethality when the mutant allele is in the homozygous configuration. Moreover, segregation analysis in kanamycin-containing medium showed that each mutant bore one T-DNA insert with a functional *nptII* marker gene (Supplementary Table 4). The phenotypic evaluation of plants cultivated *in vitro* on MB3 basal medium or in the greenhouse (Table 1) showed that the mutants more affected in vegetative and reproductive development were those showing an alteration in the process of adventitious bud differentiation (*tdb-1*, *-2*, *-3*). The mutant defective in callus proliferation (*tdc-1*) only differed from WT plants in fruit size and early seed germination and, surprisingly, the phenotype of the mutant altered in adventitious shoot development (*tds-1*) was indistinguishable from that of WT.

3.3. Characterization of a tomato mutant defective in callus proliferation

During the scrutiny of T0 progenies it was observed that some cotyledon explants of the T-DNA line *2225-etmm* failed to proliferate and give rise to a callus on TSIM culture medium (Fig. 2a). Similarly, no callus growth was observed when leaf segments were sown on the same culture medium (Fig. 2b). After corroborating the results in three

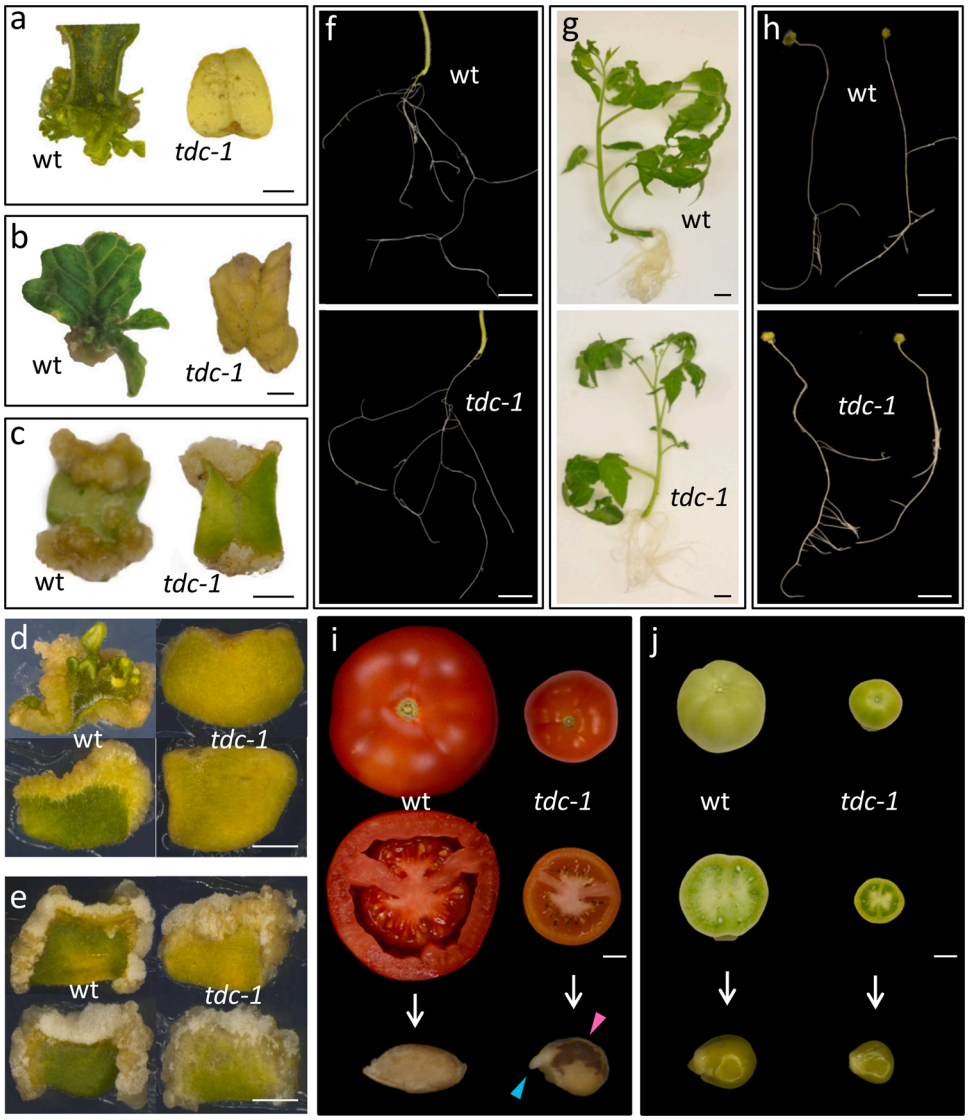

Fig. 2. Characterization of the tomato mutant *tdc-1* defective in callus proliferation. (a) Cotyledon explants of WT (left) and *tdc-1* (right) cultivated on TSIM culture medium in photoperiod conditions. (b) Leaf explants of WT (left) and *tdc-1* (right) cultivated on TSIM medium in photoperiod conditions. (c) Cotyledon explants of WT (left) and *tdc-1* (right) cultivated on CIM^{dtm} culture medium in darkness. (d) and (e) Cotyledon explants of WT (left) and *tdc-1* (right) in photoperiod conditions (up) and darkness (down) on TSIM (d) and CIM^{dtm} (e) culture media. Note that, irrespective of environmental conditions no callus was formed on TSIM while cell proliferation was observed on CIM^{dtm}. (f) The root of *tdc-1* (down) and WT (up) seedlings developed similarly. (g) No differences were detected in the aerial part and adventitious root system of *tdc-1* axenic plants (down) and WT (up). (h) Hypocotyl segments of *tdc-1* (down) and WT (up) sown on ERM medium developed an equivalent adventitious root system. (i) Seeds of mature red fruits of *tdc-1* mutant (right) germinated precociously within the fruit (blue arrow) and showed necrotic symptoms (pink arrow) in the external seed integument. (j) Seeds from immature green fruits of *tdc-1* mutant (right) did not show necrotic symptoms. Scale bars: 0,5 cm (a-e), 1 cm (f-j). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

independent experiments, the mutant was named *tdc-1* (*tomato defective in callus proliferation 1*). The evolution of wild-type and *tdc-1* cotyledon segments in the TSIM culture medium was monitored throughout the incubation period and compared to that of WT (Fig. 1b). At two days of incubation, there were almost no symptoms of anthocyanin accumulation in the adaxial side of *tdc-1* cotyledon segments and just a slight change of color on the abaxial side. At four days, wound reaction symptoms were seen in the proximal zone of *tdc-1* explants but, in contrast to the WT explants, cell proliferation stopped. At nine-ten days, the *tdc-1* cotyledon explants began to acquire a yellowish color on the adaxial side and a whitish color in the abaxial. After fifteen-sixteen days of incubation, the callosity of the proximal zone turned brown and necrotic areas began to appear on the surface of the *tdc-1* explants. A similar evolution was observed when monitoring leaf explants on TSIM culture medium. Neither anthocyanin accumulation nor callus formation was observed and, as happened with cotyledon explants, the adaxial side of the leaf segments turned yellow and the abaxial acquired a whitish color (not shown).

To overcome the lack of ability of *tdc-1* explants to proliferate and give rise to a callus several culture media were assayed. Despite TSIM medium being relatively rich in auxin (22,5 μM IAA) and cytokinins (19 μM K and 4,5 μM Z), we doubled the concentration of each growth regulator as well as that of all of them together (Supplementary Table 1). No callus formation was observed in these modified TSIM culture media which suggested that the problem of *tdc-1* explants is not related to a deficiency in the concentration but to the lack of response to these growth regulators. For this reason, we subsequently assayed different types and combinations of other growth regulators and found that by incubating *tdc-1* cotyledon explants in darkness in a culture medium supplemented with 4,5 μM 2,4-D, 11 μM NAA and 4,5 μM BAP (henceforth named CIM^{dtm}; *callus inducing medium in defective tomato mutants*) the cell proliferation continued; and also that, despite a lower growth rate than that of WT explants, those of *tdc-1* mutants were able to give rise to a disorganized callus (Fig. 2c). In a subsequent experiment *tdc-1* cotyledon explants were sown in TSIM and CIM^{dtm} culture media under two growth conditions (photoperiod and darkness). Results showed that irrespective of environmental conditions no callus was formed on TSIM (Fig. 2d) while cell proliferation was observed on CIM^{dtm} culture medium (Fig. 2e). Overall, results indicated that the capacity for cell proliferation and callus formation in *tdc-1* explants depends on the type of growth regulators in the culture medium.

The development of the root system in *tdc-1* mutant plants was carefully studied, as previous reports in *Arabidopsis* suggested that the ectopic activation of a lateral root development program is a common mechanism in callus formation from multiple organs [5,6]. Results showed a similar seedling root development in *tdc-1* and WT. (Fig. 2f). In

the same way, no differences in adventitious root development were observed from shoot apexes cultivated on basal culture medium (Fig. 2g), or in hypocotyl segments sown on the same medium supplemented with 22,5 µM IAA (Fig. 2h). Thus, in the *tdc-1* tomato mutant the deficiency in callus formation is not linked to any abnormal development of either seedling root or adventitious root system.

The vegetative development of the aerial part of *tdc-1* plants grown *in vitro* (Fig. 2g) was similar to that of WT plants and the same happened in greenhouse-grown plants (not shown). As regards reproductive development, despite the lack of statistical differences in ovary width (2.06 ± 0,18 mm in *tdc1 versus* 1,96 ± 0.08 mm in WT) and length (2.01 ± 0.15 mm in *tdc-1 versus* 1.90 ± 0.10 mm in WT), the mutant fruits showed a lower size (4.35 ± 0.12 cm in *tdc1 versus* 7.08 ± 0.09 cm in WT; Fig. 2i). Nevertheless, the most distinctive aspect was related to the *tdc-1* seeds as they germinated precociously within mature-red fruits and showed senescence or oxidation symptoms in the external seed integument (Fig. 2i). This abnormal development of mutant seeds might explain the segregation distortion (*i.e.* lower number of mutant plants than expected) in the genetic analysis mentioned above (Supplementary Table 3). The viviparous behavior of homozygous seeds for the mutant allele, which could perhaps be related to an alteration in the synthesis/perception of ABA, was a real problem since they could not be stored and used for further trials. We tried to address this problem using seeds from fruits in the mature-green stage but they already showed necrotic symptoms. Fortunately, seeds from immature-green fruits did not show such symptoms (Fig. 2j) and were able to germinate in a basal medium supplemented with 5 µM IBA (SRM medium).

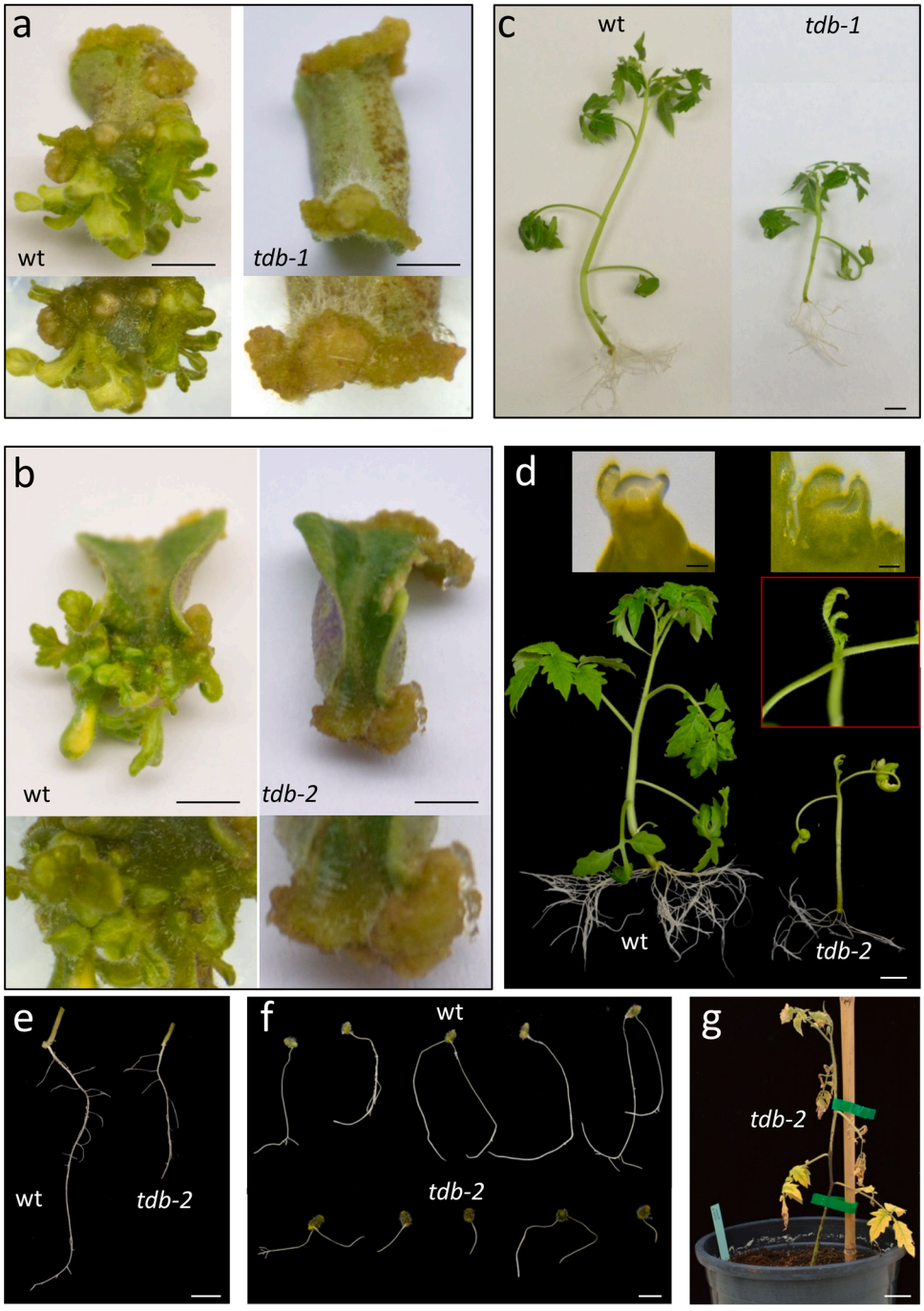

Fig. 3. Characterization of tomato mutants *tdb-1* and *tdb-2* defective in bud differentiation. (a) and (b) No adventitious buds were differentiated in cotyledon-derived callus of *tdb-1* (a) and *tdb-2* explants (b) cultivated on TSIM culture medium. (c) *tdb-1* mutant plants grown on basal medium (right) showed a lower development than WT (left). (d) *tdb-2* mutant plants showed a lower development *in vitro* as well as an abnormal development of the shoot apical meristem (see close-up); (e) and (f) *tdb-2* mutant plants showed a lower root development, though the architecture of seedling root (e) and adventitious root system (f) was unaffected. (g) *tdb-2* mutant plants were able to get over the acclimatization *ex vitro*, but after one-two months in the greenhouse leaves experienced a senescence process and the plant collapsed. Scale bars: 0,5 cm (a-b), 1 cm (c-f), 0,1 mm (meristems in d), 5 cm (g).

3.4. Characterization of tomato mutants altered in adventitious shoot-bud differentiation

Three mutants capable of forming calli in TSIM culture medium but defective in adventitious shoot-bud differentiation (*tdb-1*, *-2*, *-3*) were characterized. Calli that proliferated from *tdb-1* and *tdb2* cotyledon explants were not able to differentiate adventitious buds in the usual incubation period (Fig. 3a,b) nor in longer culture periods (not shown). Both mutants were similar in the behavior of the root system but differed in the development of the aerial part. Root growth was slower in *tdb-1* and *tdb-2* though the architecture of the seedling root and adventitious root system was unaffected (Fig. 3c–f). The aerial part of *tdb-1* also showed slower growth *in vitro* (Fig. 3c). After acclimatization in the greenhouse *tdb-1* plants developed normal leaves but grew slowly and produced smaller fruits. In contrast, *tdb-2* plants showed abnormal development of the shoot apical meristem (Fig. 3d) and, despite being able to survive the acclimatization *ex vitro*, after one or two months in the greenhouse leaves experienced a senescent process and some plants collapsed (Fig. 3g).

Cotyledon explants of *tdb-3* proliferated normally in TSIM culture medium, although no adventitious buds were differentiated (Fig. 4a). Mutant *tdb-3* plants grown *in vitro* exhibited similar behavior to WT and the same was observed during the first 30–45 days after acclimatization in the greenhouse. However, from the second month onwards, *tdb-3* greenhouse-grown plants showed severe changes in vegetative characteristics, including increased internode distance and reduced axillary branching (Fig. 4b), and leaves with an ungainly aspect and almost no serrate margins in lateral leaflets (Fig. 4c). Mutant *tdb-3* plants also showed profound changes in reproductive traits, such as an anomalous development of anthers and style (Fig. 4d), different inflorescence architecture (Fig. 4e), enlarged and curved sepals (see close-up in Fig. 4e) and smaller and seedless fruits (Fig. 4f).

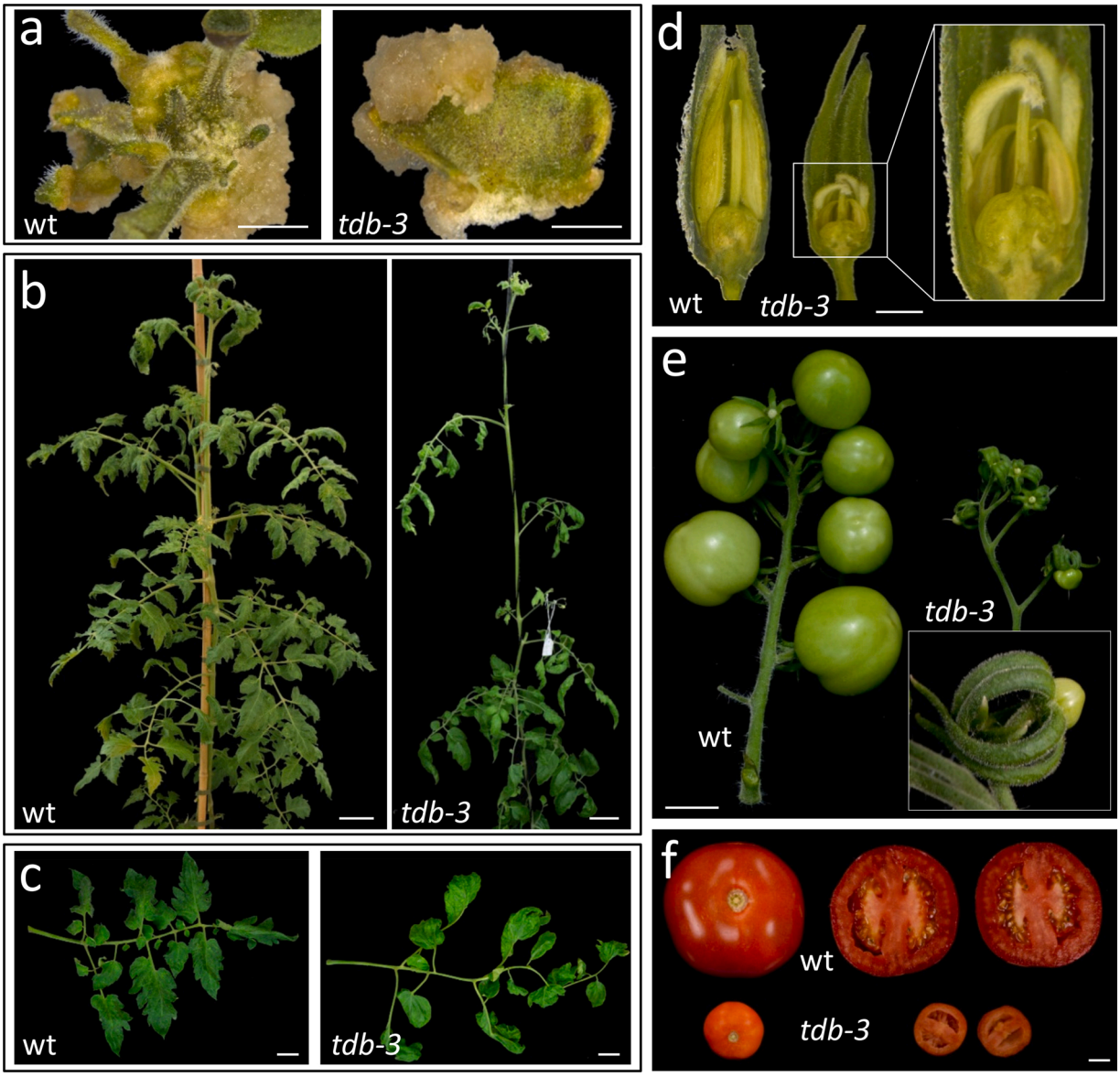

Fig. 4. Characterization of the tomato mutant *tdb-3* defective in bud differentiation. (a) Cotyledon-derived calli of *tdb3* did not develop adventitious shoot-buds on TSIM medium. (b) From the second month onwards, *tdb-3* plants showed severe changes in vegetative traits, such as increased internode distance and reduced axillary branching. (c) *tdb-3* mature leaves showed an ungainly aspect and almost no serrate margins in lateral leaflets (c). (d) *tdb-3* flowers exhibited an anomalous development of anthers and style. (e) *tdb-3* plants showed a different inflorescence architecture and the immature fruits kept enlarged and curved sepals (close-up). (e) Smaller and seedless mature fruits were developed in *tdb-3* plants. Scale bars: 0,5 cm (a), 10 cm (b), 3 cm (c and e), 2 mm (d), 1 cm (f).

3.5. Characterization of a tomato mutant defective in the development of adventitious shoots

About a quarter of the cotyledon explants of the T-DNA line *1311-etmm* cultivated in TSIM culture medium gave rise to calluses and differentiated adventitious buds but did not develop normal shoots. Unlike the WT explants only leaves or abnormal shoots were developed (Fig. 5a). We tried to restore the development of these organogenetic structures by lengthening the incubation period (45–50 days) and repeated subculture with no success. Similarly by transferring organogenetic calli to elongation or rooting culture media only leaves or abnormal shoots were developed, even when extending the incubation periods (Fig. 5b,c). Experiments were repeated not only with cotyledon but also with hypocotyl explants and just one sporadic normal shoot was obtained. In accordance with its behavior the mutant was thenceforth named *tomato defective in shoot development-1* (*tds-1*). To find out the reason for the abnormal development of organogenic structures, histological analyses were performed. Unlike wild-type organogenic calli, in which buds become shoots with a distinguishable shoot apical meristem (Fig. 5d), nodular areas containing great numbers of cells, segmented

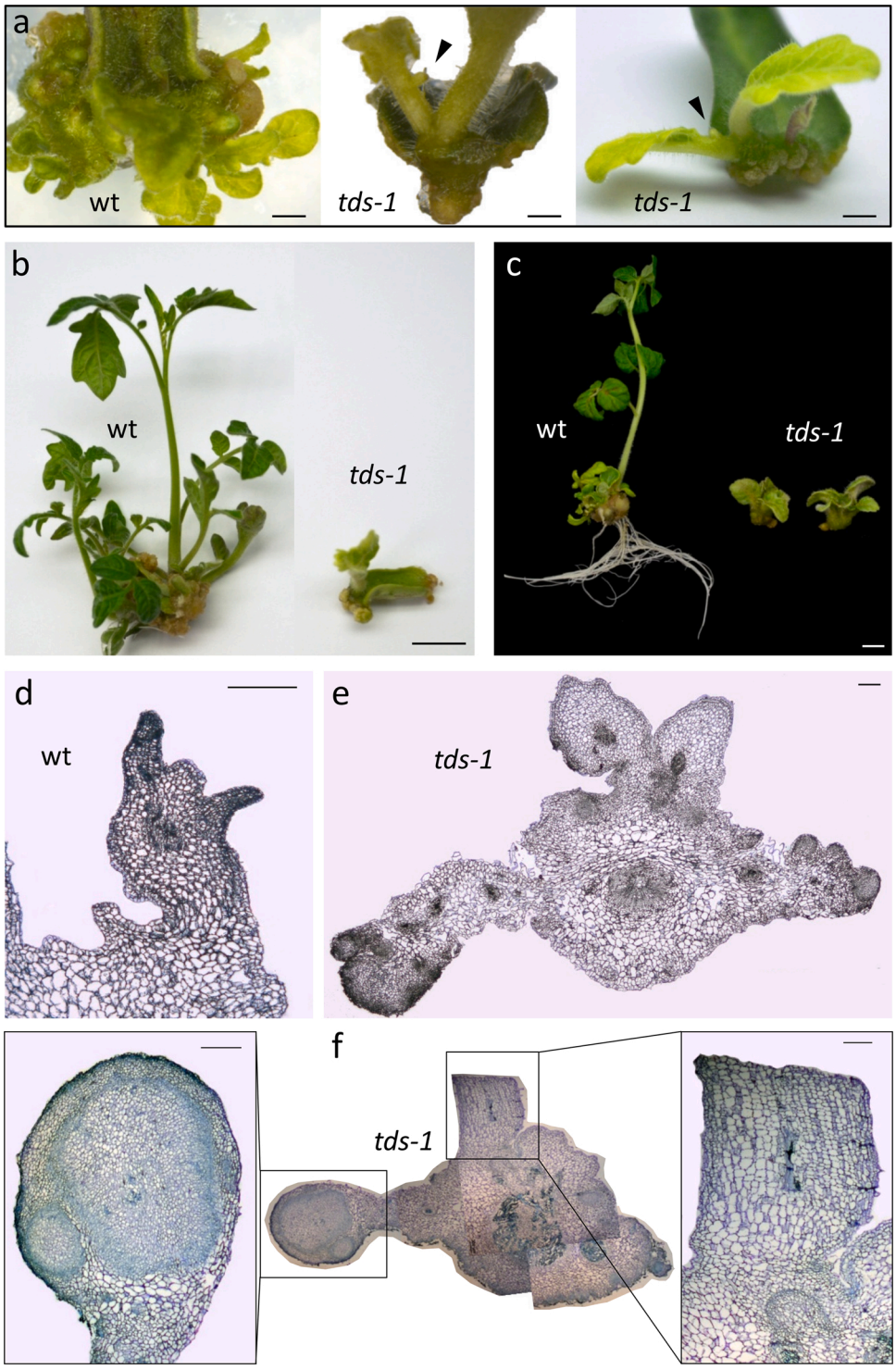

Fig. 5. Characterization of the tomato mutant *tds-1* defective in shoot-bud development. (a) Unlike WT (left) only leaves or abnormal shoots were developed in cotyledon *tds-1* explants (center and right). The black arrows point to meristem-like structures in the petioles of abnormal leaves that are not able to give rise to normal adventitious shoots. (b) and (c) No shoots were obtained by transferring *tds-1* organogenetic calli to elongation (b) or rooting (c) culture media even with long incubation periods. (d) Organization of a normal shoot adventitious meristem in WT. (e, f) *tds-1* calli develop nodular areas containing great number of cells that resemble those forming adventitious buds, segmented growth zones, modified leaves and abnormal shoots. Scale bars: 0,2 cm (a), 1 cm (b, c), 250 μm in (d, e, f).

growth zones, modified leaves and abnormal shoots appeared in *tds-1* calluses (Fig. 5e–f).

Interestingly, despite the wide range of abnormalities in the development of adventitious shoot-buds, *tds-1* mutant plants had normal development. The architecture of the aerial part of the mutant plant cultivated *in vitro* was identical to that of WT (Supplementary Fig. 1a). Root development from seedlings was similar to that of WT (Supplementary Fig. 1b) and the same happened with the development of adventitious roots from both shoot apexes cultivated in a basal culture medium (Supplementary Fig. 1c) and cotyledon explants incubated in the same medium supplemented with 22,5 μM IAA (Supplementary Fig. 1d). Similarly, the vegetative and reproductive traits of *tds-1* plants cultivated in the greenhouse were indistinguishable from those of WT plants (Supplementary Fig. 1e). All together results show that the gene altered in the *tds-1* mutant plays a specific role in the organization of adventitious shoot meristems.

3.6. Co-segregation analysis

Compared to other mutant-based approaches, one of the advantages of insertional mutagenesis is that T-DNA can tag an endogenous gene, which would facilitate its identification [38]. However, since our collection of tomato T-DNA lines was generated by *Agrobacterium*-mediated transformation, it is expected that the phenotype of some mutants is due to somaclonal variation [39]. In the first case, the identification of the altered gene in the mutant can be achieved using Anchor-PCR [31], while in the second case the use of NGS sequencing methods is required [40]. For this reason, before deciding the best approach to address the cloning of the altered gene, it is necessary to determine whether there is an association between the mutant phenotype and a T-DNA insert. Co-segregation analysis in tomato mutants defective in different phases of adventitious organogenesis was performed *in vitro* by studying the association between the mutant phenotype and the expression of the *nptII* marker gene, as this method proved to be reliable in previous studies with other mutants [29]. In the case of *tdc-1*, *tdb-1*, *tdb-2* and *tds-1*, the detection of mutant seedlings sensitive to kanamycin showed an absence of co-segregation. By contrast, the analysis of 76 plants of the T1 progeny of the mutant *tdb-3* indicated the existence of co-segregation with a probability higher than 99 % (Table 2).

3.7. Identification of a gene involved in adventitious shoot-bud differentiation in tomato

So as to isolate the gene harbouring *tdb-3* mutation, anchor-PCR assays were performed to clone the genomic regions flanking the T-DNA insertion site. Results revealed that T-DNA integration was located on chromosome 2, at position 39,865,245 bp (ITAG4.0), in the promoter region of two adjacent genes transcribed in opposite direction, one of which encodes an abscisic acid receptor homologous to the Arabidopsis *PYL6* (*SlPYL6*, *Solyc02 g076770*), and the other one a mitogen-activated protein kinase kinase kinase (*Solyc02 g076780*) previously designated as *SlMAPKKK17* [41]. More precisely, the T-DNA was inserted 1978 bp upstream of the translation start codon of the *SlPYL6* receptor, and 1716 bp upstream of the 5′-untranslated region of the *SlMAPKKK17* gene (Fig. 6a). Characterization of this insertional mutation revealed a complex T-DNA integration pattern. Thus, two copies of T-DNA in a tandem inverted orientation separated by 9768 bp of the pD991 vector backbone were inserted in the *tdb-3* mutant (Fig. 6a). To support the insertional nature of the *tdb-3* phenotype, an additional co-segregation analysis was performed by PCR in 48 plants of the T2 progeny using allele-specific primers designed from both the T-DNA and the genomic flanking sequences tagged in the *tdb-3* mutant (Supplementary Table 2). The results showed that all mutant plants carried T-DNA insertion in the homozygous state, whereas wild plants were hemizygous for insertion or did not carry T-DNA (Fig. 6b), supporting the conclusion that the *tdb-3* phenotype was caused by the T-DNA integration.

The effects of the T-DNA insertion on gene expression were determined by qRT-PCR assays, which showed a significant downregulation of the *SlMAPKKK17* gene in *tdb-3* tissues compared with wild-type tissues (Fig. 6c). However, transcripts of the *SlPYL6* gene were not detected in either the wild-type or mutant plant tissues analyzed here (Supplementary Fig. 2), suggesting that *SlPYL6* might be a pseudogene in tomato.

As the T-DNA of the enhancer trap binary vector pD991 contains close to the right border (RB) a minimal promoter fused to the *uidA* gene, a histochemical GUS assay was performed assuming that the GUS expression is due to the activity of endogenous regulatory elements that promote gene reporter transcription. Since the insertional mutation is a tandem insert in inverted orientation with the RB of each T-DNA oriented toward plant genomic DNA, the observed GUS expression pattern might be explained by regulatory elements of both *SlMAPKKK17* and *SlPYL6* genes. However, qRT-PCR analysis did not detect transcripts for *SlPYL6*, which suggests that *SlMAPKKK17* regulatory elements should presumably be responsible for the tissue specificity of GUS gene expression. Strong and reproducible GUS staining was detected in vegetative organs of the mutant, such as stem, rachis and leaf blade (Supplementary Fig. 3a) in which we had previously observed phenotypic changes (see Fig. 4). Regarding adventitious organogenesis, since the cotyledons of the mutant are unable to differentiate shoot-buds and the expression of the reporter gene is dominant, we carried out some preliminary analyses not on homozygous (mutant phenotype), but hemizygous (wild type, kanamycin resistant) seedlings. In this way, we were able to verify that the reporter gene is expressed in adventitious shoot-buds (Supplementary Fig. 3b). Further analyses to determine the temporal expression pattern of the tagged gene showed that it is not expressed in the phase of callus proliferation, but only from the moment when the differentiation of adventitious buds begins (Supplementary Fig. 3c). Overall, the results showed a close association between the GUS expression pattern and the phenotype of the *tdb-3* mutant.

By using an interference RNA approach, six RNAi lines for the *SlPYL6* gene and ten for the *SlMAPKKK17* gene were obtained. As expected, all six RNAi lines for the *SlPYL6* gene showed WT phenotype, providing further evidence that this is not the gene responsible for the traits of the *tdb-3* mutant. By contrast, of the ten RNAi lines for the *SlMAPKKK17* gene, two showed a WT phenotype (RNAi-3 and -5), seven showed some traits resembling the mutant but not as extreme (RNAi-1, -2, 4, -6, -11, 13, and -16) (Supplementary Fig. 4a), and the last one (RNAi-17) a phenotype almost identical to that of the *tdb-3* mutant (Supplementary Fig. 4b-c). The evaluation of the morphogenetic response in cotyledons from kanamycin resistant seedlings of T0 progenies of RNAi lines showed a decrease in the frequency of regeneration, although significant differences were only found in two lines with very low levels of gene

Table 2
Co-segregation analysis between phenotype and a T-DNA insert with a functional *nptII* gene in mutants defective in adventitious organogenesis.

	Observed segregation [a]					Phenotype–*nptII* association [b]
Mutant	WT – R	WT – S	M – R	M – S	Total	
tdc-1	66	18	10	5	99	No co-segregation
tdb-1	35	7	7	2	51	No co-segregation
tdb-2	36	10	4	2	52	No co-segregation
tdb-3	44	15	17	0	76	Co-segregation (P > 99 %)
tds-1	34	11	9	3	57	No co-segregation

[a] WT: wild-type phenotype; M: mutant phenotype; R: kanamycin resistant; S: kanamycin sensitive.

[b] The way to carry out the co-segregation analysis to reach a significant conclusion in statistical terms (probability > 99 %) is indicated in Section 2.4. of Material and Methods.

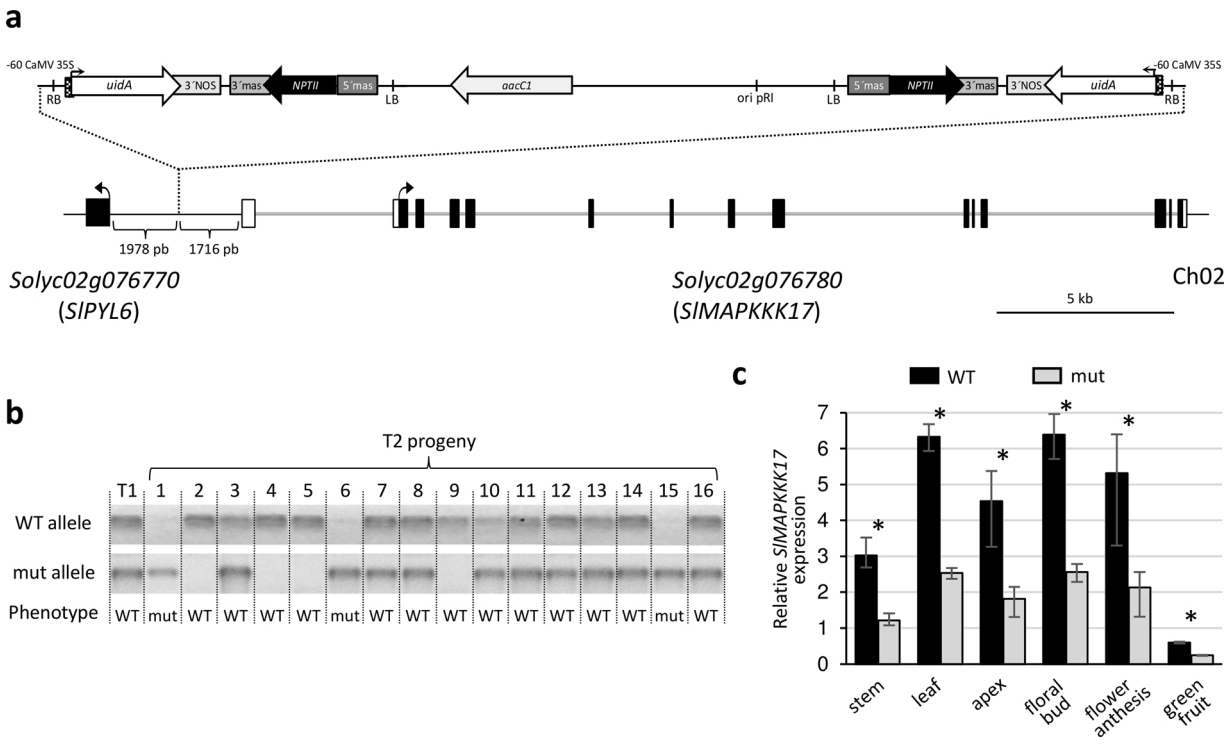

Fig. 6. Molecular characterization of the *tdb-3* mutant. (a) Genomic organization of the *Solyc02 g076770* (*SlPYL6*) and *Solyc02 g076780* (*SlMAPKKK17*) genes and the localization of the two copies of T-DNA in a tandem inverted orientation separated by a fragment of the pD991 vector backbone in the *tdb-3* mutant. Coding sequences and untranslated regions (UTR) are depicted as black and white boxes, respectively. *SlMAPKKK17* introns are shown as a bold grey line. Transcription start sites are indicated as arrows. (b) Co-segregation analysis of the T-DNA insertion and the *tdb-3* mutant phenotype in T1 and 16 plants of the T2 population. T2 plants heterozygous (3, 7, 8, 10, 11, 12, 13, 14 and 16) and homozygous for the wild-type (WT) allele (2, 4, 5 and 9) showed WT phenotype, while T2 plants homozygous for the mutant (mut) allele (1, 6 and 15) displayed *tdb-3* phenotype. (c) qRT-PCR assay for *SlMAPKKK17* gene. Asterisk denotes significant differences (Student's t-test, $P < 0.05$).

expression (RNAi- 6 and -16) (Supplementary Fig. 4d–e). In the case of the RNAi-17 line, which had the lowest level of gene expression, it was not possible to evaluate the morphogenetic response in cotyledons of T0 progeny since, as with the *tdb-3* mutant, it developed abnormal flowers and seedless fruits. However, experiments with leaf explants from the original T0 plant showed an absence of regeneration (Supplementary Fig. 4f). Overall, the results suggest that the inability to differentiate adventitious shoot-buds in *tdb-3* explants is due to downregulation of the *SlMAPKKK17* gene caused by the integration of T-DNA in an upstream regulatory region.

4. Discussion

To tackle the genetic dissection of the indirect organogenesis process in the tomato we are conducting systematic screening of a collection of T-DNA lines generated in our laboratory. The scrutiny of T1 progenies from these T-DNA lines allowed us to discover several tomato mutants impaired in different phases of the organogenetic process: callus formation (*tdc-1*), shoot-bud differentiation (*tdb-1, -2, -3*) and shoot-bud development (*tds-1*). The availability of these mutants may be particularly valuable to perform the molecular dissection of the organogenetic process in the tomato.

Cotyledon and leaf explants of the tomato mutant *tdc-1* failed to proliferate and give rise to a callus on TSIM culture medium. Interestingly the vegetative development of the *tdc-1* mutant plants was indistinguishable from that of WT plants both *in vitro* and *in vivo*, indicating that *tdc-1* mutation does not affect fundamental aspects of cell growth *in planta* but its effect is just restricted to the process of cell proliferation from cut zones of explants. It is worth mentioning that the lack of cell proliferation from the cut areas of explants is not absolute as, after a systematic search, we found a culture medium (CIM^{dtm}) with a different combination of growth regulators in which the *tdc-1* explants were able to proliferate and form a callus. Thus, results indicate that the alteration in the *TDC-1* gene (most probably loss-of-function) blocks cell division in a culture medium with IAA, K and Z (TSIM) but does not in a culture medium with 2,4-D, NAA and BAP (CIM^{dt}). The influence of different types and combinations of growth regulators on the cultural and morphogenetic response of explants is well established in plant tissue culture but the knowledge of how the cells perceive and respond to them is still fragmentary. It is to be expected that further studies with the *tdc-1* mutant should shed light on the matter and that the identification of the *TDC-1* gene will unravel a key regulator providing competence for the process of shoot-bud differentiation.

Several lines of evidence suggested that callus induction and lateral root primordium initiation are somehow linked in *Arabidopsis* [5,6,42]. It has also been reported a difference in cell proliferation driving to root or shoot development in tomato, marked by a different time course and a different expression of the *LESK1* gene [22]. Taking into account these previous reports we carefully analyzed root development in the *tdc-1* tomato mutant, but were unable to find any abnormality in either the primary and lateral root development from seedlings, or in adventitious root development from shoot apexes cultivated in basal medium, or hypocotyl explants sown in the same medium supplemented with 22,5 μM of IAA. Together our results indicate that in the tomato the connection between callus induction and lateral root development is not so obvious as it apparently is in *Arabidopsis*.

Root explants from the *shoot meristemless-1* mutant of *Arabidopsis* formed calli and green nodules when transferred from callus- to shoot-inducing medium but developed only leaves or abnormal shoots [43]. Besides, the anatomy of *stm-1* embryos indicated that the *stm-1* mutation completely blocked the initiation of the shoot apical meristem (SAM). The failure of *shoot meristemless* calli to regenerate shoots in tissue

culture and the lack of a normal SAM in seedlings suggested that the gene encoded by the *STM* locus, a member of the *KNOTTED* class, regulates adventitious shoot meristem formation, as well as embryonic SAM formation [44]. The *tomato defective in shoot development1* (*tds-1*) behaved like the *Arabidopsis stm-1* mutant in tissue culture conditions. Cotyledon and hypocotyl explants from *tds-1* seedlings were able to form calli and differentiated buds but they did not develop normal shoots. Instead, leaves or abnormal shoots were developed in TSIM or upon transference to an elongation culture medium. Histological analysis of *tds-1* callus indicated that the abnormal development of adventitious structures is due to the failure in the organization of a normal adventitious shoot meristem. However, it's worth pointing out that *tds-1* mutant plants cultivated *in vitro* showed no phenotypical alteration in the aerial part or the radical system; and similarly, when cultivated in the greenhouse there were no appreciable differences in vegetative and reproductive traits to those of WT plants. Thus, results indicate that unlike the *Arabidopsis STM* gene, which regulates the formation of both adventitious and embryonic shoot meristems, the tomato *TDS-1* gene plays a specific role in the organization of adventitious shoot meristems.

As occurs with the cotyledon explants of tomato mutants defective in shoot-bud differentiation (*tdb-1*, *tdb-2*, *tdb-3*), the root explants of three temperature-sensitive mutants of *Arabidopsis* (*shoot redifferentiation defective*; *srd-1*, *srd-2*, *srd-3*) are able to give rise to a callus, but are affected in the organogenic response [45,46]. Despite this similarity, *tdb* and *srd* mutants differ in all other characteristics both *in vitro* and *in planta*. As regards their behavior *in vitro*, the lack of ability for shoot-bud differentiation in *tdb* tomato mutants is manifested in normal growth conditions for wild-type explants, whilst shoot differentiation in *srd Arabidopsis* mutants is only impaired in restrictive temperature (27 °C) but not in permissive (22 °C). Regarding phenotypical traits *in planta*, no major aberrations were found in *srd* mutant plants grown on soil at permissive temperature. Exposure to the restrictive temperature interfered with the growth of *srd1* and *srd3* only when seedlings were exposed to 27 °C during the first 7 days after sowing, otherwise *srd* mutant plants appeared almost normal in morphology even at the restrictive temperature [45,46]. Unlike *srd Arabidopsis* mutants, the *tdb* tomato mutants displayed a wide spectrum of anomalous phenotypes, especially when plants were grown in greenhouse conditions: *e.g.* lower development (*tdb-1*), slow growth and further collapse (*tdb-2*), and several alterations in vegetative and reproductive development (*tdb-3*).

Cosegregation analysis revealed the insertional nature of the *tdb-3* mutant. Anchor-PCR assays showed that T-DNA was located in the upstream regulatory regions of two adjacent genes transcribed in opposite direction, encoding an ABA receptor homologous to the *Arabidopsis PYL6* (*SlPYL6*), and a MAPKKK previously designated as *SlMAPKKK17* [41]. Expression analysis by qRT-PCR showed that the *SlMAPKKK17* gene was significantly down-regulated in *tdb-3* tissues. By contrast expression of the *SlPYL6* gene was not detected in both the wild-type and mutant tissues analyzed, suggesting that *SlPYL6* might be a pseudogene in tomato. Some pseudogenes have recently been described as performing regulatory functions in plants [47,48], and so it cannot be excluded that *SlPYL6* may have some role of this nature. However, the results we have obtained indicate that down-regulation of the *SlMAPKKK17* gene is the most likely cause of the phenotypic changes observed in the *tdb-3* mutant both *in vivo* and *in vitro*.

Mitogen-activated protein kinase (MAPK) cascades are universal signal transduction modules in plants. These proteins mediate the intracellular transmission of extracellular stimuli, resulting in the induction of cellular responses. Plant MAPK cascades are thought to play an important role in abiotic- and biotic-stress responses [49–51], hormone responses [52], the maintenance of the shoot apical meristem [53,54] and in several other aspects of plant growth and development, [55–57]. Genomic analysis has shown that the tomato genome contains 17 *MPK*, 5 *MPKK* and 89 *MAPKKK* genes [52]. Despite their biological significance, very little is known about the functions of tomato MAPK. In this regard, it is worth mentioning that the comparison of some studies on Arabidopsis MAPK and the data we have obtained in the characterization of greenhouse-grown plants of both the tomato *tdb3* mutant and the RNAi lines in which *SlMAPKKK17* was downregulated suggest some putative functions of this gene. For example, the total sterility of the *tdb3* mutant could be related to the functions of the *Arabidopsis* MAPK3 and MAPK6 kinases in the development of the ovule [58] and the anther [59]. The plants of the *tdb-3* mutant have very long internodes and less lateral branching, which could be related to an alteration in the synthesis/perception of gibberellins. Alternatively, it has been reported that the MKK7-MAPK6 cascade regulates shoot branching in Arabidopsis [60] and, depending on the phenotype of the *tdb-3* mutant, it cannot be ruled out that *SlMAPKKK17* could fulfill some related function in tomato. It has also been noted that two *Arabidopsis* MAPK cascades, MKK9-MPK6 [61] and MAPKKK17/18-MKK3-MPK1/2/7/14 [62,63] play a role in leaf senescence. Plants from the *tdb-3* mutant and RNAi lines exhibit changes in leaf development, yet these changes do not affect senescence but leaf architecture, suggesting that *SlMAPKKK17* plays a different role in tomato.

It has been reported that the expression level of *MAPK* change significantly in response to hormone treatments [41], implying that some mitogen-activated protein kinases could act as intermediaries for the organogenic response in explant cells through the transduction of signals from an adequate combination of growth regulators and other components of the culture medium. In this regard, it is worth mentioning that *LESK1*, a gene encoding a putative serine/threonine kinase, which is located on a different chromosome than *SlMAPKKK17*, was proposed as a marker of the competence phase of organogenic response in hypocotyl and cotyledon explants of tomato [22,23]. As far as we know, no functional analysis of this gene has been reported but these interesting results point to the involvement of some of these proteins in the organogenic response in tomato tissue cultures.

In a similar way to *LESK1*, *SlMAPKKK17* may play a role in the acquisition of cellular competence for the organogenic response of tomato explants. However, it is also possible that *SlMAPKKK17* is involved in the organization and/or maintenance of adventitious meristems. In this regard it has been recently noted that MPK3 and MPK6 are required for stem cell maintenance of the shoot apical meristem in Arabidopsis [53] and that the MKK7-MPK6 MAP kinase module is a regulator of meristem quiescence or active growth in the same model species [54]. It is therefore possible that the downregulation of the *SlMAPKKK17* gene interferes with the maintenance of adventitious meristems in tomato explants which would prevent the normal development of shoot-buds. In support of this hypothesis, expression analyses of the *uidA* reporter carried by the enhancer trap revealed that the *SlMAPKKK17* gene is not expressed during the callus formation phase but coincides with the appearance of glomerular zones that will later lead to the differentiation of adventitious buds. *In vitro* studies with explants of RNAi lines of intermediate phenotype merely showed a decrease in the frequency of regeneration with respect to WT explants, possibly due to an insufficient level of gene silencing. However, in the three RNAi lines with the highest degree of gene silencing, a significant decrease in the frequency of regeneration or even a lack of morphogenetic response was observed. These results suggest that the frequency of morphogenetic response could be determined by a threshold of expression of the *SlMAPKKK17* gene. Overall, our results show that the *SlMAPKKK17* gene seems to play an essential role in the organogenic response in tomato explants, most probably in the competence phase or at the beginning of the adventitious shoot-bud differentiation process.

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CRediT authorship contribution statement

Jorge Sánchez-López: Investigation. **Alejandro Atarés:** Conceptualization, Investigation, Formal analysis, Writing - review & editing. **Marybel Jáquez-Gutiérrez:** Investigation, Formal analysis. **Ana Ortiz-Atienza:** Investigation, Formal analysis. **Carmen Capel:** Investigation. **Benito Pineda:** Conceptualization, Investigation, Formal analysis, Writing - review & editing. **Begoña García-Sogo:** Investigation. **Fernando J. Yuste-Lisbona:** Conceptualization, Investigation, Formal analysis, Writing - review & editing. **Rafael Lozano:** Conceptualization, Formal analysis, Writing - review & editing. **Vicente Moreno:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

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