The *TORNADO2* gene of *Arabidopsis thaliana* affects cellular decisions in the shoot apical meristem.

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INTRODUCTION

1. INTRODUCTION

1.1 The shoot apical meristem (SAM) of *Arabidopsis thaliana*

1.1.1 The organization and the properties the SAM

The aerial structures of higher plants are dynamically generated throughout the life cycle by the activity of pluripotent stem cells that are located at the growing shoot tip, the SAM. During the vegetative stage in *Arabidopsis*, plants have an indeterminate SAM that generates leaves repetitively in a stereotypical pattern known as phyllotaxy, and can, theoretically, grow indefinitely. When the floral transition process begins, the shape of the SAM changes (both in width and height) and it generates floral meristems and a bolting inflorescence shoot decorated with cauline leaves. Floral meristems, in contrast with SAMs, are determinate—they will be terminated after the formation of flowers and do not grow indefinitely. The SAM of *Arabidopsis thaliana*, and most other dicotyledonous plants, is organized into distinct layers and zones. The first level of SAM organization is the stratification of the cells into tunica and corpus layers. The tunica consists of the L1 layer (the outermost layer that is just one cell thick) and the L2 layer (also one cell thick and lies beneath L1). In both L1 and L2, cell divisions are anticlinal, that is, the new cell walls are formed perpendicular to the surface of the meristem, thus maintaining the organization of these layers. The L1 layer gives rise to the epidermis and the L2 layer forms the subepidermal layer and the gametes. The corpus, or the L3 layer, which lies beneath the tunica, has variable patterns of division and it transitions into the stem at the meristem base. The SAM is further organized into three functionally distinct zones. The peripheral zone (PZ) and the rib zone (RZ) contain cells that will become incorporated into lateral organ primordia and the stem core, respectively. The central zone (CZ), which is surrounded by the PZ and characterized by a lower mitotic activity, constitutes the self-renewing pluripotent stem-cell reservoir. Cell divisions in the CZ cause displacement of daughter cells outward into the PZ. Once the progeny of the stem cells have left the CZ, they are recruited for organogenesis and eventually differentiate. Cells in the CZ also divide downward into the RZ, which contributes to the meristem pith. The CZ concomitantly replenishes itself through these cell divisions. In this way, the SAM mediates plant growth and sustains itself as a stable structure, in spite of the constant flow of cells passing through it (for reviews, see: Brand *et al.*, 2001; Carles and Fletcher, 2003; Doerner, 2003).
1.1.2 Maintenance of the homeostasis of the stem cell niche in the SAM

Genetic analyses in *Arabidopsis thaliana* have identified a number of genes involved in meristem function. SAM maintenance is disrupted by a loss-of-function mutation in the *WUSCHEL (WUS)* locus. The *WUS* gene encodes a homeodomain protein that is expressed in a small group of cells located in the meristem center underneath the presumed position of the stem cells (Mayer *et al.*, 1998). *wus* mutants do form an embryonic SAM. However, shoot meristems initiate repetitively in these mutants, but prematurely terminate in aberrant flat structures during the vegetative phase. In addition, *wus* inflorescence meristems produce fewer flowers compared with wild-type plants and those flowers usually terminate prematurely in a single stamen (Laux *et al.*, 1996). It has been proposed that this defective meristem phenotype results from a loss of stem cells in the CZ that, in turn, cannot sustain the meristem (Laux *et al.*, 1996). In contrast with *wus*, which causes premature meristems, loss-of-function mutations at the *clavata* loci (*clv1, clv2* and *clv3*) lead to enlarged meristem phenotypes. *clv* mutants not only have enlarged meristems but also have an increased number of organ primordia with an apparent altered phyllotaxy and supernumerary carpels at the floral center (Clark *et al.*, 1993). Similar phenotypes of all three *clv* mutants suggest that wild-type *CLV* genes function in the same genetic pathway. The *CLV1* gene encodes a receptor-like kinase that contains an extracellular domain composed of 21 tandem leucine-rich repeats (LRR) and a predicted cytoplasmic domain that acts as a serine kinase, suggesting a role in signal transduction. *CLV1* transcripts are detected in a patch of cells across the center of the meristem in the L2 layer and predominately in the L3 layer (Clark *et al.*, 1997). The *CLV2* gene encodes a receptor-like protein with LRRs; however, its cytoplasmic tail is short and lacks a kinase domain (Jeong *et al.*, 1999). *CLV2* transcripts have been detected in shoots and flowers based on RNA gel blot analysis (Jeong *et al.*, 1999), but its precise domain of expression in meristems has yet to be determined. The *CLV2* protein is required for the accumulation of *CLV1* and its assembly into protein complexes, indicating that *CLV2* may form a heterodimer with *CLV1* to transduce extracellular signals (Jeong *et al.*, 1999). The *CLV3* gene encodes a protein of 96 amino acids and an 18-amino acid long, NH2-terminal hydrophobic region in this protein may function as a signal peptide to direct the protein into the secretory pathway (Fletcher *et al.*, 1999). *CLV3* transcripts are detected in the upper two layers of the CZ and in a few underlying L3 cells, and its expression domain is proposed to be a molecular marker for stem cells (Fletcher *et al.*, 1999). Based on analysis of expression domains, *CLV3* transcripts are largely found beneath the
CLV1 expression domain, suggesting that CLV1-expressing cells may communicate with CLV3-expressing cells through a signal transduction pathway. Consistent with this, it has been reported that CLV3 is localized to the extracellular space, and that this apoplastic localization is required for CLV3 to activate a hypothesized CLAVATA signaling pathway (Rojo et al., 2002). Furthermore, it has also been shown that CLV3 signaling occurs through a CLV1/CLV2 receptor complex (Brand et al., 2000).

In an enlarged meristem of a clv3 mutant, the WUS expression domain is expanded, and ectopic expression of WUS is observed in clv3 embryos and floral meristems (Schoof et al, 2000). On the other hand, in an arrested meristem (reminiscent of the wus phenotype) of a plant overexpressing CLV3, WUS transcripts were not detectable by RNA in situ hybridization (Brand et al., 2000). These results indicate that CLV signaling negatively regulates WUS expression. The wus clv double mutants have a phenotype indistinguishable from wus single mutants during vegetative development, suggesting that these genes act in a common pathway and WUS is required for the clv phenotype (Laux et al., 1996; Schoof et al., 2000). WUS expression under the control of the CLV1 promoter leads to expansion of the meristem and the expression of CLV3, suggesting that CLV3 is controlled by WUS (Schoof et al. 2000). These observations lead to the development of a model for homeostasis within the stem cell niche. WUS and CLV comprise a feedback loop, such that WUS acts cell-nonautonomously to promote stem-cell identity and CLV3 expression through an as yet unknown signal, while the CLV3 polypeptide, in turn, acts as a ligand to bind to the CLV1/CLV2 receptor complex thus activating the signaling pathway that represses WUS expression. Within this loop, stem cell identity is established and maintained by the signal from the organizing center (OC) where WUS is expressed, and the OC size is then limited by the signal gave back from stem cells where CLV3 is expressed.

1.2 The SHOOT MERISTEMLESS (STM) gene

1.2.1 STM functions in initiating and maintaining the SAM

In addition to the stem-cell homeostasis in the CZ described above, another important question is how the entire SAM is initiated and maintained. In maize, the homeobox gene KNOTTED1 (KN1) has proved to be a useful molecular marker for SAM (Smith et al., 1995). The onset of KN1 expression during embryogenesis coincides with the first histological features that
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characterize SAM formation in maize (Smith et al., 1995) and expression persists in the vegetative SAM, axillary meristems, terminal and lateral inflorescence meristems (tassel and ear, respectively), and in both male and female floral meristems (Smith et al., 1995). Loss-of-function mutations in knl gene are defective in shoot-meristem maintenance (Kerstetter et al., 1997). In Arabidopsis, the KNOTTED-like homeobox (KNOX) genes, which are defined by homology to the maize KN1 gene, comprise eight members. The best characterized of these is the STM gene. Like other KNOX genes, the STM gene encodes a homeodomain protein belonging to a superfamily — the three amino acid extension (TALE) family. However, in addition to the TALE homeodomain (TALE-HD), the STM protein (and other KNOX proteins) has a conserved ELK domain and a MEINOX (Cole et al., 2006) domain that may function in protein-protein interactions. STM expression is first apparent in early to mid-globular stage embryos, where it is found in one or two cells (Long et al., 1996). By the early heart stage of embryogenesis, expression is found in a continuous band between the presumptive cotyledons and by the torpedo and walking-stick stages of embryogenesis, the expression is confined to the tip of the embryo, where the primary meristem is located (Long et al., 1998).

A loss-of-function allele, stm-1, was identified and its seedlings were shown to lack a SAM but were otherwise healthy and viable, suggesting that STM functions specifically in the establishment of the SAM (Barton and Poethig, 1993). Furthermore, the configuration of cells in the apical position of stm-1 end-stage embryos and young seedlings was similar to that seen in torpedo stage embryos, revealing that the stm-1 completely blocks the initiation of the SAM at, or just after, the torpedo stage of embryogenesis (Barton and Poethig, 1993). Tissue cultures from stm-1 seedlings grown in medium that promotes shoot regeneration only produced abnormal leaves or shoots and gave rise to fewer such structures than wild-type tissue (Barton and Poethig 1993). stm-1 mutation thus affects the initiation of the SAM in culture (Barton and Poethig 1993). These results indicate that STM locus is required for SAM initiation both embryonically and postembryonically (Barton and Poethig 1993). Taken together, these results indicate that STM is required for the establishment of a functional primary SAM.

STM expression persists into the seedling and adult plant, where it is present in all SAMs: vegetative, axillary, inflorescent and floral (Long et al., 1996). Analysis of the defects in an allelic series of stm mutants showed that all postembryonic structures can be formed in all alleles, however, stm mutation often leads to fused primordia (Endrizzi et al., 1996). stm floral
meristems also displayed similar defects and terminated prematurely in central stamens (Endrizzi et al., 1996), which are reminiscent of \textit{wus} mutants, thus implying loss of the meristem activity. The persistence of \textit{STM} expression and \textit{stm} phenotypes suggest that \textit{STM} activity is required for SAM maintenance, as well as initiation.

1.2.2 \textit{STM} maintains the indeterminate state of cells in meristems and is downregulated in lateral organ primordia

\textit{STM} is expressed throughout the shoot tip, yet there are regions that lack expression. These \textit{STM}-negative regions, which coincide with early steps (P0) in the development of lateral organ primordia, indicate that \textit{STM} is downregulated while organ primordia are initiated (Long et al., 2000). Thus, \textit{STM} expression is specifically confined to indeterminate cells within shoot meristems. Furthermore, loss-of-function \textit{stm} mutant seedlings develop cotyledons and leaves that are fused at their bases, suggesting that cells in the center of \textit{stm} shoot meristems, which in wild-type plants remain undifferentiated, appear to become incorporated into ectopic primordia and undergo differentiation (Endrizzi et al., 1996). Therefore, the indeterminate state of cells within the SAM is dependent on the \textit{STM} gene. In addition, downregulation of \textit{STM} in lateral organ primordia has been shown to be a critical event in organ development, as ectopic expression of \textit{STM} disrupts normal leaf development (Chuck et al., 1996; Williams, 1998). Hence, lack of \textit{STM} expression is a useful molecular marker for lateral organ primordia.

In \textit{Arabidopsis}, two other \textit{KNOX} genes, \textit{KNAT1} and \textit{KNAT2}, have similar expression patterns to \textit{STM} (Lincoln et al., 1994; Serikawa et al., 1996); both genes are expressed throughout the vegetative SAM but are specifically excluded from initiating leaf primordia and mature leaves. Based on the similarity of residues within the homeodomain of the proteins, the intron positions and the expression patterns (Hake et al., 2004; Reiser et al., 2000), \textit{STM}, \textit{KNAT1} and \textit{KNAT2} are referred to as class I \textit{KNOX} genes. Overexpression of \textit{KNAT1} in transgenic \textit{Arabidopsis} altered leaf development and caused the formation of ectopic meristems (Chuck et al., 1996; Lincoln et al., 1994). Thus, exclusion of class I \textit{KNOX} gene activity from peripheral founder cells appears to be crucial in leaf cell fate acquisition.

Plants with a loss-of-function mutation in \textit{ASSYMETRIC1 (AS1)} or \textit{AS2} have a phenotype similar to \textit{KNAT1} overexpression, suggesting that \textit{AS1} and \textit{AS2} genes negatively regulate \textit{KNAT1}
expression (Byrne et al., 2000; 2002). The AS1 gene encodes a Myb domain transcription factor and its transcripts are first detectable in late globular stage, maintained in developing cotyledons, but absent in cells that subsequently form the SAM (Byrne et al., 2000). Later, its transcripts are detected in leaf founder cells from the time of primordium initiation until stage P4 (Byrne et al., 2000). Genetic interactions between as1 and stm demonstrate that as1 can rescue the stm phenotype in embryonic and vegetative meristems (Byrne et al., 2000), and AS1 expression spreads throughout the apical region in stm embryos (Byrne et al., 2000). These results suggest that STM negatively regulate AS1 expression. as1 leaf phenotype is comparable with as2 mutants, which have class I KNOX genes also miss-expressed (Ori et al., 2000; Semiarti et al., 2001). AS2 is also negatively regulated by STM and it is likely that it interacts with AS1 and negatively regulates other class I KNOX genes (Byrne et al., 2002). These observations generate a model of mutual negative interactions between MYB and class I KNOX genes. In the wild-type meristem STM is on and it keeps AS1 and AS2 off. This allows the class I KNOX genes and other targets required for meristem function to be on. In leaf founder cells, all class 1 KNOX genes are downregulated by some unknown mechanism. In slightly older leaf primordia (P2 stage and beyond) the activity of AS1 and AS2 maintains KNAT genes repression while another yet unknown factor maintains STM repression (Barton, 2001; Byrne et al., 2002). Together, this pathway functions to distinguish organ founder cells from meristem cells in the SAM, allowing for tightly controlled regulation of lateral organ cell fate.

1.2.3 STM and its interacting partners

TALE homeobox genes are found in plants, animals and fungi (Hake et al., 2004; Reiser et al., 2000), and the interactions between TALE-HD proteins, which have important functions in the developmental processes of both animal and fungi, have been explored (Bellaoui et al., 2001). It has been shown that the Arabidopsis KNOX TALE-HD proteins interact with BELL1-like homeodomain (BLH) proteins, also TALE-HD proteins, through the N-terminal MEINOX domain (Bellaoui et al., 2001; Muller et al., 2001; Smith et al., 2002). The KNOX/BLH complex mediates high affinity DNA binding in vitro and this interaction is selective, suggesting that KNOX proteins have a greater affinity for certain BLH proteins than for others (Bellaoui et al., 2001; Smith et al., 2002). The overlapping patterns of BEL1 and STM expression within the inflorescence meristem suggest a function for the BEL1/STM complex in maintaining the indeterminate status of the inflorescence meristem (Bellaoui et al., 2001). Three BLH proteins,
ATH1, BLH3 and BLH9, were identified as interacting partners of STM in yeast two-hybrid studies (Cole et al., 2006). As a potential transcriptional factor, STM would be expected to be localized to the nucleus. However, fusion proteins of STM and GFP are cytoplasmic when they are expressed in leek, onion or Arabidopsis hypocotyl epidermal cells (Cole et al., 2006), suggesting that the STM protein is not located in the nucleus by default. Conversely, bimolecular fluorescence complementation (BiFC) substantiates the nuclear localization of STM through heterodimerization with the previously mentioned BLH partner proteins (Cole et al., 2006). ATH1, BLH3 and BLH9 are expressed in the inflorescence meristem (IFM) in discrete domains, suggesting that the IFM is divided into zones, from the center to the periphery (Cole et al., 2006). Furthermore, overexpression of these BLH genes affected flowering time, and when combined with inducible STM expression of STM-GR (Brand et al., 2002), additive phenotypes of early flowering were observed in the pre-conditioned ectopic ATH1 or BLH3 background, and a lobbed leaf phenotype was observed in the pre-conditioned ectopic BLH9 background (Cole et al., 2006). Taken together, these results suggest that STM may interact with specific BLH proteins in the center and periphery of the shoot apex to contribute to different functions.

1.3 Plant hormone auxin

The plant hormone auxin has divergent functions in various developmental processes. Auxin is actively distributed within the plant by efflux- and influx-dependent cell-to-cell movement. The direction of auxin flow was suggested to be mediated by the asymmetric cellular localization of efflux carriers, probably represented by plant-specific PIN-FORMED (PIN) proteins.

During embryogenesis, the auxin response gradient is first established basipetally and at about the 32-cell stage, is reversed to become acropetal (Friml et al., 2003). Application of auxin-efflux inhibitors perturbs the polar distribution pattern of auxin during embryogenesis, suggesting that the polar distribution of auxin in embryos is mediated by auxin efflux (Friml et al., 2003). The polar distribution of auxin establishes an apical-basal axis that is crucial for embryogenesis. Disruptions of the polar distribution of auxin are always accompanied by embryo defects (Friml et al., 2003). Auxin transport and its polar distribution also have key functions in shoot organogenesis. During the establishment of the lateral organ primordia of shoots, auxin is transported to, and accumulated at, the primordium tip to establish an auxin gradient prior to lateral organ formation (Benková et al., 2003). Treatment of the SAM with auxin-efflux
inhibitors results in a pin-like inflorescence, without any lateral organ (Benková et al., 2003).

PIN proteins are membrane proteins and are known as auxin-efflux facilitators (Galweiler et al., 1998). There are 8 members of PIN protein family in Arabidopsis (Friml, 2003; Friml et al., 2003), but only 4 of them, PIN1, PIN3, PIN4 and PIN7, are expressed in embryos and are implicated in auxin transport (Friml et al., 2003). During embryogenesis, the initial auxin-dependent specification of the apical cell relies on the efflux activity of the PIN7 protein that is localized on the apical face of the basal cell. At approximately the 32-cell stage, auxin flux is reversed following retargeting of both PIN1 and PIN7 proteins to the basal faces of provascular and basal cells, respectively (Friml et al., 2003). Multiple combinations of pin mutants revealed functional redundancy among PIN proteins (Friml et al., 2003). The PIN efflux-carrier activity is also required during the formation of lateral organ primordia. Recent data provide evidence that auxin is transported towards founder cells of new organ primordia involving changes in the distribution of PIN proteins at the plasma membrane (Reinhardt et al., 2003).

In addition to embryogenesis and organogenesis, auxin also has a central role in vascular patterning. Throughout leaf development, auxin accumulation is restricted to subepidermal cell layers (Mattsson et al., 2003). Auxin is strongly accumulated at the distal tip in very early stage leaf primordia and is weakly accumulated in the incipient primary vein (Mattsson et al., 2003). As leaf primordia develop, auxin accumulation disappears in the mature primary vein but becomes apparent where incipient secondary veins emerge, and then in a series in tertiary and quaternary veins, and finally completely disappears in mature vessels (Mattsson et al., 2003). As the primary midvein develops acropetally, and secondary veins develops in a basipetal sequence, it has been proposed that auxin induces the formation of a vascular strand along which auxin is subsequently transported in a polar fashion (Reinhardt, 2003). Thus, auxin accumulation precedes oriented cell divisions, which produce continuous files of narrow procambial cells, and disappear at later differentiation stages.

1.4 Tetraspanin proteins

Extracellular stimuli, such as CLV3 ligands and some plant hormones, have to interact with membrane proteins to transduce signals through distinct signaling pathways. This crosstalk between cell-surface stimuli and signaling pathways needs to be regulated. In animals, the
regulation of cell-surface stimuli and signaling pathways can be modulated by tetraspanin proteins. Tetraspanins are a large family of transmembrane proteins that form ‘tetraspanin-enriched microdomains’ (TEMs) on the cell membrane and function as specific membrane docks — they cluster associated membrane proteins and interact with intracellular signaling proteins.

### 1.4.1 Tetraspanin proteins are implicated in divergent biological processes

To our knowledge, there are at least 33 tetraspanins in mammals, 36 in *D. melanogaster* and 20 in *C. elegans* (Hemler, 2005; Levy and Shoham, 2005; Huang, 2005). In humans and mice, CD81, which was originally identified as a target of an anti-proliferation antibody on human B cells (Oren et al., 1990), is present in nearly all cell types. The regulation of cell proliferation by CD81 has been shown to be positively associated with the recruitment of the adaptor protein, Shc, to the plasma membrane and the activation of ERK/MAPK pathway (Carloni, 2004; Hemler, 2005). CD81 at the surface of B cells directly associates with CD19, a signaling molecule, and CD19 associates with the complement receptor CD21, which lacks a signaling domain. Moreover, CD81 is required for the membrane reorganization that is induced in response to co-engagement of the B-cell receptor (BCR) and CD19/CD21 complex (Levy and Shoham 2005) and this co-engagement of associated proteins is sufficient to increase the activity of the B-cell response (Levy and Shoham 2005). This did not occur in *Cd81*−/− mice (Levy and Shoham 2005). CD81 has also been identified as a cell surface receptor for HCV (Pileri et al., 1998). However, CD81 is required, but not sufficient, for HCV infection (McKeating et al., 2004). In *D. melanogaster*, a mutant of tetraspanin gene, *late bloomer (lbm)* has been unambiguously linked to the formation of synaptic contacts at neuromuscular junctions in the embryo (Kopczynski et al., 1996), and two additional tetraspanin genes also contribute to timely embryonic synapse formation (Hemler, 2005). However, deletion of 9 of the 36 tetraspanin genes in *D. melanogaster* did not cause severe developmental abnormalities (Huang et al., 2005), suggesting redundancy among tetraspanins. In *C. elegans*, the TSP-15 tetraspanin protein functions to maintain epithelial integrity (Hemler, 2005). In addition to those tetraspanins found in animals, tetraspanins are also found in fungi. In the fungal plant pathogens *Magnaporthe grisea* and *Botrytis cinerea*, Mgpls1 and Bcpls1, respectively, are required for fungal penetration into the leaves of the host plant (Gourgues et al., 2004; Clergeot et al., 2001).
The tetraspanins described above provide a general overview of the functions of tetraspanin proteins. These proteins are widely expressed in divergent cell and tissue types, and are implicated in diverse biological processes. A phylogenetic tree of tetraspanins has been established and shows that all tetraspanins rise from a common ancestor (Huang et al., 2005), suggesting that tetraspanins are highly conserved during evolution, and moreover, that tetraspanins have a uniform architecture, suggesting that they may function with similar mechanisms.

1.4.2 Tetraspanins structural properties and the TEM

Tetraspanins are proteins containing 200-350 amino acids. The conventional tetraspanin structure consists of four transmembrane (TM) domains, two extracellular loops and short cytoplasmic N- and C-tails. The first, third and fourth TM domains often contain polar residues of unknown function, and almost all tetraspanins are post-translationally modified by the addition of palmitate to the cystine residues proximal to the membrane. TM domains 1 and 2 flank a small extracellular loop (SEL) containing 13-30 amino acids, whereas TM domains 3 and 4 flank a large extracellular loop (LEL) containing 61-150 amino acids. The structure of the SEL has not yet been determined, but analysis of the crystal structure of a recombinant soluble form of the LEL of CD81 has determined some properties of LEL. The LEL is subdivided into a constant region that contains three α-helices (termed A, B and E), and a variable region (located between the helices B and E) that contains an almost 100% conserved CCG motif and two other absolutely conserved cysteins. The CCG motif in the LEL is characteristic of tetraspanins and is often called the ‘tetraspanin signature’. The number of cystein residues in the LEL varies considerably among tetraspanins; most contain four or six cystein residues, and a few contain eight cystein residues, which allows the formation of two, three or four disulfide bridges, respectively. The variable region of LEL is folded, as a result of disulfide bridges, to form a mushroom-like structure, and this region contains nearly all known tetraspanin protein-protein interaction sites (for reviews, see: Hemler, 2001; Hemler, 2003; Levy and Shoham 2005; Huang et al., 2005). Distinct tetraspanin structural domains are associated with specific functions. In general, LELs mediate protein-protein interactions with laterally associated proteins and a few known ligands, TM domains mediate the stability and the dimerization of tetraspanins, and the cytoplasmic regions provide links to cytoskeletal and signaling molecules (Levy and Shoham, 2005).
The first level of tetraspanin interactions is primary interaction — homozygous or heterozygous proteins interacting with each other directly. The CD81 LEL crystals consist of homodimers, which implies that homodimerization occurs through the LEL. In addition, the results obtained by covalent crosslinking of CD81 and CD9 indicate that tetraspanin homodimers (as well as trimers and tetramers) may be the fundamental primary units within TEMs (Levy and Shoham, 2005; Hemler, 2005). Furthermore, tetraspanin homophilic crosslinking far exceeds heterophilic crosslinking (Hemler, 2005), and tetraspanin homodimers are reported to be formed in the Golgi and have been suggested to be the common core unit for the assembly of multicomponent tetraspanin-tetraspanin and tetraspanin-partner complexes (Levy and Shoham, 2005).

The next level of tetraspanin interactions involves the assembly of primary, direct homophilic and heterophilic complexes into a network of secondary interactions (Hemler, 2005). The tendency for heterophilic association between tetraspanins is essential for this assembly. In this manner, different partner proteins can be recruited, through tetraspanins, into functionally important complexes (Hemler, 2005). Therefore, tetraspanin proteins and their associated proteins form TEMs in the plasma membrane to provide a scaffold for the transmission of external stimuli to intracellular-signaling components and to modulate biological processes (Levy and Shoham, 2005).

1.5 The aim of this project

*STM* plays an important event to establish the primary SAM during embryogenesis and to maintain meristem activity throughout the plant lifecycle, and it has to be shut down during the initiation of lateral organ primordia; however, little is known about its regulation. In this study, the analysis of a mutant obtained from a transcriptional effector-screen performed with a *STM:GUS* marker line following EMS mutagenesis is described. The mutant is characterised by an altered *STM:GUS* expression domain and SAM morphology. The responsible mutation was shown to be allelic to *tornado2* (*trn2*) and provides evidence that plant tetraspanin-like proteins such as TRN2 contribute to SAM function. Molecular markers indicate that the size of the central stem cell zone is minor affected although the SAM is significantly enlarged compared to wild type.
2. MATERIALS AND METHODS

2.1 Plant materials and growth conditions

Arabidopsis thaliana seeds were sowed on the soil and kept at 4°C in the dark for 3 days and then transferred to a greenhouse. When elongated hypocotyls could be seen (two days after transferred to a greenhouse), the day was defined as 0 day-after-germination (0 DAG). Plants were grown on soil under 16-h-light/8-h-dark regime (long-day conditions) at 22°C.

Columbia-0 (Col) ecotype was used as the wild-type in all experiments. clavata3-2 (clv3-2), wuschel-1 (wus-1), shoot meristemless-1 (stm-1) and shoot meristemless-5 (stm-5) seeds were obtained from The European Arabidopsis Stock Center (NASC). tornado1-1 (trn1-1), tornado2-1 (trn2-1) and tornado2-2 (trn2-2) seeds were a kind gift from Cnops G..

The STM-GUS transgenic line was created and their seeds were treated with methanesulfonate (EMS). 0.3g STM-GUS transgenic seeds were treated with 0.1% Tween 20 for 15 minutes and then immersed in 15 ml H2O contains 37 µl 100% EMS overnight (Koenig 1999).

2.2 Chemicals and enzymes

All chemicals of analytical quality have been purchased from Bilmol (Hamburg), Biozyme (Hameln), Fluka (Neu-Ulm), Life Technologies (Karlsruhe), Merck-Eurolab (Darmstadt), Pharmacia (Freiburg), Roche Dianostics GmbH (Mannheim), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (Dewasenhof).

Enzymes were purchased from Firmen Gencraft (Muenster), Invitrogen GmbH (Karlsruhe), New England Biolabs (Frankfurt am Main), Promega GmbH (Mannheim), Roche Diagnostics GmbH (Mannheim) and Stratagene (Heidelberg). Concentrated (10 x or 5 x) buffers were supplied with each enzyme and used according to the manuscript suggests.

Expendable materials were from Roth (Karlsruhe) and Sarstedt (Rommelsdorf).
2.3 Buffers, solutions and media

All routine used solutions and buffers for culturing E. coli were described before by Ausubel et al. (1996). All buffers, solutions and media were prepared with double distilled H$_2$O (ddH$_2$O, Milli-Q, Water-Clinging-System, Millipore Eschborn). Solutions used for molecular biology experiments were autoclaved or sterilized with filters (0.2 µm filter, Roth).

2.4 Bacteria and vectors

2.4.1 Bacteria strains

DH10B  
F, endA1, Δ(mrr-hsdRMS-merBC), Φ80dlacZΔM15, ΔlacX74, deoR, recA1, araD139, Δ(ara, leu)7697, galU, galKX$, rpsL, nupG
(Invitrogen)

TOP 10  
F, mrcA, Δ(mrr-hsdRMS-merBC), Φ80dlacZΔM15, ΔlacX74, recA1, deoR, araD139, Δ(ara, leu)7697, galU, galK, rpsL(Str$^R$), endA1, nupG
(Invitrogen)

2.4.2 Vectors

pCR$^®$II-TOPO  
(Invitrogen)

2.5 Oligonucleotides

Oligonucleotides were obtained from Eurogentec (Seraing, Belgien) or Sigma (Deisenhof).

Primers used in this study of co-dominant cleaved amplified polymorphic sequences (CAPS) markers were described before by Konieczny and Ausubel (1993) or in the TAIR databank (http://www.arabidopsis.org).
MATERIAL AND METHODS

Primer sequences for amplifying specific gene fragments for making probes of RNA in situ hybridization:

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Oligonucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TRN2</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>ATG CCT TTA AGC AAC AAT GTA ATT</td>
</tr>
<tr>
<td>Reverse</td>
<td>TCA AGT ATA ACC CTG CTT GTA CT</td>
</tr>
<tr>
<td><strong>WUS</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GCA TCA GCA TCA TCA TCA TCA A</td>
</tr>
<tr>
<td>Reverse</td>
<td>CTA GTT CAG ACG TAG CTC AAG AGA</td>
</tr>
<tr>
<td><strong>CLV3</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CAG TCA CTT TCT CTC TAA AAA TGG</td>
</tr>
<tr>
<td>Reverse</td>
<td>TCA AGG GAG CTG AAA GTT GTT T</td>
</tr>
<tr>
<td><strong>LFY</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>ATG GAT CCT GAA GGT TTC ACG AG</td>
</tr>
<tr>
<td>Reverse</td>
<td>CTA GAA ACG CAA GTC GTC GC</td>
</tr>
</tbody>
</table>

For *STM* RNA in situ hybridization, the probe was described before by Long et al., (1996).

Primer sequences for amplifying gene fragments for genotyping:

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Oligonucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>trn2_3010</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CAC TTA ATC AGT CAC GCC GAA CTA C</td>
</tr>
<tr>
<td>Reverse</td>
<td>GAC GAT AAT CAA TCC GAT AAG C</td>
</tr>
<tr>
<td><em>stm-1</em></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GTC AAT TCA AAT CCC TCT CTC TA</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCT TTC CTT TCT TCC TCT TCT TC</td>
</tr>
<tr>
<td><em>stm-5</em></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GAA GAA GAG GAA GAA AGG AAA GC</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCA ATG CCA ACA TGA GCT AAC</td>
</tr>
</tbody>
</table>
Primers for real-time PCR were designed by Applied Biosystems (TaqMan® Gene Expression Assays). A non-inventoried assay At02329915_s1 (ACTIN 2) was used as an endogenous control, but the sequences of primers were not given.

**Primer and probe sequences for real-time PCR:**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Oligonucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>STM</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GCC GCT TAT GTC AAT TGT CAG AA</td>
</tr>
<tr>
<td>Reverse</td>
<td>GAC GAG CAT GCC TCC TCT AG</td>
</tr>
<tr>
<td>Probe</td>
<td>FAM CCA CCG GAG GTT GTG NFQ</td>
</tr>
<tr>
<td>WUS</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GGA TCA TCA TTA CTC ATC TGC ACC TT</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCC ACC ACA TTC TTC TTC TTG A</td>
</tr>
<tr>
<td>Probe</td>
<td>FAM TTC GAT AGA GCA AAG CCT NFQ</td>
</tr>
<tr>
<td>CLV3</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CAG ATC TCA CTC AAG CTC ATG CT</td>
</tr>
<tr>
<td>Reverse</td>
<td>CCA ACC CAT TCA CTT TCC ATT TTC A</td>
</tr>
<tr>
<td>Probe</td>
<td>FAM ACG TTC AAG GAC TTT CC NFQ</td>
</tr>
</tbody>
</table>

FAM, 6-carboxy-fluorescein.
NFQ, nonfluorescent quencher.
MATERIAL AND METHODS

2.6 Genetic mapping

The mapping work was carried out by using CAPS markers (Konieczny and Ausubel, 1993).

Heterozygotes of the interesting line (Colombia ecotype), which was isolated from the mutagenesis screen, were crossed to Landsberg erecta plants. 30 mutant plants of the F2 population were picked up, and the genomic DNA was extracted. DNA fragments of CAPS markers were amplified by polymerase chain reaction (PCR) by using specifically designed primers distributed over the genome of Arabidopsis (Konieczny and Ausubel, 1993, TAIR databank). To identify restriction fragment length polymorphism (RFLP) between the amplified Col and Ler sequences, the PCR products were digested with specific endonucleases (Konieczny and Ausubel, 1993, TAIR database), and the ecotype of origin of these sequences could thus be determined. The mutant locus then could be unambiguously mapped to one of the 10 Arabidopsis chromosome arms.

2.7 Molecular biology methods

2.7.1 Standard molecular biology methods

All molecular biology standard methods were described before (Ausubel et al., 1996). Other materials (enzymes and chemicals) were described above.

2.7.2 Transformation of bacteria (E. coli)

Electrocompetent E. coli was used for transformation (Muehlhardt, 2000). 50 µl Electrocompetent E. coli with 1 µl vector was added into a pre-cooled 0.1 cm electroporation cuvette and then placed in a GenePulser™ (BIORAD). The condition for electroporation was: capacity 25 µF, voltage 1.8 kV, and resistor 200Ω.
2.7.3 Preparation of plasmid DNA

“Alkaline lysis protocol” (Sambrooke et al., 1989) was used for plasmid-mini preparation. Large amount of DNA or DNA-probes for sequencing or in situ hybridization were prepared by the kits (Plasmid Midi or Maxi kit, Qiagen).

2.7.4 The extraction of the genomic DNA from Arabidopsis thaliana

Up to 200 mg plant tissues (1-2 young leaves or 3-4 inflorescences) were harvested and placed in a 1.5 ml tube and frozen immediately with liquid nitrogen. The tissue was crushed by a micropistil against the tube wall. After crushing, 600 µl EB-buffer (100 mM Tris pH 7, 50 mM EDTA, 500 mM NaCl. Add 2 µl RNase (10µg/µl) and 12 µl β-mecaptoethanol into the EB buffer before use) was added and the tube was vigorously vortexed to suspend the sample powder. After vortexing, added 40 µl 20% SDS (mixed well, did not vortex) and 200 µl 5M KAc (mixed well). The tube was incubated at 68°C for 15 minutes to homogenize the tissue and then placed on ice for 20 minutes. Centrifuged the tube at full speed for 2 minutes. Removed the supernatant to a new 1.5 ml tube and added 0.6 volume isopropanol into the supernatant and mixed well. To precipitate genomic DNA, centrifuged the tube at full speed for 5 minutes, and then washed the pellet by 70% EtOH. Slightly dried the pellet and dissolved it in 50 µl TE buffer. The genomic DNA was then stored at 4°C for several months.

2.7.5 The extraction of total RNA from Arabidopsis thaliana and the synthesis of cDNA

The RNeasy Plant Mini Kit (QIAGEN) was used to extract total RNA of Arabidopsis thaliana. On-column DNA digestion was performed during RNA purification. 500 ng – 2 µg total RNA was used to synthesize cDNA. Oligo(dT) was used to hybridize to 3’ poly(A) tails, which were found in the vast majority of eukaryotic mRNAs. SuperScriptII™ reverse transcriptase (Invitrogen) was used to synthesize cDNAs at 48°C for one hour. After RNaseH treatment, cDNA was used as templates for real-time PCR.
2.7.6 Polymerase chain reaction (PCR)

In a standard PCR reaction, 100 pg to 10 ng DNA was used as the template. 1-2 U Taq-polymerase (Invitrogen) was added to the reaction mixture (1X PCR reaction buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.2-1.0 μM primers). The final volume of the reaction mixture was 50 μl. The polymerase chain reaction was taken place in a MJ Research Thermoblock (Biozyme, Hameln) using the following program:

1. Initial denaturation: 1 minute 94°C
2. Denaturation: 30 seconds 94°C
3. Annealing: 30 seconds at the appropriate temperature of the primer pairs
4. Elongation: 1 minute/kb PCR fragment at 72°C
5. Cycle number: repeat 2-4 for 39 times
6. Final elongation: 10 minutes at 72°C
7. Pause 4°C forever

2.7.7 Real-time RT-PCR

2.7.7.1 The principle of real-time PCR

The real-time PCR system is based on the detection and quantitation of a fluorescent reporter. This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value measured during the 3-15 cycles (usually the exponential phase) indicates the detection of accumulated PCR product.

A fixed fluorescence threshold is set significantly above the baseline that can be altered by the operator. The parameter Ct (cycle threshold) is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold. Ct is the most important parameter for quantitation. The higher the initial amount of genomic DNA, the sooner accumulated product is
MATERIAL AND METHODS

detected in the PCR process, and the lower the Ct value. The threshold should be placed above any baseline activity and within the exponential increase phase (which looks linear in the log transformation). The log phase provides the most useful information about the reaction. Some software allows determination of the cycle threshold (Ct) by a mathematical analysis of the growth curve. This provides better run-to-run reproducibility. A Ct value of 40 or higher means no amplification and this value cannot be included in the calculations. Besides being used for quantitation, the Ct value can be used for qualitative analysis as a pass/fail measure. To calculate the relative change of a gene in different samples, Ct values have to be normalized. A simple way to normalize Ct values is that using the Ct value of the targeted gene to minus the Ct value of the reference gene, and the normalized Ct is called delta Ct. Delta Ct can be used to compare changes of gene expression directly or to calculate the relative expression change of a gene between two samples (Livak and Schmittgen, 2001).

TaqMan probes are oligonucleotides longer than the primers (20-30 bases long with a Tm value of 10°C higher) that contain a fluorescent dye usually on the 5′ base, and a quenching dye typically on the 3′ base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing (this is called FRET = Förster or fluorescence resonance energy transfer) (Hiyoshi, 1994; Chen, 1997). Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5′ exonuclease activity cleaves the probe (Holland, 1991). This ends the activity of quencher (no FRET) and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye (note that primers are not labeled). TaqMan assay uses universal thermal cycling parameters and PCR reaction conditions. Because the cleavage occurs only if the probe hybridises to the target, the origin of the detected fluorescence is specific amplification. The process of hybridization and cleavage does not interfere with the exponential accumulation of the product. One specific requirement for fluorogenic probes is that there be no G at the 5′ end. A 'G' adjacent to the reporter dye quenches reporter fluorescence even after cleavage. Well-designed TaqMan probes require very little optimisation.

Relative gene expression comparisons work best when the gene expression of the chosen
endogenous/internal control is more abundant and remains constant, in proportion to total RNA, among the samples. By using an invariant endogenous control as an active reference, quantitation of an mRNA target can be normalised for differences in the amount of total RNA added to each reaction. For this purpose, the most common choices are 18S RNA, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β-actin. Because the 18S mRNA does not have a poly-A tail, cDNA synthesis using oligo-dT should not be used if 18S RNA will be used as a normaliser. GAPDH is severely criticised as a normaliser (Dheda, 2004). Caution should also be exercised when 18S RNA is used as a normaliser as it is a ribosomal RNA species (not mRNA) and may not always represent the overall cellular mRNA population. Since the chosen mRNA species should be proportional to the amount of input RNA, it may be best to use a combination as normaliser. It is desirable to validate the chosen normaliser for the target cell or tissue. It should be expressed at a constant level at different time points by the same individual and also by different individuals at the target cell or tissue. It is important to choose a normalizer whose expression will remain constant under the experimental conditions designed for the target gene. The strategy of using multiple and variable normaliser genes depending on the cell and tissue type is validated for general use.

2.7.7.2 Preparation and manipulation of real-time RT-PCR

In this study, primers and probes for target genes were ordered, and designed by Applied Biosystems (TaqMan® Gene Expression Assays) (see nucleotides part). Primers and probes were mixed in the stock solutions. Oligonucleotides were at 100mM and had to be diluted to 1/20 when used. The stock solution should be aliquoted, frozen and kept in the dark. cDNAs were diluted to 1/8 for real-time PCR reactions to quantify STM and LFY expressions and to 1/4 for real-time PCR reactions to quantify WUS and CLV3 expressions. ACT2 was used as an endogenous control for each reaction.

Fifteen 11-DAG-old seedlings of Columbia and mutant plants were collected, and the total RNA was extracted. The amount of the total RNA was measured by a spectrophotometer using UV-260, and the ratio of A260/A280 was determined and should be between 1.8 and 2.2 to qualify the total RNA for making cDNA. Same amounts of the total RNA of wild-type and mutant plants were taken, and reverse transcriptions were carried out to make cDNAs. The cDNA was first tested by a normal PCR reaction using designed ACT2 primers, and the PCR product was
observed by an electrophoretic gel. There had to be a single band of the PCR product of ACT2 and no obvious differences between the wild-type and the mutant. The cDNA was then ready for real-time PCR reactions. The reaction mixture was prepared as follows: 4 µl TaqMan® Universal PCR Master Mix, diluted primer solution and appropriately diluted cDNA. The final volume of the reaction mixture is 8 µl. The reaction mixture was added into a 384-well plate, and real-time PCR reactions were taken place in an ABI Prism HT7900 machine (TaqMan®). SDS software version 2.2.1 (TaqMan®) was used for controlling each real-time PCR reaction. Ct values comparisons and t-tests were done by Excel software (version 11.1.1, Microsoft® Excel 2004 for Mac). Relative gene expression changes were compared by using the formula described before (Livak and Schmittgen, 2001). Real-time PCR reactions were repeated 4 times using 4 independently prepared cDNAs from 4 independently collected wild-type and mutant plants.

2.7.8 Non-radioactive RNA in situ hybridization

Full length cDNA fragments (TRN2, STM, CLV3, WUS and LFY) were amplified by primers described above and then cloned into pCR®II-TOPO (invitrogen) vectors. Vectors were linearized by appropriate enzymes and anti-sense RNA probes were then transcribed by SP6 or T7 polymerase.

Nonradioactive in situ hybridization experiments were performed essentially as described before (Bradley et al. 1993).

2.8 Histology

2.8.1 Fixation, embedment and sections of the plant tissue

2.8.1.1 Preparation of the fixative

4 g paraformaldehyde was dissolved in 100 ml pre-heated 1XPBS (pH=11) (10X PBS: 1.3 M NaCl, 0.07 M Na2HPO4, 0.03 M NaH2PO4). Placed the solution on ice till it decreased to room temperature, and then adjusted the pH value to 7 with concentrated H2SO4. Added 200 µl Tween20 to the fixative and kept it on ice.
2.8.1.2 Fixation of samples

To fix young seedlings or inflorescences, leaves must be removed. 20 samples could be putted into a 20 ml glass bottle filled with the fixative. 3 X 10 minutes vacuum-infiltration was performed till all samples were sunk, and then putted the fixed samples at 4°C overnight.

2.8.1.3 Embedment of samples

After fixation, exchanged the fixative for cold EtOH and performed series dehydration in 50%, 70%, 85% cold EtOH, each for 90 minutes. After serious dehydration, exchanged EtOH for 0.1% Eosin Y (dissolved in 100% EtOH), and putted the solution at 4°C overnight. In the next day, exchanged the solution for fresh 0.1% Eosin Y and putted it on ice for 90 minutes. After Eosin Y, exchanged the solution for 100% EtOH, 50% EtOH/50% Rotihistol and three times 100% Rotihistol, each for 60 minutes. After 100% Rotihistol, exchanged the solution for solution of 50% (v/v) fresh Rotihistol and 50% (v/v) Paraplast Pius®, and then putted the solution at 50°C overnight. In the next three days, exchanged the solution for fresh molten Paraplast Pius® in the mornings and evenings, and putted the solution at 60°C.

After three days solutions exchanges, samples had to be embedded into a wax block. A model within some molten Paraplast Pius® was placed on a warm plate (60°C). Molten Paraplast Pius® within samples was then poured into the pre-warmed model, and fresh molten Paraplast Pius® was added to cover all samples. Used a pre-heated tweezers to adjust samples to appropriate orientations, and then carefully putted the model in cold water. After solidification, the wax block was stored at 4°C for several months.

2.8.1.4 Sections

The sample-containing wax block was cut to be a cube with 1.5 cm side length. 7 µm thick sections were made by the microtome (Leica: Disposable microtome blade 819), and ribbon pieces were floated on a Superfrost plus slide (Menzel-Glässer / KEBO 113.720-0) covered with distilled H₂O. Placed the slide on a slide warmer (42 °C) for 5 minutes to allow the ribbon to
2.8.2 Microscopic technique

Light microscopy was performed with an Axioskop microscope with Nomarsky-Optik (Zeiss, Heidelberg, Germany) and an Axiocam camera (Zeiss, Heidelberg, Germany) was used. The DISKUS software package (Carl H. Hilgers-technwascches Buero, Koenigswinter, Germany; version 4.30.578) was used to take digital photos.

2.8.3 GUS staining

GUS-activity was assayed according to Sessions and Yanofsky, 1999. To allow complete penetration of the X-gluc-solution, plants were vacuum-infiltrated in staining buffer (0.2% Triton X-100, 50mM NaPO₄ pH 7.2, 2mM potassium-ferrocyanide K₄Fe(CN)₆*H₂O, 2mM potassium-ferricyanide K₃Fe(CN)₆ containing 2mM X-Gluc) for 15 to 30 minutes and afterwards incubated at 37°C from 5 minutes to overnight (the staining time was dependent on each individual transformed line). Clearing was performed by series dehydration in 50%, 70%, 85%, 95% and 100% Ethanol, each for 1 hour.

2.9 Computer analysis

The analyses of DNA and protein sequences were done by programs offered by Biology Workbench 3.2 (http://seqtool.sdsc.edu/CGI/BW.cgi). Primers were designed by the program Launch NetPrimer (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html). Chi squarer values were calculated by the web Chi squarer calculator (http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html). Sequences were obtained from databanks of NCBI (http://www.ncbi.nlm.nih.gov/) and TAIR (http://www.arabidopsis.org).
3. RESULTS

3.1 Identification of the mutant

To search for *STM* effector genes, an EMS-mutagenesis screen was previously carried out on a homozygous *STM:GUS* line (Kirch et al., 2003) where glucuronidase (GUS) activity reflected the activity of the *STM* promoter in the shoot meristem. The GUS activity in shoot tips represented the *STM* expression (Fig. 1), and changes in the GUS pattern therefore reflected affected *STM* activity. After self-fertilization of M1 progeny, the M2 generation was screened for differences in GUS expression at the early seedling stage. Among 4,900 independent M2 lines, two lines, line 3010 and 145 showed expansion of GUS activity domains in shoot tips of young seedlings, and they were subjected to further analyses.

![Figure 1](image.png)

**Figure 1.** The GUS activity in the shoot tip represents *STM* expression of a *STM:GUS* transgenic plant. (A) A *STM:GUS* young seedling shows the GUS stain in the shoot tip (red arrow). In this figure, one leaf in front was removed to make the shoot tip be clearly seen. The domain of GUS activity is consistent with the *STM* expression pattern, which is confined in the meristem.

Mutant plants of line 3010 displayed a unique phenotype of twisted organs (see the description of the phenotype below) and the population showed segregation ratios conforming to the 3:1 pattern that would be expected for a recessive single mutation. Furthermore, this phenotype was linked to the expanded GUS domain in mutant shoot tips (Fig. 2A). To understand the effects on the mutant SAM, serially longitudinal sections of wild-type and mutant seedlings were made and compared. The SAM of seedlings that are younger than one week old did not show obvious
discrepancies between wild-type and mutant seedlings. However, sections of 11-DAG-old mutant seedlings showed vast effects in the morphology of the SAM (Fig. 2C). Seventy-five percent of 11-DAG-old mutants had an enlarged and protruding SAM at the apex of the shoot, whereas the wild-type SAM was smooth and doom-shaped (Fig. 2B and C).

Figure 2. GUS stains in shoot tips of wild-type and homozygous 3010 mutant young seedlings. (A) A 7-DAG-old homozygous 3010 mutant seedling shows expanded GUS activity at the shoot tip. (B) The longitudinal section of a 11-DAG-old STM:GUS seedling reveals that the GUS stain is confined in the dome shaped SAM. (C) The longitudinal section of a 11-DAG-old homozygous 3010 mutant seedling reveals that the GUS stain is confined in the aberrant SAM, which is protruding and enlarged compared to wild-type (B).

Like homozygous mutants of the line 3010, mutant plants of the line 145 also had expanded GUS domain readily detected in shoot tips of young mutant seedlings (Fig. 3 A). However, at least two phenotypes, tiny-white plants and three cotyledons plants (Fig 3 B and C), were segregating in 145 populations during propagation. In addition, the linkage between mutant phenotypes and the expanded GUS domain was uncertain. Because the line 145 did not show a segregation pattern compatible with a single locus trait or a double mutant combination, further analysis therefore focused on the line 3010, which is described in this study.
Figure 3. Two phenotypes segregating in the line 145. (A) The young mutant seedling of the line 145 shows the GUS activity at the shoot tip not restricted to the SAM but expanded to leaf primordia. (B) and (C) at least two phenotypes segregating in the M3 population.

3.2 The mutant phenotype of the line 3010

3.2.1 The morphology of the mutant

Homozygous mutant 3010 plants are severely dwarfed, have twisted and malformed organs, and are sterile. Because of the sterility, this line was maintained and propagated heterozygously. Although homozygous 3010 seedlings did not show any obvious phenotype that could be observed by the naked eye in hypocotyls and cotyledons, mutant seedlings could be unambiguously identified after the emergence of the first pair of true leaves (Fig. 4A and B). Mutant leaves had irregular margins and asymmetric leave blades, and were reduced in size (Fig. 4C and D). In some cases, rosette leaves rotated 180° during development and hence the abaxial and adaxial sides were turned to the opposite directions. Interestingly, leaves usually lost half of the lamina, and in the most severe cases they lost their entire lamina and became pin-like structures (Fig. 4D, arrow head). Unlike wild-type leaves that have centrally located midribs, mutant leaves had thicker midribs, located near the center, or bifurcated ribs located marginally (Fig. 4F).

The twisted phenotype was not restricted to the vegetative phase but involved cauline leaves, the inflorescence stem, flowers and siliques (Fig. 4H-K). Mutant flowers were also twisted, asymmetric and smaller when compared with wild-type, and were accompanied by reduced numbers of floral organs in the outer 3 whorls (Table 1). This effect was least pronounced in the
whorl of sepals (3.2 as compared with 4 in wild-type), but the number of petals was reduced to 2.4 (compared with 4 in wild-type) and, on average, mutant flowers had only 3.5 stamens compared with 6 in wild-type (summarized in Table 1). In contrast with the outer three whorls, there were two carpels at the mutant flower center, same as the wild-type flower, suggesting that the number of carpels was unaffected. However, 52% of the mutant flowers had carpels that were not fused on their upper parts, whereas wild-type flowers always had a central style with well-fused carpels. Mutant stamens occasionally generated little sterile pollen, and thus, both male and female sex organs are defective in mutant flowers.

<table>
<thead>
<tr>
<th>Sepals</th>
<th>Petals</th>
<th>Stamens</th>
<th>Carpels</th>
<th>Flower having style with unfused Carpels</th>
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<tr>
<td>WT (n=31)</td>
<td>4.0 ± 0</td>
<td>4.0 ± 0</td>
<td>6.0 ± 0</td>
<td>2.0 ± 0</td>
</tr>
<tr>
<td>trn2_3010 (n=39)</td>
<td>3.2 ± 0.8</td>
<td>2.4 ± 0.8</td>
<td>3.5 ± 1.0</td>
<td>2.0 ± 0</td>
</tr>
</tbody>
</table>

Figure 4. The homozygous 3010 mutant plant shows pleiotropic phenotype. (A) A 7-DAG-old Columbia wild-type seedling compared to (B), a 7-DAG-old homozygous 3010 mutant seedling, which shows the first pair of true leaves smaller and twisted (see enlargements in (A) and (B)). (C) The first to sixth rosette leaf of Columbia wild-type compared to (D), the first to sixth rosette leaf of the homozygous 3010 mutant. The mutant leaves are twisted, reduced in size, and half of leaf laminas are usually missing. In the most severe case, leaves developed to a pin-like structure (red arrow head).
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Figure 4. (Continued) (E) The wild-type leaf has a midrib located centrally. (F) The mutant leaf usually has a broadened midrib located centrally or a bifurcated midrib as shown in the figure located marginally. (G) One-month-old Columbia wild-type plant. (H) The one-month-old mutant plant is dwarf compared with wild-type. (I) The shoot of the homozygous 3010 mutant is also twisted and with small and malformed flowers. (J) The mutant flower is asymmetric and has fewer floral organs in the first to third whorl, and a style with unfused carpels at the floral center (red arrow). (K) The mutant silique is also twisted and occasionally produces few sterile seeds.

3.2.2 The plastochron index and the flowering time of the mutant

The plastochron index was calculated from 11 wild-type and mutant plants for 9 days. On average, wild-type plants took 1.81 ± 0.14 days to initiate two leaf primordia successively, whereas mutant plants took 2.38 ± 0.24 days (Fig. 5A). When plastochron indexes were compared, the Student’s $t$-test gave a $p$ value of 0.026, suggesting that mutants initiate leaves significantly more slowly than wild-type plants. Despite the leaf initiation rates, mutants
RESULTS

flowered earlier than wild-type plants. On average, wild-type plants flowered at 25.1 DAG, whereas mutants flowered at 20.7 DAG. Furthermore, wild-type plants had 12 leaves, whereas mutants had only 9.1 leaves, while bolting (Fig. 5B). The Student’s t-test gave p values much smaller than 0.001, suggesting that mutants flower significantly earlier than wild-type plants.

Figure 5. The average leaf number and flowering time of the wild-type plants and the homozygous 3010 mutant plants. (A) The average leaf number of mutant plants was smaller than of wild-type. The plastochron indices were calculated for both wild-type and mutant plants and showed that mutants initiated leaf primordia slower than wild-type. (B) Mutants start to flower at 16 DAG and have a wide window of time to flower. The average time to flower of mutants is earlier than of wild-type plants, in addition, the average leaf number of mutants is smaller than of wild-type plants while flowering (see text).

To summarize, the homozygous mutant 3010 plants have a pleiotropic phenotype that affects stature, leaves, inflorescence shoots, flowers, leaf initiation rates and flowering time. The pleiotropic phenotype implies that the mutated locus may affect meristem function or the gene is widely expressed and implicated in different developmental processes.

3.3 Genetic mapping and allelism test

Genetic mapping was carried out by using CAPS markers that were distributed across the entire Arabidopsis genome. Four CAPS markers (LMYC6, MDA7, EG7F2 and ATTED2) on the long arm of chromosome 5 were tightly linked with the mutant phenotype. The marker LMYC6 showed 90% linkage with the mutant phenotype and the marker MDA7, which is more telomeric than the marker LMYC6, showed 100% linkage with the mutant phenotype (Fig. 6A and B). This
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result indicated that the mutant locus should be located in the area between markers *LMYC6* and *MDA7* (Fig. 6C). Therefore, the chromosomal position of the mutant locus was assigned to the long arm of chromosome 5, close to the CAPS marker *MDA7*.

**Figure 6. Genetic mapping of the mutant locus.** (A) and (B) show the segregation of the mutant locus in F2 progeny from the heterozygote of the mutant line 3010 (Col background) X Landsberg *erecta* crosses. PCR amplified CAPS marker *LMYC6* was digested by *HindIII* (A) and *MDA7* was digested by *AluI* (B). The most left two lanes in both figures correspond to Col (C) and Ler (L) ecotype. The doublet bands are readily apparent in the heterozygotes. The marker *LMYC6* shows 90% linkage and the marker *MDA7* shows 100% linkage to the mutant locus. (C) The mutant locus was assigned to the area between CAPS markers *LMYC6* and *MDA7* (red line). *TRN1* and *TRN2* genes were mapped to the same area close to the marker *MDA7*. The RFLP marker m233 (blue) is near the CAPS marker *MDA7*. Centromere is to the left.

The phenotype of homozygous 3010 mutant plants was reminiscent of the previously described mutants, *trnl* and *trn2* (Fig. 7A and B). *TRN1* was mapped to the bottom half of the long arm of
the chromosome 5, close to the visible marker tz and the RFLP marker m233 (Cnops et al., 1996). Genetic linkage between TRN2, and the DFR, AthPHYC and Ath50191 markers, indicated that TRN2 is also located on the same area of the chromosome 5 — most likely on the left of TRN1 (Fig. 6C) (Cnops et al., 1996; 2000). Based on the genetic map of chromosome 5, the RFLP marker m233 is near the CAPS marker MDA7 (Fig 6C). Therefore, both TRN1 and TRN2 genes should be located in the area enclosed by the markers LMYC6 and MDA7 (Fig. 6C). As the homozygous 3010 mutant and the trn mutants had similar phenotypes, and the mutant loci were mapped to the same area, it was then examined whether the 3010 mutant locus is allelic to trn1 or trn2.

Figure 7. trn mutants show similar phenotypes to the homozygous 3010 mutants. (A) and (B) are one-month-old trn1-1 and trn2-1 mutants, respectively. Both mutants show dwarf statures, twisted organs and sterility. The phenotypes are similar to homozygous 3010 mutant plants (compared to Fig 4 H).

To test for allelism, heterozygotes of line 3010 were crossed to heterozygotes of trn1-1 and trn2-1. Based on Mendelian genetics, it is expected that a quarter of plants in the F1 population will show the mutant phenotype when mutations in two different strains reside in the same gene. The F1 population of line 3010 X trn1-1 crosses revealed that there were no plants showing the mutant phenotype, indicating that the mutant 3010 locus and trn1-1 are not allelic. Conversely, a quarter plants of the progeny of line 3010 X trn2-1 crosses exhibited the mutant phenotype, strongly suggesting that the mutant 3010 locus and trn2-1 are allelic. To further confirm that line 3010 contains a mutant trn2 gene, both the TRN1 and TRN2 coding sequences were amplified by PCR from the homozygous mutant 3010 genomic DNA and subjected to DNA sequence analysis (the DNA sequence analysis was done by Gerda Cnops, Gent). The sequencing results revealed
that the homozygous mutant 3010 plants contain a wild-type \textit{TRN1} gene but the \textit{trn2} gene has a point mutation that is identical to one previously described in \textit{trn2-2} (Cnops \textit{et al}., 2000). These results suggested that the mutant locus is an allele of \textit{TRN2}, and the allele is hereafter referred to as $\textit{trn2}_{3010}$.

### 3.4 The \textit{TRN2} gene encodes a tetraspanin-like protein

The \textit{TRN2} coding region consists of two exons that are separated by a large intron of 595 bp and encodes a protein of 269 amino acids. NCBI blastp and Pfam HMM searches revealed that \textit{TRN2} has homology to the putative senescence-associated protein 5 of day lily (\textit{Hemerocallis fulva}) and a conserved domain related to tetraspanin family at N-terminus (this study and Cnops \textit{et al}., 2006). Further protein sequence analysis revealed that \textit{TRN2} possesses typical features of tetraspanins: short cytoplasmic N- and C- termini, four transmembrane domains, a small extracellular loop (SEL), and a large extracellular loop (LEL) within a set of conserved cysteins (see Fig. 8A; this study and Olmos \textit{et al}., 2005, Cnops \textit{et al}., 2006). Although it contains many structural properties of tetraspanins, almost no similarity alignments could be made between \textit{TRN2} and tetraspanins. The most notable difference between \textit{TRN2} and tetraspanins is the absence of a so-called tetraspanin signature CCG in the LEL (Fig. 8A). Instead, \textit{TRN2} has a GCC motif conserved in plant homologues in the LEL (Huang \textit{et al}., 2005; Fig. 8B). Despite this discrepancy, \textit{TRN2} is closer to tetraspanins than to other TM4 proteins (proteins with four transmembrane domains). Therefore, \textit{TRN2} is considered to be a plant tetraspanins (Huang \textit{et al}., 2005) — a so-called tetraspanin-like protein.

$\textit{trn2}_{3010}$ has a G to A nucleotide exchange that is consistent with a point mutation induced by EMS. This nucleotide exchange in the second exon leads to a single amino acid substitution (Gly to Glu) at position 81 of \textit{TRN2}, and this substitution is located on the variable region of LEL (just after a conserved cystein, which is six amino acids after the conserved GCC motif).
Figure 8. Schematic diagram of tetraspanin and TRN2 structures. (A) A cartoon represents schematic structure of tetraspanin proteins (Huang et al., 2005). The conserved cysteines are indicated; 100% conserved cysteines are labeled; ‘‘CCG’’ is the so-call tetraspanin signature. (B) TRN2 protein possesses typical features of tetraspanins. In the EC2, there are three helices (blue bars), termed A, B and E, which are known as the constant region. Conserved Cysteins are showed in green circles. The conserved motif GCC is located on the variable region where multiple protein-protein interaction sites are. The amino acid exchange in trn23010 and the truncated site of trn2-1 are presented by blue arrows.

LEL was known to have crucial roles in tetraspanin function (Hemler, 2005; Seigneuret, 2001). The point mutation in the trn23010 leads to the replacement of an uncharged glycine with a negatively charged glutamate (Fig. 8B), and this may affect the scaffold formed by disulfide bonds between conserved cysteins in the variable region. trn2-1, an allele previously described (Cnops et al., 2000), has a G to A nucleotide exchange that introduces a stop codon at the beginning of exon 2 and leads to a truncated protein that lacks EC2, the last transmembrane domain and the cytoplasmic tail. Lack of the entire LEL (Fig. 8B) may severely disrupt the function of the protein. Therefore, both trn2-1 and trn23010 alleles encode loss-of-function proteins.
3.5 RNA *in situ* hybridization of *TRN2* and other meristem-related genes

3.5.1 The *TRN2* expression pattern

The expression pattern of *TRN2* was examined by RNA *in situ* hybridization using a full-length cDNA probe that is specific to *TRN2*. During vegetative development, *TRN2* transcripts were detected in the SAM through the L1 to L3 layer (Fig. 9) and preferentially in the distal tip of the leaflet and the incipient vascular tissues (Fig. 9, arrow and arrow head, respectively).

![Figure 9](image)

**Figure 9.** *TRN2* is expressed in the SAM, leaf primordia and incipient vascular strands. The longitudinal section of a 11-DAG-old wild-type seedling hybridized with the antisense RNA probe of *TRN2*. *TRN2* is expressed in the SAM through L1 to L3 layer. *TRN2* RNA accumulates also in the tip of the leaflet (red arrow) and in the incipient vascular strand (red arrow head).

After floral transition, *TRN2* transcripts accumulated in inflorescence and floral meristems (Fig. 10A). Continuous *TRN2* expression was detected through the floral meristem into the flanking sepal primordia in a stage 5 flower (Fig. 10B). In a stage 8 flower, the transcripts were detected preferentially in developing petals, anthers and the gyneocium (Fig. 10C). In later stages, strong expression of *TRN2* was observed in haploid pollen but not in tapetum layers (Fig. 10D and E). After floral organs were differentiated, *TRN2* transcripts were not detectable (Fig. 10F).
Figure 10. *TRN2* is expressed in the inflorescence meristem and developing flowers. (A) *TRN2* is expressed in inflorescence meristems, floral meristems and developing flowers. (B) A close look to a stage 5 flower. *TRN2* is expressed in the floral meristem and sepal primordia. (C) A close look to a stage 8 flower. *TRN2* is expressed in developing petals, anthers and carpels but not in sepals, which are already differentiated. (D) and (E) In a stage 10 flower, *TRN2* is expressed in the developing haploid pollen but not in tapetum layers. (D) The longitudinal section of a stage 10 flower. (E) The cross section of a stage 10 flower. (F) In a mature flower, *TRN2* expression is not detectable by RNA *in situ* hybridization.

In summary, *TRN2* transcriptional activity was detected in all types of meristem — vegetative, inflorescent and floral. *STM* is also active in these meristems (see below, Fig. 11A and B). As *TRN2* and *STM* expression spatially overlaps, defects in TRN2 function may affect *STM* expression. However, in contrast with *STM*, *TRN2* transcripts are not confined to the meristems and are also detected in lateral primordia.
3.5.2 *STM* expression pattern in *trn2* mutants

As previously described, the expansion of the *STM-GUS* activity domain in the shoot tips of *trn2* seedlings implied an increased domain of *STM* expression. To gain insight into the effects of *STM* expression on *trn2* seedlings, RNA *in situ* hybridization was carried out to characterize *STM* expression in the mutant SAM. Wild-type and *trn2*<sub>3010</sub> plants were grown under long-day conditions, and 11-DAG-old seedlings and inflorescences were fixed and sectioned. The resulting serial sections were hybridized to an antisense *STM* probe. The data were collected from longitudinal sections because this allows the morphology of SAM to be easily observed.

During vegetative development, *STM* transcripts were detected in the SAM and excluded from leaf primordia in both *trn2*<sub>3010</sub> and wild-type SAMs (Fig. 11A and C). The endogenous *STM* transcripts detected by the antisense probe in the whole enlarged mutant SAM suggested that the meristem identity of cells within the SAM was maintained, despite the changes in morphology. During the reproductive phase, RNA *in situ* hybridization revealed that *STM* transcripts are confined to the inflorescence and to the floral meristems of mutants, similar to wild-type plants (Fig. 11B and D). *STM* transcripts also accumulated in stem tissues of both wild-type and mutant plants and were therefore not uniquely restricted to the SAM (Long *et al*., 1996). In mutant plants, the *STM* transcripts in stems were more strongly detected than that in the wild-type stem (Fig. 11B and D). This result may be an artifact due to the twisted architecture of the mutant stem, which means that sections may not be in the compatible plane, as in the wild-type.

![Figure 11](image-url) **Figure 11. STM expression in wild-type and trn2 meristems.** (A) The longitudinal section of a 11-DAG-old wild-type seedling hybridized with the antisense *STM* probe shows that *STM* expression is confined to the SAM. *STM* transcripts are not detected in lateral organ primordia and leaves. (B) The longitudinal section of a wild-type inflorescence hybridized with the antisense *STM* probe revealed that *STM* transcripts accumulate in inflorescence and floral meristems, and also in stem tissues. (C) The
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longitudinal section of a 11-DAG-old trn2 seedling hybridized with the antisense STM probe. STM transcripts accumulate are confined in the aberrant SAM but not in organ primordia and leaves. (D) The longitudinal section of a trn2 inflorescence hybridized with the antisense STM probe. STM transcripts accumulate in the inflorescence meristem in major like it accumulates in the wild-type inflorescence.

To summarize, in both wild-type and mutant plants, STM expression is confined in the vegetative SAM, inflorescence and floral meristems, and is downregulated in lateral organ primordia. Though the GUS activity was detected in mutant leaflets (Fig 2A), ectopic STM expressions in lateral organ primordia were not found by in situ hybridizations. No obvious evidences suggested that there is a significant change of the STM expression patterns in mutant plants; however, the endogenous STM transcripts accumulated in the enlarged mutant SAM suggests that the STM expression domain is increased.

3.5.3 WUS and CLV3 expression patterns in trn2 mutants

To gain a deeper insight into the organization of the trn2 SAM, different meristem-related molecular markers were used in RNA in situ hybridization analysis. WUS and CLV3 probes were used on 11-DAG-old trn2 mutant and wild-type seedlings. These probes should indicate differences in the CZ relative to the surrounding PZ.

In the mutant SAM, WUS transcripts were detected in few cells in the L3 layer of the CZ, similar to what was observed in wild-type plants (Mayer et al., 1998; Fig. 12A and B), suggesting that the expression pattern of WUS is not affected in the trn23010 background. CLV3 transcripts were detected in the same domain in trn2 mutant SAM as in wild-type SAM (Fletcher et al., 1999; Fig. 12C and D). However, the CLV3 expression domain in the mutant SAM is relatively reduced compared with wild-type (Fig. 12C and D).
Figure 12. *WUS* and *CLV3* expression in the wild-type and *trn2* SAM. (A)(B) *WUS* expression in the wild-type and *trn2* SAM, respectively. (C)(D) *CLV3* expression in the wild-type and *trn2* SAM, respectively. Relative expression patterns of *WUS* and *CLV3* are not affected in the mutant SAM. However, the *CLV3* expression domain is reduced in the mutant SAM (D) compared with the wild-type (C).

Although the *trn2*3010 SAM has an aberrant morphology and is enlarged relative to wild-type, both molecular markers uncovered no major differences in the relative positions of the *CLV3* and *WUS* domains. The results suggested that the organization of the SAM is not affected in the *trn2* mutant. Both of these gene markers argue against the size of the stem-cell niche in the CZ being increased by the *trn2* mutation. Therefore, the enlargement and aberrant morphology of the mutant meristem must be the consequence of an increase in the PZ.
3.5.4 *LFY* expression in *trn2* mutants

11-DAG-old *trn2* plants have a protruding and enlarged SAM compared with wild-type. However, the earlier flowering time of *trn2* mutants argues that the change in SAM shape may relate to a switch from vegetative to reproductive phase. To exclude the possibility that the change in morphology in the mutant SAM is due to the floral transition, RNA *in situ* hybridization analysis was carried out using *LEAFY (LFY)* as a molecular marker for the reproductive phase.

The expression of *LFY* in the shoot tip is an early marker for the formation of flowers. The *LFY* gene encodes a plant-specific transcriptional factor. It is expressed in the newly emerging leaf primordia, but is absent from the SAM, and older leaves, during the vegetative phase. When the floral transition process begins, *LFY* is first expressed in subsets of cells in the PZ, presumably directing these cells toward a floral fate (Weigel *et al*., 1992). Later, high levels of *LFY* transcripts are detected in flower primordia that have just formed (Blázquez *et al*., 1997). Therefore, it is proposed that *LFY* is both necessary and sufficient for the initiation of individual flowers in the transition from the vegetative to the reproductive phase (Blázquez *et al*., 1997).

As expected, in an 11-DAG-old wild-type plant (grown in long-day conditions), which was still at the vegetative phase, *LFY* transcripts could be detected only in newly emerged leaves but not in the SAM (Fig. 13A). Similar results were obtained from mutant seedlings. RNA *in situ* hybridizations revealed no detectable signals in the protruding SAM of *trn2* mutant plants, and no newly formed flower primordia were observed (Fig. 13B). These results suggested that the morphological changes in the 11-DAG-old mutant SAM were not related to the floral transition.
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Figure 13. RNA in situ hybridization with the antisense LFY probe on the wild-type and the mutant SAM. (A) LFY transcripts were not detectable in the 11-DAG-old wild-type SAM, but signals were detected in young leaves. (B) In an 11-DAG-old trn23010 seedling, LFY transcripts were not detectable in the protruding SAM. As in the wild-type, LFY transcripts accumulated in young leaves. (C) A positive control of the LFY RNA in situ hybridization. In the inflorescence, the LFY transcripts accumulated in flower primordia and floral meristems.

3.6 Real-time RT-PCR experiments

In addition to gene expression patterns, it is interesting to consider whether the level of gene expression is affected in trn2 plants. To answer this question, and to further understand meristem-related gene expression in mutant plants, real-time RT-PCR experiments were carried out to quantify the levels of STM, WUS and CLV3 expression.

The real-time RT-PCR results are summarized in Table 2. The delta Ct values of STM gene of mutants were always lower than that of wild-type plants, and a Student’s t-test gave p values less than 0.01. These results suggested that the amount of STM transcripts is significantly increased in trn2 mutants. Using a previously described formula (Livak and Schmittgen, 2001) to calculate the relative amount of change of gene transcripts substantiated an, on average, 4.1-fold increase in STM transcripts in trn2 seedlings relative to wild-type. For WUS, the delta Ct values were similar in both mutant and wild-type seedlings. The amount of WUS transcripts were increased, on average, 1.2-fold in mutant seedlings relative to wild-type. However, a Student’s t-test showed p values larger than 0.05, suggesting that the increase is not significant. Therefore, it seems that WUS expression is not affected in trn2 mutants. In contrast with WUS, the amount of CLV3 transcripts was significantly affected in trn2 mutants. The delta Ct values of CLV3 in mutant seedlings were always higher than those of wild-type seedlings, indicating that there were fewer CLV3 amplicons detected in real-time PCR reactions using the mutant cDNA as templates than using wild-type cDNA as templates. On average, CLV3 transcripts were decreased 0.5-fold in mutant seedlings relative to wild-type. Moreover, a Student’s t-test gave p values lower than 0.04, suggesting that the amount of CLV3 transcripts decreases significantly in mutant seedlings.
Table 2. Gene expression changes in \textit{trn2} plants

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<th>Gene name</th>
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<th>P value$^b$</th>
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<td>\textit{STM}</td>
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<tr>
<td>\textit{WUS}</td>
<td>1.2 ± 0.1</td>
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<tr>
<td>\textit{CLV}</td>
<td>0.5 ± 0.1</td>
<td>&lt;0.04</td>
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$^a$Fold change, the mean of changes averaged from three independent real-time PCR reactions.

$^b$P value, generated by a t-test comparing Cts of the gene in wild-type and mutant plants. P value that is less than 0.05 suggests a significant difference.

Taken together these results indicate that, \textit{trn2} mutants have increased levels of \textit{STM} expression, similar levels of \textit{WUS} and decreased levels of \textit{CLV3} expression compared with wild-type. The increased \textit{STM} transcripts in mutant seedlings are compatible with the observed \textit{STM-GUS} pattern of expression, which suggested an increased \textit{STM} domain. The decreased level of \textit{CLV3} expression can be explained in several ways. \textit{CLV3} RNA \textit{in situ} hybridization analysis revealed a decreased \textit{CLV3} expression domain, suggesting that there are fewer cells expressing \textit{CLV3}. This is consistent with the result of real-time RT-PCR. A decrease in \textit{CLV3} expression was expected to an increase of \textit{WUS} expression. However, the results of RNA \textit{in situ} hybridization and real-time RT-PCR revealed that \textit{WUS} expression is not affected in \textit{trn2} mutants.

3.7 Genetic interactions between \textit{trn2} and other meristem-related genes

To further understand \textit{TRN2} function, heterozygous \textit{trn2\textsubscript{3010}} plants were crossed with heterozygous \textit{stm-1}, \textit{stm-5} and \textit{wus-1}, and homozygous \textit{clv3-2} plants to create double mutants. Double mutants were then subjected to detailed phenotypic analysis to unravel the genetic interactions between \textit{TRN2} and other meristem-related genes.
3.7.1 Genetic interactions between trn2 and stm

3.7.1.1 stm-5 and stm-1 alleles and mutants

stm-5 contains a point mutation at the beginning of the second intron of STM gene. Mature stm-5 embryos do not exhibit a recognizable SAM, and 7 days after germination stm-5 young seedlings do not display leaves but the petioles of their cotyledons are partially fused. The production of other organs in stm-5 plants is very delayed and they rarely have flowers (Endrizzi et al., 1996).

stm-1 carries a nonsense mutation that encodes a protein missing the homeodomain. In homozygous stm-1 embryos, no primary SAM is initiated, the mutant seedlings fail to produce any organs post-embryonically and they do not express STM (Long et al., 1996).

3.7.1.2 Identification of double mutants for both trn2 and stm

To isolate trn2 stm double mutants, 4-DAG-old F2 seedlings of trn2\textsubscript{3010} X stm-1 or stm-5 crosses without visible leaf primordia were picked out while wild-type seedlings developed visible leaf primordia. The no-leaves populations were supposed to be a mixture of single mutants and double mutants. To identify stm-5 trn2\textsubscript{3010} double mutants, statistical and genetic methods were used. A chi square test generated a value 3.11 (Table 3), suggesting that the segregation ratios are conformed to Mendelian rules. To characterize the genotype of the stm-5 trn2\textsubscript{3010} double mutants, DNA fragments containing the point mutation of stm-5 and trn2\textsubscript{3010} were amplified from the genomic DNA of presumptive double mutants and the PCR products were then digested by TaqI to check stm-5 and EcoRI to check trn2\textsubscript{3010} (Fig 14). The results revealed that those presumptive double mutants were homozygous of both stm-5 and trn2\textsubscript{3010}. Based on the statistical test and the genotyping, stm-5 trn2\textsubscript{3010} double mutants were confirmed unambiguously.

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RESULTS

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<th>trn\textsubscript{23010} X wus-1 F2</th>
<th>394</th>
<th>141</th>
<th>121</th>
<th>35</th>
<th>1.62</th>
<th>Accepted</th>
</tr>
</thead>
<tbody>
<tr>
<td>trn\textsubscript{23010} X clv3-2 F2</td>
<td>404</td>
<td>143</td>
<td>150</td>
<td>42</td>
<td>0.73</td>
<td>Accepted</td>
</tr>
</tbody>
</table>

| Expected ratio | 9 | 3 | 3 | 1 |

\textsuperscript{a}Wt, including wild-type plants and heterozygotes of each mutant.

\textsuperscript{b}Single mutant, homozygotes of \textit{stm-5}, \textit{stm-1}, \textit{wus-1} or \textit{clv3-2}.

\textsuperscript{c}\textit{trn\textsubscript{23010}}, homozygotes of \textit{trn\textsubscript{23010}}.

\textsuperscript{d}Null hypothesis, the null hypothesis is the expected ratio.

---

\textbf{Figure 14. Check of \textit{stm}-5 and \textit{trn\textsubscript{23010}} fragments.} (A) Intact \textit{stm}-5 fragment (left lane) and \textit{stm}-5 fragment digested by \textit{TaqI}. (B) Intact \textit{trn\textsubscript{23010}} fragment (left lane) and \textit{trn\textsubscript{23010}} fragment digested by \textit{EcoRI}.

The same methods were also used to identify \textit{stm-1 \textit{trn\textsubscript{23010}} double mutants. Statistical tests indicated that one-in-sixteen plants in the population were presumptive double mutants (Table 3). DNA fragments carrying the point mutation of \textit{stm-1} and \textit{trn\textsubscript{23010}} were amplified from the genomic DNA of presumptive double mutants by specifically designed primers. \textit{trn\textsubscript{23010}} fragments were checked by \textit{EcoRI} digestion, and \textit{stm-1} fragments were subjected to sequencing analysis (sequence data not shown). Taken together, the results of the statistical analysis and the genotyping unambiguously confirmed that \textit{stm-1 \textit{trn\textsubscript{23010}} double mutants were homozygous.}
3.7.1.3 Phenotypic analyses of trn2 stm double mutants

In contrast with STM-5 single mutants, in which only 3% plants developed leaves by 8 DAG (Table 4), 51.7% of trn2_3010 STM-5 double mutants developed at least one leaf before 8 DAG, and all double mutants developed leaves during a 20-day interval (Table 4). In addition to developing leaves more rapidly than the STM-5 single mutant, only 20% of trn2_3010 STM-5 double mutants exhibited leaves with fused bases (Fig. 15A). During their lifetime, the STM-5 trn2_3010 double mutant plants continued to develop leaves from the shoot tip and bolting shoots and flowers were never observed.

A similar result was obtained from crosses of trn2_3010 and STM-1. 75.0% of STM-1 trn2_3010 double mutants began to develop leaves before 8 DAG and they all developed leaves by 20 days (Table 4), whereas STM-1 plants did not develop leaves even one month after germination. However, trn2_3010 STM-1 leaves had fused petioles, which is a typical phenotype of STM mutants (Endrizzi et al., 1996). In addition, 66% of double mutants developed leaves from the hypocotyl, implying the formation of an adventitious SAM (Fig. 15B). Furthermore, trn2_3010 STM-1 double mutants also developed leaves repetitively from the same site (shoot tips or the side of the hypocotyl) during their lifetime and bolting shoots and flowers were never observed.

Therefore, in both double mutant combinations, the trn2_3010 background can partially compensate for lost or reduced STM function. Unfortunately, none of the double mutant plantlets developed flowers which would have allowed phenotypic analysis during the reproductive phase.
**RESULTS**

**Figure 15. Genetic interactions between trn2 and stm.** (A) The 11-DAG-old trn2<sub>3010</sub>stm-5 double mutant seedling. A pair of trn2-like leaves developing from the tip of the shoot (red arrow). Note that the bases of cotyledons and leaves are not fused whereas stm-5 single mutants have leaves with fused petioles. (B) The 11-DAG-old trn2<sub>3010</sub>stm-1 double mutant seedling. The bases of cotyledons and leaves were always fused. Half of the double mutants developed leaves from the side of the hypocotyls (red arrow), suggesting the formation of an adventitious meristem.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>8 DAG&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No leaves&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>stm-1 (Ler)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>49</td>
<td>0.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>stm-5 (Ler)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29</td>
<td>3.0%</td>
<td>45.0%</td>
</tr>
<tr>
<td>stm-1 (Ler/Col)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>38</td>
<td>5.3%</td>
<td>60.5%</td>
</tr>
<tr>
<td>stm-5 (Ler/Col)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>38</td>
<td>24.2%</td>
<td>18.1%</td>
</tr>
<tr>
<td>trn2&lt;sub&gt;3010&lt;/sub&gt;stm-1 (Ler/Col)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>40</td>
<td>75.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>trn2&lt;sub&gt;3010&lt;/sub&gt;stm-5 (Ler/Col)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>54</td>
<td>51.7%</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

<sup>a</sup>8 DAG, the percentage of plants developing leaves 8 days after germination.

<sup>b</sup>No leaves, the percentage of plants did not develop leaves 20 days after germination.

<sup>c</sup>(Ler), plants in the Ler background.

<sup>d</sup>(Ler/Col), plants in the background of Col X Ler.

As stm-1 and stm-5 mutants are both in the Landsberg erecta background, whereas trn2<sub>3010</sub> mutants were created in the Columbia ecotype, the effect of timing to generate leaves may be a consequence of the cross of both ecotypes. To exclude this possibility, both stm alleles were back crossed to the Columbia background, and the time required for leaf generation in the F2 populations were examined.

Only 3% of stm-5 single mutants (Ler background) developed leaves before 8 DAG. On the other hand, stm-1 single mutants (Ler background) never developed leaves (Table 4). In populations of backcrosses, 24.2% of stm-5 plants and 5.3% of stm-1 plants began to develop leaves before 8
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DAG. However, 18.1% of *stm*-5 and 60.5% of *stm*-1 plants did not develop leaves even after 20 DAG (Table 4). Although in the Ler X Col background both *stm*-1 and *stm*-5 mutant seedlings initiated leaves slightly more frequently than in the Ler ecotype, the difference was nowhere near as large as when combined with the *trn2*3010 mutant background, where essentially all plants developed leaves 20 days after germination (Table 3). In conclusion, in both double mutant combinations, the *trn2* background can compensate for the reduced or lost STM activity and thus partially rescue the *stm* phenotype.

3.7.2 *trn2* and *wus*

3.7.2.1 The *wus*-1 allele and mutant

*wus*-1 is a strong loss-of-function allele of *WUS* gene, which contains a point mutation in 5’-exon-intron boundary of intron 2 where the nucleotides were changed from GG to GA, replacing a G that is highly conserved at plant gene splice sites. The failure to remove intron 2 results in a translational stop after a few codons within the intron (Mayer et al., 1998). *wus*-1 plants do not develop leaves 7 DAG while the wild-type shoot meristem gave raises to a rosette of leaves. After floral transition, to the inflorescence, *wus* mutants repetitively initiated defective shoot meristems, which give rise to only a few leaves and then discontinue primordia initiation. Eventually, disorganized bunches of leaves are observed at the base and the tip of several stems in mutants. Mutant flowers are rare and differed from wild-type in that they lack most central organs (carpels) and terminate most often in a single to few (but never six) central stamens (Laux et al., 1996) (Fig 16 C), and it was proposed that precocious consumption of the central region during definition of the third-whorl domain appears to be the basis of the *wus* floral phenotype (Laux et al., 1996).

3.7.2.2 Genetic interactions between *wus*-1 and *trn2*3010

To identify *wus*-1 *trn2*3010 double mutants, plants that did not develop leaves by 7 DAG were selected from the F2 population, and these presumptive double mutants developed twisted leaves several days later.

To confirm these presumptive double mutants, DNA sequence analyses and statistical methods
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were used as described above. The calculated chi-squared value was 1.62, suggesting that the segregation ratio of the F2 population fitted the Mendelian ratio (Table 3). The genomic DNA of several presumptive double mutants was extracted, and the trn23010 fragment was amplified and checked. The wus-I fragment was also amplified by a specifically designed pair of primers and subjected to sequence analysis (sequence data not shown). Based on the phenotype, the statistical analysis and the genotype, these plants were confirmed to be homozygous for both wus-I and trn23010.

During vegetative development, despite the dwarf stature and twisted organs of double mutants, the behavior of wus-I plants and double mutants was indistinguishable. The time for double mutants to develop leaves was as delayed as that of wus-I plants. After floral transition, a novel phenotype was observed in double mutant flowers. All wus-I trn23010 flowers developed 2 carpels, whereas in wus-I mutants, carpels were completely absent (Fig. 16C) and only 1.6 stamens (compared with 6 in wild-type) developed in the third floral whorl (Table 5). Therefore, the trn2 mutation may compensate for a premature consumption of stem cells in wus-I flowers, which is also consistent with the increased number of stamens (4.2 on average; Table 5) in the third floral whorl. In contrast, the number of sepals and petals in the outer two whorls was reduced and was closer in number in trn23010 than in wus-I single mutant flowers.

![Image A](image1.jpg)  ![Image B](image2.jpg)  

![Image C](image3.jpg)  ![Image D](image4.jpg)
RESULTS

Figure 16. Genetic interactions between trn2 and wus. (A) The one-month-old wus-l plant. (B) The one-month-old trn23010 wus-l double mutant. The double mutant has dwarf stature like the trn2 single mutant. The red arrow indicates flowers. (C) The wus-l flower. (D) The trn23010 wus-l flower, which is like the trn23010 flower. Note that there is a severe twisted style with two unfused carpels at the floral center (red arrow head).

<table>
<thead>
<tr>
<th>Table 5. The mean of floral organs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Sepals</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>WT (n=31)</td>
</tr>
<tr>
<td>trn23010 (n=39)</td>
</tr>
<tr>
<td>wus-l (n=31)</td>
</tr>
<tr>
<td>trn23010 wus-l (n=27)</td>
</tr>
</tbody>
</table>

aND, not detectable.

In conclusion, there was no additive phenotype observed in double mutants during the vegetative phase, but surprisingly, double mutant flowers were completely trn2-like. wus-l flowers lacked the most central organs, carpels, and generally had zero to three stamens, but the numbers of organs in the outer two whorls were on average only slightly reduced compared with wild-type (Table 5). In contrast, the double mutant flowers had fewer trn2-like organs in the outer three whorls compared with wild-type and two carpels at the center, which are never found in wus-l flowers, suggesting that the trn2 background compensates for the prematurated floral meristem of wus-l flowers. Furthermore, double mutants have typical twisted trn2 lateral organs, suggesting that TRN2 acts epistatically to WUS.

3.7.3 trn2 and clv3

3.7.3.1 The clv3-2 allele and mutant

clv3-2 was identified during a gamma-irradiation mutagenesis of Landsberg erecta seeds and
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contained a deletion in the second intron of the gene. clv3-2 plants have enlarged meristems throughout their lifetime. The enlarged inflorescence meristems of clv3-2 result in thicker shoots compared with wild-type. The larger clv3-2 floral meristem develops into a flower with additional organs in each whorl, and the number of stamens and carpels are more dramatically increased than petal and sepal number (Clark et al., 1996).

3.7.3.2 Genetic interactions between clv3-2 and trn23010

It was difficult to identify clv3-2 trn23010 double mutants in the F2 population on the basis of their phenotypes during the vegetative phase, suggesting that there was no additive phenotype for these double mutants. When flowering, some plants in the F2 population were considered to be presumptive double mutants because of their twisted organs (typical of trn2) and thicker shoots (typical of clv3).

The same methods described above were used to confirm that these plants were homozygous for both trn23010 and clv3-2. The chi-squared test indicated that the segregation ratio fitted to the Mendelian ratio (Table 3). The trn23010 fragment was amplified and checked as described above, and the clv3-2 fragment was amplified by a specifically designed pair primers and subjected to sequence analysis (sequence data not shown). The phenotype, the statistical test and the genotype together confirmed that these presumptive double mutants were homozygous for both trn23010 and clv3-2.

During the reproductive phase, an additional phenotype not observed in single clv3-2 or trn23010 mutants was identified in double mutant flowers. Double mutant flowers had reduced organ numbers in the outer whorls of sepals, petals and stamens (Table 6), reminiscent of trn23010 single mutant flowers, and in contrast with clv3-2 single mutant flowers (Fig. 17A), there was no increase in the number of stamens (Table 6). Also, when compared with the outer three whorls, there were supernumerary carpels at the floral center in double mutant flowers, as was observed in clv3-2 flowers (Fig. 17B), and in 27% of double mutant flowers these carpels were unfused at their upper parts (Table 6). The reduced number of floral organs and the twisted morphology of double mutant flowers suggested that TRN2 acts epistatically to CLV3 in the outer three whorls. On the other hand, the supernumerary carpels and the increased percentage of carpel fusion may be due to prolonged WUS expression caused by the clv3-2 background (see discussion).
Figure 17. Genetic interactions between \textit{trn2} and \textit{clv3}. (A) The \textit{clv3}-2 flower. \textit{clv3}-2 flowers always have supernumerary floral organs. In this \textit{clv3}-2 flower, there are 5 sepals and petals, 8 stamens and a gynoecium within supernumerary carpels. (B) The \textit{trn2}_{3010}\textit{clv3}-2 flower. The flower has reduced number of floral organs in the outer three whorls (see table 5) but a gynoecium with supernumerary carpels fused together.

\begin{table}[h]
\centering
\begin{tabular}{lcccc}
\hline
 & Sepals & Petals & Stamens & Carpels & Carpels not fused \\
\hline
WT (n=31) & 4.0 ± 0 & 4.0 ± 0 & 6.0 ± 0 & 2.0 ± 0 & 0.0% \\
\textit{trn2}_{3010} (n=39) & 3.2 ± 0.8 & 2.4 ± 0.8 & 3.5 ± 1.0 & 2.0 ± 0 & 52.0% \\
\textit{clv3}-2 (n=34) & 4.3 ± 0.4 & 4.1 ± 0.5 & 7 ± 0.9 & \textsuperscript{a}Many & 0.0% \\
\textit{trn2}_{3010}\textit{clv3}-2 (n=33) & 2.1 ± 1 & 1.9 ± 1.1 & 2.8 ± 2.8 & \textsuperscript{a}Many & 27.0% \\
\hline
\end{tabular}
\caption{The mean of floral organs}
\end{table}

\textsuperscript{a}Many, multi-carpels-fused style.
4. DISCUSSION

4.1 The TRN2 gene encodes a tetraspanin-like protein

Starting from a genetic screen designed to identify changes in STM promoter activity, a new trn2 mutant allele was isolated, trn2_{3010}. It carried the same G-A transition in the beginning of the second exon as the previously identified trn2-2 allele (Cnops et al., 2006). trn2 mutants have dwarf stature, asymmetric leaves with aberrant vascular patterning and malformed inflorescences and flowers. The TRN2 gene has been examined in many aspects, including root and leaf development (Cnops et al., 2000; 2006); however, its role in the SAM has not been described. This study revealed that one of the peculiarities of trn2 mutants is an aberrant SAM protruding and enlarged compared to wild-type, readily detected 11 DAG. The aberrant SAM morphology and malformed lateral organs suggest that the TRN2 gene is implicated in SAM functions. In addition to careful inspection of the SAM histology, molecular markers were used to examine the change of gene expressions and the organization of the trn2 SAM, and double mutants were also created to test genetic interactions between trn2_{3010} and stm-1, stm-5, clv3-2 and wus-1.

The TRN2 gene encodes a protein of 269 amino acids that contains a tetraspanin domain at the N-terminal. Therefore, TRN2 can be considered as a plant tetraspanin protein. However, TRN2 protein contains a GCC motif in the LEL, instead of the conserved CCG motif that is characteristic of tetraspanin proteins, and in addition, TRN2 and its homologues among other plant species are grouped in one clade of a phylogenetic tree that is separate from the tetraspanins of animals, fungi, and amoeba (Huang et al., 2005). Thus, TRN2 and its homologues are referred to as tetraspanin-like proteins.

Comparison of secondary structure between plant tetraspanin-like proteins and animal tetraspanin proteins revealed both groups of proteins were of similar size and shared the same topology, indicating that the plant tetraspanin-like proteins share structural homology with tetraspanin proteins (Cnops et al., 2006). Specifically, the LEL found in animal tetraspanins seems to be relevant for complex formation with other proteins, as follows from genetic evidence and chimeric protein analysis (Hemler, 2001; 2003; Stipp et al., 2003). Previous studies revealed that the homodimerization through LEL should be a basic unit in forming the TEM (Levy and Shoham, 2005; Hemler, 2005), and mutations in the LEL of tetraspanin CD151 disrupt direct
primary interaction with integrin and result in protein malfunction (Hemler, 2003). The point mutation in the \textit{trn2}_{3010} allele leading to a G to E amino acid exchange at position 81 in the LEL of the protein. Therefore, the amino acid exchange in the LEL of TRN2 might interfere the correct folding of the protein and interactions with associated proteins and disrupt the TRN2 normal function. Considering another previously identified allele, \textit{trn2-1} (Cnops \textit{et al.}, 2000), which lacks the entire LEL and has similar phenotype with \textit{trn2}_{3010}, LEL of TRN2 may play critical roles as of animal tetraspanin proteins.

Animal tetraspanins are found in nearly all tissue and cell types and they participate in divergent biological processes (Hemler, 2003). For example, CD81 is present in all types of cells within the immune system, has been implicated in both B cell and T cell immune responses and may serve as a receptor for HCV (Levy and Shoham 2005; McKeating \textit{et al.}, 2004). TRN2 is widely expressed in \textit{Arabidopsis}, including in the SAM, in lateral organ primordia, in the incipient vasculature and in roots, suggesting that TRN2 tetraspanin-like proteins are involved in divergent biological processes in \textit{Arabidopsis}, similar to the tetraspanin proteins in animals.

\textbf{4.2 trn2 mutation affects cell-fate decisions in the SAM}

Previous studies (Cnops \textit{et al.}, 2000) indicated that the radial patterning of the root meristem is incomplete in \textit{trn2-2} mutant plants. Cell fates are inappropriately specified, with most irregularities seen in determined or differentiated cells such as the epidermal or lateral root-cap cell files. These data suggest that TRN2 contributes to cell-fate decisions in the root meristem. The cotyledons of \textit{trn2} plants develop relatively normal, but the lateral organs are twisted and malformed, arguing that the \textit{trn2} phenotype may be due to a malfunction of the SAM. Twisted \textit{trn2} rosette leaves usually rotate 180°; however, dorsalventrality of leaves is normal (Cnops \textit{et al.}, 2006), suggesting that the \textit{trn2} mutation does not affect the dorsal-ventral polarity of leaves. In contrast with dorsalventrality, \textit{trn2} leaves are severely defective in symmetry and size. \textit{trn2} leaves are smaller compared with wild-type and have asymmetric laminas, and the most conspicuous defect is the loss of half leaf blade. Furthermore, these defects are not restricted to the vegetative development; \textit{trn2} flowers are small, asymmetric and have fewer floral organs in outer three whorls than wild-type. Both leaf lamina and floral organ missing may reflect that early steps of lateral organ anlagen development are disturbed in \textit{trn2} mutants. \textit{STM RNA in situ} hybridization revealed that the expression pattern of \textit{STM} was confined to meristems in both
wild-type and trn2 shoot tips. However, real-time RT-PCR experiments indicated that the amount of STM transcripts in trn2 mutants is, on average, 4.1 times higher than in the wild-type, suggesting that excess STM transcripts accumulated in the trn2 SAM. As STM maintains cells in an undifferentiated state (Long et al., 2000), excess STM activity may inhibit cells from undergoing proper differentiation. Accordingly, the excess amount of STM transcripts detected in trn2 seedlings suggests that cells in the PZ of the SAM, which should undergo differentiation in wild-type seedlings, cannot differentiate properly. Therefore, TRN2 is required for proper cell differentiation in the SAM as well as in the root meristem. However, down-regulation of STM is still the earliest available signifier for the acquisition of the primordial fate (P0) (Long et al., 1996). In the absence of additional markers, it cannot be discriminated whether cells at the periphery of the trn2 SAM are released for terminal differentiation in an appropriate timely or spatial fashion, or whether cells within the PZ of the SAM over-proliferate.

4.3 TRN2 may contribute to another pathway rather than to STM pathway directly

To understand genetic interactions between STM and TRN2, heterozygotes of trn23010 were crossed to heterozygous plants of stm-1, which is a null allele, and stm-5, which is a strong allele. Double mutant analyses revealed that trn2 mutation promotes leaf development in either an stm-1 or stm-5 mutant background during early seedling development. It has been suggested that stm-5 shoot meristems have cells that seem to prematurely undergo differentiation (these cells should remain undifferentiated in wild-type plants), and hence, the bases of the cotyledons and leaves of stm-5 mutants are fused (Endrizzi et al., 1996). However, not only do they form leaves more rapidly than stm-5 single mutants, trn23010 stm-5 double mutants rarely have cotyledon and leaves with fused bases. The double mutant phenotype suggests a partial rescue of stm-5 phenotype by trn23010, and this rescue may arise from the accumulation of indeterminate cells at the shoot tip, which compensates for the premature stm-5 meristem.

Similar results were obtained from trn23010 stm-1 double mutants. stm-1 single mutants have cotyledons with fused bases and fail to form postembryonic organs because of the failure to establish a primary SAM during embryogenesis, whereas trn23010 stm-1 double mutants started to form leaves by 8 DAG. These results suggest that trn2 mutation promote stm-1 mutants to regain the meristematic activity. However, these double mutants always develop leaves with fused petioles. Therefore, the stm-1 phenotype rescued by trn23010 is not as complete as that observed
DISCUSSION

in *trn2* *stm* double mutants with respect to fused organs. In addition, half of *trn2* *stm* double mutants form adventitious meristems, suggesting that *trn2* may promote *stm* mutants to form a functional primary SAM during embryogenesis and to form meristems adventitiously while no functional primary SAMs are formed.

These results revealed that partial rescues of *stm* phenotype by the *trn2* mutation are not dependent on the strength of *stm* alleles with respect to the time to develop leaves, suggesting that the promotion of leaf development in the *trn2* background is not dependent on *stm* activity. Conversely, *stm* phenotype rescued by the *trn2* background is dependent on the strength of *stm* alleles with respect to organ fusion. Therefore, the *trn2* mutation may affect the contribution of meristem activity, independent of *STM* activity, to the promotion of leaf development. However, the effect of the *trn2* mutation is antagonistic to that caused by the *stm* mutation during the initiation of lateral organ primordia. These results suggest that *TRN2* may contribute to another pathway that crosslinks with the *STM* pathway.

Loss-of-function mutations in the *TRN1* locus result in similar phenotype to *trn2* mutants. It is predicted that the TRN1 is a large cytoplasmic protein that contains a putative LRR ribonuclease inhibitor–like (LRR-RI) subfamily domain at the N-terminal region (Cnops et al., 2006). However, the kinase domain is absent. Other LRR proteins lacking kinase domains, such as CLV2, have been shown to be required for cellular communication processes that decide between stem-cell fate and a differentiated fate (Nadeau and Sack, 2003). Given that TRN2 is a tetraspanin-like protein, and may act in a similar manner to the animal tetraspanin proteins that are implicated in divergent signaling pathways (Hemler, 2003; 2005), and also recent studies that revealed that both *TRN1* and *TRN2* act in the same pathway (Cnops et al., 2006), TRN1 is likely to be a component in the TRN2 pathway. The meristem phenotype and changes in gene expression caused by the *trn2* mutation suggest that the TRN2 pathway may contribute to normal meristem function. In addition, both *TRN2* and *TRN1* genes are conserved in angiosperm and gymnosperm (Cnops et al., 2006), suggesting a presumptive signaling pathway established by the membrane bounded TRN2 protein and the cytoplasmic TRN1 protein is conserved during plant evolution.
DISCUSSION

4.4 *TRN2* affects auxin distribution and vasculature patterning in leaves

The dwarf stature and aberrant vascular patterning in leaves of *trn2* mutants are reminiscent of defects in auxin transport. Although the concentrations of endogenous IAA and the capacity to actively transport auxin were not altered in *trn2* plants, auxin distribution is severely altered in the leaves of *trn2* plants (Cnops *et al*., 2006). Auxin distribution is correlated with the polar distribution of auxin efflux carrier PIN proteins and the direction of auxin transport can be monitored by the distribution of these proteins (Benková *et al*., 2003; Friml *et al*., 2003). PIN proteins are asymmetrically distributed in cellular membranes (Galweiler *et al*., 1998), and in the SAM, the PIN1 protein is oriented toward those cells newly recruited for the $P_0$ primordia (Reinhardt *et al*., 2003). Moreover, the basal localization of PIN1 is gradually established in the incipient vasculature below the primordia (Benková *et al*., 2003). In this manner, auxin transport in the SAM and in the lateral organ primordia is dynamically directed towards new primordia and incipient vasculatures, respectively. However, how the orientations of PIN proteins are regulated is still unclear. TRN2 belongs to the tetraspanin-like family and may establish a tetraspanin-like protein enriched microdomain (TLEM) on the plasma membrane (similar to animal tetraspanin proteins) that then recruits associated proteins and facilitates their functions. In this way, TRN2 may contribute to the process of polar distribution of PIN proteins and affect the transport of auxin.

PIN1 also has a central role in mediating auxin distribution for patterning of phyllotaxis (Reinhardt *et al*., 2003). However, phyllotactic patterns defects are not found in *trn2* plants, therefore TRN2 may not contribute a major role to auxin distribution in the SAM. In contrast, *TRN2* is preferentially expressed in the tips of leaflets — where the highest auxin concentration is detected during the leaf development (Mattsson *et al*., 2003), and in the incipient midvein. Therefore, TRN2 may be implicated in the vascular patterning of leaves.

It was proposed that in both *as1* and *as2* mutants, the initial asymmetric placement of auxin at the leaf tip would give rise to later asymmetries in the internal auxin sources. This asymmetry would subsequently result in asymmetrical cell differentiation and division patterns (Zgurski *et al*., 2005). *ASI* expression may be depressed due to the prolonged *STM* expression in *trn2* mutants during the initiation of lateral organ primordia. In this manner, loss of TRN2 activity may indirectly cause a decrease in *ASI* expression and subsequently cause the aberrant patterning of leaves.
the vasculature network. In compatible with this suggestion, it was reported recently that TRN2 is synergistic with AS1 with respect to venation patterning (Cnops et al., 2006); and moreover, an altered auxin response pattern was observed in young leaflets of trn2 mutants and trn2 mutants also exhibited an ectopic and asymmetric distribution of auxin along the leaf margins of expanding leaves (Cnops et al., 2006). These results suggest that TRN2 is required for normal auxin distribution and vasculature patterning in leaves.

4.5 trn2 mutation compensates meristem defects of stm and wus mutants

Double mutant analyses revealed that trn23010 partially rescues stm phenotype, suggesting that trn2 mutation compensates for a loss-of-function SAM in stm mutants. Based on the WUS RNA in situ hybridization and real-time RT-PCR experiments, the expression domain of WUS and the amount of WUS transcripts are not affected in trn2 mutants. These results suggest that the stem-cell organizing center is not altered in the mutant SAM. In contrast with trn23010 stm double mutants, combinations of both trn23010 and wus-1 did not exhibit obviously additive phenotypes during the vegetative stage. Nevertheless, trn23010 wus-1 flowers are trn2-like, and surprisingly, these double mutant flowers develop carpels at the floral center, which are lacking in wus-1 single mutants (Laux et al., 1996). Reminiscent of the enlarged SAM in the vegetative trn23010 seedling, the trn23010 wus-1 double mutant floral meristem may contain an increased number of meristematic cells, which are not completely consumed through the precursor of outer whorl organs (as was the case in the wus-1 single mutant; Laux et al., 1996). If supernumerary meristem cells remain competent for the development of reproductive organs in the inner whorls, this could explain both the increase in stamen number and carpel development.

Taken together, these results indicate that trn2 mutation promotes meristem activity and compensates for the stm and wus meristem phenotypes. Both the enlarged vegetative shoot apex and the partial rescue of the stm mutant phenotype in the trn23010 background suggest that TRN2 may be involved in restricting meristem identity. This assumption is compatible with the lack of leaf segments observed (this study; Cnops et al., 2006) when cells that are normally recruited as leaf founder cells (according to their position) are not released for primordial fate in a timely fashion. The twisted and malformed lateral organs of these double mutants suggest that TRN2 acts epistatically to STM and WUS.
DISCUSSION

4.6 Genetic interactions between trn2 and clv3

CLV3 RNA in situ hybridizations revealed that the CLV3 expression domain is decreased in trn2 seedlings and real-time RT-PCR experiments indicated that the amount of CLV3 transcripts is half of the wild-type. Therefore, it is suggested that CLV3 expression is decreased in trn2 mutants. However, an expanded OC would be expected on a background of decreased CLV3 expression, yet this is not observed in trn2 mutants. A possible explanation is that although CLV3 expression is decreased, the amount of the gene product, the CLV3 peptide ligand, is still above the threshold required to maintain the homeostasis of the OC. A Student’s t-test gave a p value of less than 0.04 on average, suggesting a significant difference in the amount of CLV3 transcripts between trn2 mutants and wild-type plants. However, the decrease in CLV3 transcripts in trn2 seedlings is not as marked as the increase of STM transcripts (Table 2). Nevertheless, the possibility that TRN2 participates in the regulation of CLV3 expression cannot be unambiguously excluded.

Double mutant analyses revealed that no additive phenotype was observed in the trn23010 clv3-2 double mutants during vegetative development. Conversely, during reproductive development, trn23010 was epistatic to clv3-2 and organ numbers in the three outer whorls of sepals, petals and stamens resembled those observed in trn23010 mutant flowers, and not the increased numbers observed in clv3-2 flowers. In addition, instead of 2 mostly unfused carpels in trn23010 single mutants, the trn23010 clv3-2 double mutant flowers all contained supernumary carpels, which were generally fused (>70%) to a single twisted style. Consequently, double mutant flowers of trn23010 clv3-2 plants showed trn23010-specific reductions in organ number in the outer whorls of sepals and petals, and even decreased stamen number relative to clv3-2 alone, whereas additional organs were formed in the inner carpel whorl in double mutant combinations.

Supernumerary carpels at the center of trn23010 clv3-2 flowers suggest that clv3-2 phenotype is not rescued by the trn2 background in the innermost whorl of the flower, and this is reminiscent of the phenotype of prolonged WUS-expressing flowers (Lenhard et al., 2001). The activity of stem cells in the floral meristem has to be terminated after all organ primordia are formed, otherwise more than four whorls will be formed in Arabidopsis flowers. WUS expression is discontinued after stage 6 when carpel primordia are initiated (Mayer et al., 1998; Lenhard et al., 2001). Prolonged WUS expression in whorl 4, in a wild-type background, was shown to be
sufficient for floral meristem indeterminacy and resulted in massively proliferated gynoecium (Lenhard et al., 2001). AGAMOUS (AG) is a direct target of WUS and it was shown to be an inhibitor of WUS expression, thus creating a negative feedback to WUS in the floral meristem (Lenhard et al., 2001; Lohmann et al., 2001). However, AG alone is not sufficient to terminate WUS expression completely (Mizukami and Ma, 1997). WUS expression is more strongly increased in ag-1 clv1-4 double mutants compared with ag-1 single mutants (Lohman et al., 2001). Components of the CLV pathway, therefore, are candidates for cooperation with AG to terminate WUS expression (Schoof et al., 2000). Thus, in trn2 mutants, CLV and AG pathways are both active and WUS expression can be properly terminated after the formation of carpel primordia. However, in the trn23010 clv3-2 background, CLV3 activity is depleted and only the AG pathway is active. WUS expression is therefore prolonged in floral meristems and causes supernumerary carpels at the flower center.

4.7 TRN2 contributes to maintain a normal meristem function

A peculiarity of trn2 mutants is an enlargement of the shoot apex, which is readily detectable in the STM:GUS background at 11 DAG. However, floral buds are not observed at this stage and the expression of the gene LFY, a molecular marker for floral transition, was not detectable by RNA in situ hybridization in the trn2 SAM as in the wild-type SAM. This result indicates that the morphology change of the mutant SAM is not related to the floral transition. In addition, the discrepancy between the wild-type and trn2 SAM is not evident before 7 DAG, suggesting that the enlargement of the trn2 SAM is progressively accumulated during development. Based on RNA in situ hybridizations, WUS and CLV3 expression domains are not increased relative to the enlarged SAM, indicating that the stem-cell niche in the CZ is not increased in the mutant SAM. Therefore, an enlargement of the PZ could account for the enlarged SAM.

Genetic analyses of double mutants of trn23010 and stm or wus-1 indicated that mutation of trn2 may promote meristem activity. The progressively increased size, and the final aberrant morphology, of the trn2 SAM may reflect the gradual accumulation of indeterminate cells in the PZ. Excess number of indeterminate cells in the PZ may be the consequence of increased cell division rates in the CZ, which serves as a reservoir for stem cells and the PZ, or the proper recruitment of the cells in the PZ by lateral organ primordia is inhibited, or both. Increased rates of cell division in the CZ may cause an expansion of the CZ. However, this was not observed
when the stem-cell marker, CLV3, was used. Conversely, the trn2 phenotype of delayed leaf initiation, lamina missing in leaves and fewer floral organs implies that this mutation causes defects in the early steps of lateral organ primordia development. Moreover, these defects are reminiscent of mgoun1 (mgo1) and mgo2 mutant phenotypes. Both mgo1 and mgo2 plants have an enlarged SAM, which progressively enlarges between 8 and 12 days (Laufs et al., 1998a), and are defective in delayed leaf initiation compared with wild-type (Laufs et al., 1998a), have asymmetric leaves and flowers that are missing organs (Laufs et al., 1998a). These defects are the consequences of perturbation in initiation of primordia at the meristem periphery and the meristem outgrowth in mgo mutants may be a secondary effect of the perturbed primordia formation — instead of cells being recruited by the primordia they remain in the PZ (Laufs et al., 1998a; 1998b). In addition, although the trn2 SAM is larger than wild-type SAM, the vegetative meristem produces fewer leaves compared with wild-type, despite the fact that there are enough cells. trn2 mutants are different from mutants that perturb the stem cell niche (such as clv or wus), where the number of organs is positively correlated with the number of cells produced by the meristem. Taken together, these data strongly suggest that cells in the PZ of the SAM are not properly recruited by lateral organ primordia in trn2 mutants. Instead, they are maintained in an indeterminate state and remain in the PZ.

Excess accumulation of indeterminate cells in the PZ may be related to the higher activity of STM, suggesting that excess STM activity in trn2 mutants disturbs normal meristem function — even it is not ectopically expressed in lateral organ primordia. Double mutant analyses revealed that meristem activity in stm-1 mutants is recovered in a trn2 background, suggesting that the major function of the wild-type TRN2 gene is to limit the meristem identity. Considering that both TRN2 and STM are expressed in overlapping patterns in the SAM, and that TRN2 may act in another pathway rather than STM pathway, it is therefore suggested that TRN2 may contribute to maintain a normal function of the SAM by regulating STM activity, either directly or indirectly.

In conclusion, an EMS mutagenesis effector screen performed with the STM:GUS marker line in Arabidopsis thaliana identified the trn23010 allele. The lesion in trn23010 was identical to that in the trn2-2 allele, which carries a single G to A transition in the coding region of the second extra cellular loop of the TRN2 tetraspanin protein. Histological and genetic analyses described here implicate TRN2 in SAM function, as the peripheral zone appears enlarged relative to the central stem-cell zone in trn2 mutants. The trn23010 allele can partially rescue stm mutant phenotypes.
during seedling development, but is epistatic to wus-1 and clv3-2 alleles during the vegetative phase and in the outer floral whorls. The development of carpels in trn23010 wus-1 flowers suggests that pluripotent cells persist, in absence of the TRN2 function, to the end of floral development. These data indicate a role for a membrane bound plant tetraspanin-like protein meristem function.
5. SUMMARY

(In English)
The aerial part of higher plants is derived from the SAM. In *Arabidopsis thaliana*, *STM* is the gene required to initiate and maintain the meristem. To understand how *STM* is regulated during developmental processes, an EMS-mutagenesis screen was carried out to isolate the gene(s) that affects *STM* expression. A line containing a quarter of plants with expanded GUS activity in shoot tips was isolated, and based on the genetic mapping, the mutant locus was confirmed to be allelic to *tornado2* (*trn2*). Previous studies (Cnops, et al., 2000; Olmos et al., 2003) showed that *TRN2* gene encodes a tetraspanin-like protein and its mutation causes dwarf plants, twisted lateral organs and inflorescence shoots, and abnormal development of roots. However, its functions in the SAM have not been described. In this study, it was showed that *trn2* mutants have an enlarged SAM within excess *STM* transcripts. *TRN2* is expressed not only in all types of meristems, which overlaps with *STM* expression patterns, but also in lateral organ primordia and in incipient vascular tissues. This expression pattern of *TRN2* is compatible with the higher accumulation of *STM* transcripts in mutants and the pleiotropic phenotype of *trn2* plants. Besides, *WUS* expression is not affected whereas the amount of *CLV3* transcript is decreased in mutants. Genetic interactions showed that *trn2* partially rescued *stm* phenotype and acts epistatically to *wus* and *clv3*, though there was no additive phenotype found in *trn2* and *wus* or *clv3* double mutants during the vegetative phase, *trn2* mutation restored the carpels development in *wus-1* background and made flowers to become *trn2*-like but still have supernumerary carpels in the *clv3-2* background. Since the central zone (CZ) is not increased relative to the enlarged SAM, the increase of the periphery zone (PZ) could account for the enlargement of the SAM. Taken together, these results suggest that *TRN2* functions in limiting the meristem activity, and loss of function of TRN2 protein causes more cells undifferentiated and increases the meristematic activity.
6. ZUSAMMENFASSUNG

(Auf Deutsch)


In dieser Arbeit konnte gezeigt werden, dass *trn2* Mutanten ein vergrößertes SAM mit einem Überschuss an *STM* ausbilden. TRN2 wird nicht nur in allen Arten von Meristemen überlappend zum *STM*-Expressionsmuster angeschaltet, sondern zusätzlich in den Primordien der lateralen Organe und in sich entwickelndem vaskulärem Gewebe.

Dieses Expressionsmuster von TRN2 ist kompatibel zu der stärkeren Anhäufung des *STM*-Transkripts in Mutanten und dem pleiotropen Phänotyp der *trn2*-Pflanzen. Zusätzlich konnte gezeigt werden, dass die *WUS*-Expression nicht beeinflusst wird, während die Menge des *CLV3* Transkripts reduziert ist. Genetische Interaktionen zeigen, dass *trn2* teilweise den *stm* Phänotyp retten kann und epistatisch zu *wus* und *clv3* wirkt. Obwohl kein zusätzlicher Phänotyp während der vegetativen Phase in *trn2 wus* oder *trn2 clv3* Doppelmutanten gefunden wurde, konnte *trn2* die Entwicklung der Fruchtblätter im *wus*-1 Hintergrund wiederherstellen und führte zu Blüten ähnlich denen in *trn2*-Singlemutanten, im *clv3*-2 Hintergrund blieb allerdings die Anzahl der Karpellen erhöht. Da die zentrale Zone (CZ) relativ zum vergrößerten SAM nicht zunimmt, ist anzunehmen, dass die Ausdehnung des SAM mit der Vergrößerung der peripheren Zone (PZ) zusammenhängt.
ZUSAMMENFASSUNG

Zusammenfassend lässt sich aus diesen Ergebnissen schließen, dass TRN2 bei der Begrenzung der Meristemidentität mitwirkt und ein Verlust des TRN2 Proteins zur Folge hat, dass die Anzahl der undifferenzierten Zellen erhöht wird und somit die Region meristematischer Aktivität vergrößert wird.
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7. BIBLIOGRAPHY


ERKLÄRUNG


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