Functional Analysis of the Arabidopsis TETRASPANIN Gene Family in Plant Growth and Development

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TETRASPANIN (TET) genes encode conserved integral membrane proteins that are known in animals to function in cellular communication during gamete fusion, immunity reaction, and pathogen recognition. In plants, functional information is limited to one of the 17 members of the Arabidopsis (Arabidopsis thaliana) TET gene family and to expression data in reproductive stages. Here, the promoter activity of all 17 Arabidopsis TET genes was investigated by pAtTET::NUCLEAR LOCALIZATION SIGNAL-GREEN FLUORESCENT PROTEIN/β-GLUCURONIDASE reporter lines throughout the life cycle, which predicted functional divergence in the paralogous genes per clade. However, partial overlap was observed for many TET genes across the clades, correlating with few phenotypes in single mutants and, therefore, requiring double mutant combinations for functional investigation. Mutational analysis showed a role for TET13 in primary root growth and lateral root development and redundant roles for TET5 and TET6 in leaf and root growth through negative regulation of cell proliferation. Strikingly, a number of TET genes were expressed in embryonic and seedling progenitor cells and remained expressed until the differentiation state in the mature plant, suggesting a dynamic function over developmental stages. The cis-regulatory elements together with transcription factor-binding data provided molecular insight into the sites, conditions, and perturbations that affect TET gene expression and positioned the TET genes in different molecular pathways; the data represent a hypothesis-generating resource for further functional analyses.

During embryogenesis in plants, the fertilized egg cell develops gradually by consecutive, but partially overlapping, processes, such as cell division, patterning, and growth, into the rudimentary body plan of the mature embryo. Early pattern formation in the embryo generates the body plan polarity, with apical and basal stem cell progenitor domains, and the different concentric progenitor tissue layers, in which cells interpret their position, acquire cell fate, and differentiate into specific morphologies and functions after germination. Then, the apical meristems are activated to generate the primary root and the aboveground vegetative structures (Murray et al., 2012; Perilli et al., 2012). In the seedling, patterning processes occur in the epidermis to generate specialized cells such as trichomes and stomata, in the shoot apical meristem (SAM) upon leaf initiation and in the primary root pericycle upon lateral root initiation. Developmental programs such as germination, photomorphogenesis, and floral transition occur in response to environmental stimuli, such as light, circadian clock, and temperature, and hormonal stimuli that require cellular communication and signaling. Membrane proteins, located at the plasma membrane, are the most upstream components in signaling perception and transduction during cellular communication. It is estimated that 20% to 30% of all genes in most genomes encode integral proteins (Krogh et al., 2001), which are transmembrane proteins.

Tetraspanins are a distinct class of conserved integral proteins with four transmembrane domains, a small extracellular loop, and a large, Cys-rich extracellular loop, which is important for interacting with each other and other proteins to form tetraspanin-enriched microdomains that participate in cell-to-cell communication.
processes during cell morphogenesis, motility, and fusion (Yañez-Mó et al., 2009). Tetraspanins comprise large gene families in multicellular organisms: 33 in human and 36 in Drosophila melanogaster (Huang et al., 2005). Mutations in animal tetraspanins result in severe defects, such as incurable blindness and impaired fertility (Kaji et al., 2002; Goldberg, 2006). In plants, phylogenetic studies identified 17 tetraspanin genes in the Arabidopsis (Arabidopsis thaliana) genome, most of which are duplicated (Cnops et al., 2006; Wang et al., 2012; Boavida et al., 2013). Mutants of the Arabidopsis TETRASPANIN1/TORNADO2/EKEKO (TET1/TRN2) show defects in leaf lamina symmetry and venation pattern, root epidermal patterning (Cnops et al., 2000, 2006), peripheral zone identity of the SAM (Chiu et al., 2007), and megasporogenesis (Lieber et al., 2011). A number of Arabidopsis tetraspanins are expressed in reproductive tissues and at fertilization, and are localized at the plasma membrane of protoplasts (Boavida et al., 2013). However, a systematic analysis of all members of the plant TET gene family in embryonic and vegetative development and upon hormonal and environmental stimuli is lacking.

Here, the 17 members of the Arabidopsis TET gene family were analyzed for their cellular expression during embryonic and vegetative development by pAtTET1-17::NUCLEAR LOCALIZATION SIGNAL (NLS)-GFP/GUS reporter lines, which was instrumental for further mutational analyses. Indeed, a role for TET1 in primary root growth and lateral root development and redundant roles for TET5 and TET6 in leaf and root growth were identified, as suggested by the site of their expression. Complementary experimental and computational regulatory data sets were integrated to identify putative cis-regulatory elements in the TET promoters. A transcription factor (TF)-TET gene regulatory network was delineated, and regulatory interactions were confirmed by perturbation expression data.

RESULTS

Expression of TET Genes in Specific Tissues, Domains, and Cells throughout the Plant’s Life Cycle

The promoter activities of the 17 TET gene family members were analyzed by pAtTET::NLS-GFP/GUS lines throughout the life cycle in order to reveal their site and timing of activity and help in designing further mutational analyses. Per TET gene, at least three independent T2 pAtTET::NLS-GFP/GUS lines, with a single-locus insertion, were analyzed by 5-bromo-4-chloro-3-indoly-
\[\beta-D\]-glucuronide (X-Gluc) histochemical staining in embryo, root, leaf, and flower organs. None of the TET genes was expressed in all organs, and only a few had similar expression patterns (Fig. 1). Moreover, within a specific organ, TET gene expression was not constitutive but restricted to specific domains, tissues, or cell types.

Nine TET genes (i.e. TET1, TET3, TET4, TET5, TET8, TET10, TET13, TET14, and TET15) were expressed in the early globular and heart stage embryo, in which patterning takes place (Fig. 2). TET3 was expressed in the SAM progenitor domain of all embryo stages, TET13 in the hypophysis, and TET15 in the basal part of the embryo, including the hypophysis, suggesting that TET3, TET13, and TET15 might participate in apical-basal patterning. TET1 was asymmetrically expressed in vascular tissue precursor cells of the heart stage embryo. TET5 expression was restricted to vascular tissue progenitor cells in the center of the globular, heart, and torpedo stage embryo, a pattern similar to that of TARGET OF MONOPTEROS5, which is necessary for periclinal cell division and vascular tissue initiation (De Rybel et al., 2013). TET4 and TET10 were expressed in the central part of the embryo, including the vascular bundle progenitor region; TET14 expression was restricted to the vascular progenitor strand in the cotyledons, suggesting that TET1, TET4, TET5, TET10, and TET14 might participate in radial patterning. TET8 was expressed in the apical domain of the heart stage embryo and in the tip regions of the cotyledons of the torpedo and mature stage embryo (Fig. 2). After germination, expression remained at the apical domains and vascular tissues: TET3 in the SAM-organizing center

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Figure 1. Schematic overview of TET expression patterns in different organs. Duplicated gene pairs are indicated with square brackets. (The length of the square brackets does not represent the evolutionary distance.) E, Embryo; R, root; m, primary root meristem and root tip; d, primary root differentiation zone; lrp, lateral root primordia; C, cotyledon; L, rosette leaf; F, flower; se, sepal; pe, petal; st, stamen; ca, carpel.
Remarkably, TET2 was specifically expressed after germination in the stomatal guard cell lineage during early leaf development. TET2 expression was not detected in the meristemoid mother cell that originates from a protodermal cell, but it was in the small triangle-shaped daughter cell (called the meristemoid) generated after the first asymmetric division (Fig. 3A) and not in the large daughter cell (called the stomatal lineage ground cell). TET2 expression remained in the guard mother cell (Fig. 3D) after the first and subsequent asymmetric divisions (Fig. 3B and C) and in mature guard cells (Fig. 3E).

TET gene expression in progenitor tissues, domains, and cells remained in stem cells of shoot or root apical meristems or in their derived differentiated tissues or cell types in the seedling, suggesting that their function is required at different developmental stages. The variation in spatial and temporal expression patterns of the 17 TET genes suggests a range of functions in the plant’s life cycle.

**Divergent TET Expression Patterns within Clades, But Overlapping among Clades**

The TET gene family consists of different clades with duplicated or triplicated members (Cnops et al., 2006; Wang et al., 2012; Boavida et al., 2013), showing diverging expression profiles (Figs. 1–3). Indeed, the duplicated genes TET1 and TET2 had strikingly divergent gene expression patterns. The TET1 asymmetric gene expression pattern was found in vascular tissue precursor cells in the embryo, in the columella cells and vascular tissues of the primary root (Fig. 2), in the vascular tissues of cotyledons and rosette leaves, and in the stigma and transmitting tissue of the female gametophyte (Supplemental Figs. S1, C, J and N, and S2, A and B), which correlated with tet1 mutant phenotypes observed in root and leaf (Cnops et al., 2000, 2006), in SAM (Chiu et al., 2007), in embryo (Lieber et al., 2011), and in fertility, which was strikingly different from TET2 expression specifically in the stomatal cell lineage (Fig. 3). The duplicated genes TET3 and TET4 also had divergent expression patterns. TET3 was expressed in the SAM precursor cells during embryogenesis (Fig. 2) and in the SAM-organizing center of the seedling (Supplemental Fig. S1A). The TET3-GFP protein moved toward and along the plasma membrane and

**Figure 2.** TET expression patterns during embryogenesis and in the primary root meristem after germination. Transgenic plants shown are as follows: pAtTET1::NLS-GFP/GUS, pAtTET3::NLS-GFP/GUS, pAtTET4::NLS-GFP/GUS, pAtTET5::NLS-GFP/GUS, pAtTET8::NLS-GFP/GUS, pAtTET10::NLS-GFP/GUS, pAtTET13::NLS-GFP/GUS, pAtTET14::NLS-GFP/GUS, and pAtTET15::NLS-GFP/GUS. Columns represent consecutive developmental stages: globular, heart, torpedo, and mature. Dotted lines delineate the outlines of the young embryos. Arrows in TET1, TET3, and TET14 images indicate gene expression sites. Bars = 0.01 mm (globular, heart, and torpedo stages) and 0.1 mm (mature embryo and root meristem).
localized itself at the plasmodesmata (Supplemental Fig. S1B; Supplemental Movie S1), which confirmed previous purification from the Arabidopsis plasmodesmal proteome (Fernandez-Calvino et al., 2011). In the primary root, TET3 was expressed in the cortex, endodermis, and pericycle at the differentiation zone (Fig. 2). TET4 was expressed in vascular tissue progenitor cells during embryonic development, in the QC and vascular tissue of the primary root (Fig. 2), in stomatic guard cells of cotyledons and anthers, and in the basal region of the flower (Supplemental Figs. S1E and S2A). Similarly, the triplicated genes TET7, TET8, and TET9 had distinct expression patterns. TET7 expression was restricted to the pollen (Supplemental Fig. S2C). TET8 was expressed in the apical domain of the embryo, starting from the heart stage (Fig. 2), in the differentiation zone of the primary root (Fig. 2), and in stipules and hydathodes of rosette leaves (Supplemental Fig. S1, F and G). TET9 was expressed in the vascular tissues of the primary root (Fig. 2), in cotyledons and rosette leaves, in the SAM, and in trichomes and surrounding pavement cells (Supplemental Fig. S1, J–L). TET10 is the only single gene within the family. It was widely expressed in globular, heart, and torpedo stages during embryogenesis (Fig. 2). In the mature embryo, it was expressed in the vascular tissue of root and cotyledons. After germination, the expression remained in the primary root meristem but not in the vascular tissue of cotyledons (Fig. 2). TET11 was expressed only in the pollen (Supplemental Fig. S2C), and its duplicated gene, TET12, was expressed in the primary root and stipules (Supplemental Fig. S1M), suggesting a thoroughly diverged function. The triplicated genes TET13, TET14, and TET15 diverged in expression patterns. TET13 was expressed in the embryonal hypophysis, in the QC, stem cells, and columella cells of the primary root, and in the lateral root primordia (LRP; Figs. 2 and 4, A and E–N). TET14 expression was restricted to vascular precursor cells of heart stage embryos and in the cotyledonary vascular tissues of the mature embryo (Fig. 2). TET15 was expressed in the basal domain of the heart stage embryo, in the lateral root cap and columella cells (Fig. 2), in the pollen tubes, and in stomatal guard cells of sepal (Supplemental Fig. S2, B and C). TET16 was expressed only in the basal region of the flower and pollen (Supplemental Fig. S2C), and no TET17 gene expression was detected in any of the organs, which was consistent with the absence of gene expression in transcriptome data of Genevestigator or the eFP browser throughout development. Nevertheless, nucleus-localized TET17-GFP protein was detected in mature pollen (Boavida et al., 2013).

However, overlapping expression patterns were observed in TET genes of different clades in pollen (TET1,

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**Figure 3.** Diagram of Arabidopsis stomatal development (left; based on Pillitteri and Torii [2012]) and the respective TET2 expression patterns at the abaxial epidermis of 6-d-old cotyledons of the pAtTET2::NLS-GFP/GUS line (right). A, TET2 expression in the meristemoid after entry division. B and C, TET2 expression in the meristemoid after one or two additional asymmetric divisions. The arrow indicates the meristemoid.
Figure 4. TET13 expression in the primary root and LRP, tet13-1 root phenotypes, and complementation. Transgenic plants shown are as follows: pAtTET13::NLS-GFP/GUS (A, E–N, and Q), tet13-1 (B–D, O, and P), C1 and C5 (tet13-1 containing one and two T-DNA loci with p35S::TET13; C and P), pDR5::GUS (R and T), and tet13-1 x pDR5::GUS (S). A, TET13 expression in the primary root tip. B, tet13-1 scheme and 12-d-old seedlings of Columbia-0 (Col-0) and tet13-1 growing vertically under the 24-h light condition. C, Primary root growth kinetics. Means ± so are presented (n = 22–33). Asterisks mark significant differences: *, P < 0.05; **, P < 0.01; and ***, P < 0.001. D, Root meristem size (defined as the number of root meristem [RM] cells) of 6-d-old seedlings. Means ± so are presented (n = 28–39). E to N, Detailed expression analysis of TET13 at different stages during lateral root development. Corresponding developmental stages I to VII and emerged lateral root (EMLR) are indicated in the top right corner. IL and OL, Inner and outer layers, respectively; c, cortex; en, endodermis; ep, epidermis; p, pericycle. Arrowheads indicate the division planes. The inset in I shows the top view of stage IV. O, Staging of LRP and EMLR densities. Means ± so are presented (n = 20). P, EMLR densities. Means ± so are presented (n = 22–33). ns, Not significant. Asterisks mark significant differences: *, P < 0.05; **, P < 0.01; and ***, P < 0.001. Q and R, NAA-induced TET13 (Q) and pDR5::GUS (R) expression. The seedlings were grown on 10 μM NPA medium for 72 h after germination and then transferred to 10 μM NAA medium for 0, 2, and 3 h. S and T, DR5::GUS expression patterns at the root basal meristem in 7-d-old tet13-1 x pDR5::GUS (S) and wild-type (T) seedlings. In Q to T, black arrowheads indicate sites of inducible expression. Bars = 0.01 mm (E–N), 0.1 mm (S and T), and 0.5 mm (Q and R).
TET2, TET3, TET4, TET7, TET8, TET9, TET10, TET11, TET13, TET14, TET15, and TET16; Supplemental Fig. S2C) and in vascular tissues of the embryo and/or the seedling (TET1, TET3, TET4, TET5, TET6, TET8, TET9, TET10, TET12, and TET14; Fig. 2; Supplemental Fig. S1), suggesting redundancy in function that might complicate functional analysis by the mutational approach. Transfer DNA (T-DNA) insertion lines in TET genes were characterized as knockout or knockdown alleles by quantitative reverse transcription-PCR, and phenotyping in the respective mutants was done with particular attention to organs in which the respective TET gene was expressed. Strikingly, only in the TET13 knockout line, tet13-1, was a clear phenotype observed in the primary and lateral root development, which correlated with its site of expression (Supplemental Table S1). Although the single mutant analysis was not exhaustive for putative phenotypes, the identification of a phenotype for only one TET gene argued for functional redundancy among the other TET genes and showed the need for double and triple mutant combinations.

**TET13 Function in Primary Root Growth and Lateral Root Development**

*TET13* expression and mutant phenotypes were studied in more detail during primary and lateral root development. Arabidopsis root identity is specified early during embryogenesis. The cells at the basal region of the globular embryo will give rise to the hypocotyl, primary root, and root stem cells. The lens-shaped cells in this region are the progenitors of the QC, which is crucial for maintaining root stem cell identity (van den Berg et al., 1997). In wild-type plants, the stem cells surrounding the QC are maintained in the undifferentiated state. In the primary root, *TET13* was predominantly detected in the QC, most of the stem cells (cortex/endodermis initials, pericycle initials, vascular tissue initials, and columella initials but not epidermis initials), the first two columella cell layers, and the two middle cells in the root cap (Fig. 4A). *TET13* promoter activity was equally present at the two QC cells of 35 seedlings, indicating that *TET13* was not cell cycle regulated. The function of *TET13* in primary root and lateral root development was studied in the T-DNA insertion line *tet13-1* (SALK_011012C), in which the T-DNA is located at the first exon, resulting in a gene knockout (Boavida et al., 2013). In addition, complementation of root phenotypes was studied in the C1 and C5 complementation lines of *tet13-1* containing one and two T-DNA loci with p35S::TET13, respectively. The primary root length was reduced significantly in *tet13-1* (Fig. 4, B and C), partially restored to wild type in the C1 line, and fully restored and even larger than the wild type in the C5 line (Fig. 4C). The primary root length is determined by several parameters such as meristem, elongation zone, and differentiation zone size and cell length at these zones. The meristem size, defined as the number of cells from the QC to the first elongated cell in the cortex cell file (Casamitjana-Martinez et al., 2003) was reduced significantly in *tet13-1* (27 ± 3 [sd] cells) as compared with the wild type (34 ± 4 cells; Fig. 4D; Supplemental Fig. S3A). Below the QC, there are one or two layers of undifferentiated columella initials that generate the differentiated columella cells containing starch that stains purple by Lugol solution. One of the functions of the QC is to keep the surrounding initials in an undifferentiated state, because defective QC function results in a differentiated cell layer below the QC instead of the columella initial cell layer (van den Berg et al., 1997; Sarkar et al., 2007). Exogenous auxin (1-naphthaleneacetic acid [NAA]) or auxin transport inhibitor (N-1-naphthylphthalamic acid [NPA]) promoted the differentiation of columella initial cells in wild-type seedlings but not in mutants in auxin biosynthesis or transport (Ding and Friml, 2010). In *tet13-1* mutants, one or two columella initial cell layers were measured in primary roots, which is comparable with the wild type, and no precocious differentiation of the columella initials was observed (Supplemental Fig. S3, B and C); hence, columella initial cell identity was maintained in the *tet13-1* mutant under normal conditions and after NAA and NPA treatment (Supplemental Fig. S3, D–F). The architecture of the QC and initials was normal in the *tet13-1* mutant, as visualized by confocal microscopy with propidium iodide staining (Supplemental Fig. S3G), indicating no function of *TET13* in cytokinesis. *tet13-1* seedlings were equally insensitive as wild-type seedlings to hydroxyurea, a replication-blocking agent, because no dead cells were observed after propidium iodide staining (Supplemental Fig. S3, H and I).

Auxin oscillation in the pericycle of the primary root basal meristem (De Smet et al., 2007) determines the lateral root founder cell fate, and their anticlinal asymmetric division results in a single-layered primordium composed of small daughter cells flanked by two large daughter cells, termed stage I. During lateral root development, *TET13* was active at the two neighboring pericycle cells before the asymmetric division (Fig. 4E). At stage I, the expression was stronger in the two newly generated, small daughter cells (Fig. 4F). From stage II to VII (Fig. 4, G–L), anticlinal and periclinal divisions form a dome-shaped primordium with two to seven cell layers. At stage II, *TET13* was expressed equally strongly in the tip cells at the outer layer as well as in the middle two cells at the inner layer (Fig. 4G). From stage III on, *TET13* expression was always stronger in the tip cells at the outer layer (Fig. 4, H–M) and comparable to that of the pDR5::GUS reporter gene (Benková et al., 2003). At stage VIII, upon lateral root emergence, *TET13* was expressed in the QC and columella (Fig. 4N).

Lateral root initiation stages and lateral root emergence were analyzed in *tet13-1* mutants; the number of stage I LRP was reduced severely (Fig. 4O), which correlated with early *TET13* expression in the pericycle founder cells before the first asymmetric division (Fig. 4E); stage IV, V, and VI LRP densities were increased significantly, whereas the number of stage II, III, and VII LRP was increased slightly in *tet13-1* (Fig. 4O); the EMLR density was reduced significantly (Fig. 4, O and P).
Because the LRP and EMLR densities, defined as the number of LRP and EMLRs per 1 cm of primary root, were significantly increased and reduced, respectively (Fig. 4O), TET13 has a potential role in restricting lateral root initiation and promoting lateral root emergence. The EMLR density in the complementation lines C1 and C5 was restored to the wild-type level (Fig. 4P). In an assay for synchronous lateral root initiation (Himanen et al., 2002), seedlings were first incubated on medium containing the auxin transport inhibitor NPA and then transferred to NAA medium, after which synchronous lateral root initiation was monitored. The TET13 promoter activity was induced after 2 h of NAA induction at the xylem pole of the pericycle, which coincides with the onset of stage I (Fig. 4Q); after 3 h of NAA induction, two clear blue lines marked the pericycle (Fig. 4Q), which was similar to the pDR5::GUS reporter gene expression pattern (Fig. 4R), indicating that TET13 expression is auxin inducible and specific for pericycle cells. In the tet13-1 mutant, the pDR5::GUS reporter gene was not or very weakly expressed at the basal meristem in eight out of 13 tested seedlings (Fig. 4S), whereas in all 11 wild-type seedlings it was expressed (Fig. 4T), indicating that auxin accumulation in the pericycle founder cells is affected. Hence, the tet13-1 mutant allele and the complementation lines showed that TET13 has a function in the primary root affecting apical meristem size and root length and in lateral root initiation and emergence, which is consistent with its expression sites in the root.

**TET5 and TET6 Have Redundant Functions in Root and Leaf Growth**

TET5 and TET6 were the only duplicated genes with similar expression patterns (i.e. in the vascular tissue of all organs, except for embryos, in which only TET5 was expressed; Fig. 1). At the globular stage, TET5 expression was restricted to the vascular tissue progenitor cells (Fig. 1). After germination, both TET5 and TET6 were expressed in the pericycle, the vascular tissues of the primary root starting from the transition zone, the hypocotyl, cotyledons, and rosette leaves (Fig. 5, A and B). Transverse sections of the primary root showed that, at the meristem-elongation transition zone, TET5 and TET6 were expressed in the phloem, a few procambial cells surrounding the phloem, and the phloem-pole pericycle but not in the protoxylem (Fig. 5A, bottom). At the differentiation zone, they were expressed in the pericycle and all vascular tissues except for the protoxylem (Fig. 5A, top). In the siliques, they were expressed in the funiculus, which has a function in guiding the pollen tube to reach the micropyle during fertilization (Supplemental Fig. S2B). The redundant expression patterns suggest redundant functions, which was confirmed by single and double mutant analyses. Several tet5 and tet6 mutant alleles were analyzed for their TET5 and TET6 gene expression: tet5-1 (GABI-Kat 290A02, insert in promoter), tet5-2 (SALK_148216, insert in first exon; Boavida et al., 2013), and tet5-3 (SALK_020009C, insert in second exon) are TET5 knockouts, tet6-1 (SALK_005482C, insert in promoter) is a TET6 up-regulated line, and tet6-2 (SALK_139305, insert in promoter) is a TET6 knockdown (Fig. 5C). No morphological alterations or patterning defects were observed in the roots, leaves, or inflorescences of the single mutants tet5-1, tet5-2, tet5-3, and tet6-2. However, morphological analysis of three different double mutant combinations, tet6-2tet5-1 (Supplemental Fig. S4), tet5-2tet6-2 (Supplemental Fig. S4), and tet5-3tet6-2 (Fig. 5, E–I), revealed synergistic phenotypes, such as an enlarged leaf size in seedlings at all stages of rosette development (Fig. 5F) due to a significantly increased total number of cells per leaf (23,238 ± 2,967 in tet5-3tet6-2 compared with 15,495 ± 1,635 in tet5-3 and 15,197 ± 2,887 in tet6-2; Fig. 5G), increased fresh weight in 21-d-old seedlings (Fig. 5H), and longer primary roots in a root growth kinetic study (Fig. 5I), confirming redundant functions of TET5 and TET6 in restricting cell proliferation during root and leaf growth.

**TET Promoter cis-Regulatory Elements to Predict Responses to Environmental and Developmental Stimuli**

The cis-regulatory elements (or motifs) were identified in the TET promoter regions using an integrative bio-informatics approach (see “Materials and Methods”) in order to further study and explain the divergence, overlap, and redundancy in TET gene expression. The cis-regulatory elements also provided information about environmental and developmental stimuli that might influence TET gene expression, which could be used for further functional analysis. Apart from mapping known cis-regulatory elements to the 2-kb promoters of the different TET genes, also coregulatory genes, evolutionary sequence conservation, and information about open chromatin regions (DNaseI-hypersensitive [DH] sites) were integrated to identify cis-regulatory elements and TFs putatively regulating TET genes. Recently, we have shown that combining complementary regulatory data sources has a positive impact on reducing the high number of false positives associated with simple mapping of cis-regulatory elements and strongly increases the likelihood that retained regulatory interactions are biologically functional (Vandepoele et al., 2009; Van de Velde et al., 2014). To identify TET cis-regulatory elements shared with coregulated genes, for each TET gene, Genevestigator was first used to identify a set of tissue and perturbation experiments in which the gene was strongly responsive (see “Materials and Methods”). Next, coregulated genes were identified under these conditions, and enrichment analysis was performed to identify cis-regulatory elements significantly shared between each TET gene and its coregulated genes, considering the motifs obtained by simple motif mapping or simple motif mapping filtered for DH sites or filtered for evolutionary conservation (indicated with analysis type motif, DH, and CM in Supplemental Table S2, respectively). In total, 128 cis-regulatory elements were identified in the TET
promoters and their respective coregulated genes, yielding regulatory information about 13 TET genes (no result for TET11, TET13, TET16, and TET17). More than half of the regulatory elements (71 of 128) were identified through the enrichment analysis using the motif-mapping files filtered for DH sites or evolutionary conservation, indicating that they are functionally related to accessible TF-binding sites in open chromatin regions or to regulatory elements that have been conserved during hundreds of millions of years of evolution (Supplemental Table S2).

Figure 5. TET5 and TET6 expression, and tet5-3, tet6-2, and tet5-3tet6-2 mutant phenotypes. Transgenic plants shown are as follows: pAtTET5::NLS-GFP/GUS (A and B), pAtTET6::NLS-GFP/GUS (A and B), tet5-3 (C–I), tet6-2 (C–I), and tet5-3tet6-2 (E–I). A, TET5 and TET6 expression in the vascular tissue of the meristem-elongation transition zone (between dashed lines) and the differentiation zone (top) of the primary root, of which part of the elongation zone is omitted; the root tip (lower images) and the differentiation zone (upper images) are shown. Transverse sections show the transition zone and differentiation zone, as indicated by the dashed lines. C, Cortex; e, endodermis; ep, epidermis; p, pericycle. Arrowheads and arrows indicate phloem pole and protoxylem, respectively. B, TET5 and TET6 expression in the rosette leaves. C, TET5 and TET6 schemes with T-DNA insertions. Thick, thin, and broken lines indicate exons, introns, and promoter, respectively. D, Relative TET5 (right) and TET6 (left) transcript levels in 7-d-old seedlings measured by quantitative reverse transcription-PCR. Means ± SD are presented (n = 3). E, Rosette and leaf series of 21-d-old seedlings. From left to right are leaf 1 to leaf 8; incisions were made to make the leaves fully expanded when necessary. F, Quantification of the rosette leaf sizes in E. Means ± SD are presented (n = 8–10). G, Total number of cells per leaf. Leaf 1 and/or 2 were used. Means ± SD are presented (n = 3). H, Fresh weight of 21-d-old seedlings. Only rosette leaves were used for the experiment. I, Primary root growth kinetics (root length). Means ± SD are presented (n = 31–39). Asterisks in all graphs mark significant differences: *, P < 0.05; **, P < 0.01; and ***, P < 0.001. Bars = 0.01 mm (A, transverse section), 0.1 mm (A, longitudinal section), and 1 mm (B).
A heat map was generated with TET differential gene expression upon a selection of perturbations in Genevestigator (Supplemental Fig. S5A) and used to verify the cis-regulatory elements in TET promoters identified in Supplemental Table S2. The cis-regulatory elements identified from TET1, TET2, TET3, TET4, TET5, TET6, TET8, TET9, and TET16 correlated with their expression patterns or inducible conditions. TET1 up-regulation by high light correlates with the enrichment in the light-regulated gene-binding sites and induction upon brassinolide treatment with a GATA promoter motif (Supplemental Table S2) that can be recognized by GATA family TFs, some of which mediate the cross talk between brassinosteroid and light signaling pathways (Luo et al., 2010). TET2 promoter elements are related mainly to stress responses, including cold, dehydration, and drought responses (Supplemental Table S2), and fit with the up-regulation of TET2 in response to abscisic acid (ABA), cold, and drought (Supplemental Fig. S5A), which can induce stomatal closure. TET2 function in the mature stomatal guard cell might relate to stomatal closure caused by stress conditions. TET3 up-regulation by ABA, cold, and drought and in the C-REPEAT/DEHYDRATION-RESPONSIVE ELEMENT-BINDING FACTOR3 (CBF3) overexpression background (Supplemental Fig. S4A) correlates with cold- and drought-responsive elements and the CBFHB element that is the binding site of CBF3 (Supplemental Table S2), a TF involved in cold response. The TET3 expression pattern in the SAM and the cold response element at the promoter region suggest a function in cold sensing at the SAM or upon floral transition. TET4 is also up-regulated by ABA, cold, and drought and in the CBF3 overexpression background (Supplemental Fig. S5A) and correlates with ABA response elements and elements in seed-specific promoters (Supplemental Table S2; Ezcurra et al., 2000; Chandrasekharan et al., 2003). TET5 and TET6, both expressed in the vascular tissue, contain distinct sugar response promoter elements that suggest a role in sugar sensing and growth (Supplemental Table S2). TET8 and TET9 promoter regions contain defense and pathogen response elements (Supplemental Table S2), which correlate with high up-regulation by pathogens and elicitors (Supplemental Fig. S5A); in addition, there are three endosperm-specific elements that fit with TET8 expression in the endosperm (Supplemental Table S2; Supplemental Fig. S1H). One of them is the MYB98-binding site; MYB98 expression is observed in the synergid cells (Kasahara et al., 2005). Presumably, TET8 is expressed in the synergid cells as well (Supplemental Fig. S1H). TET16 is 63 times up-regulated during pollen tube growth (Supplemental Fig. S5A) and is expressed specifically in pollen (Supplemental Fig. S2C), suggesting a function in this process.

In conclusion, the cis-regulatory element analysis confirmed the divergence in reporter expression of duplicated TET genes. The presence of the same regulatory elements in TET genes of different clades, such as the root-specific element in TET4 and TET10 and the vascular tissue-specific element in TET4, TET8, TET9, and TET12 (Supplemental Table S2), supported the redundancy of the reporter expression of TET genes over the different clades.

A TF-TET Gene Regulatory Network to Predict Function in Molecular Pathways

In order to obtain a better understanding of the transcriptional regulation of TETs and their positions in molecular pathways, a TF-TET gene regulatory network was built by combining different regulatory data sets. Apart from conserved TF-binding sites, also chromatin immunoprecipitation (ChIP) TF-binding data and TF expression perturbation information were integrated to generate a TF-TET gene regulatory network (data types denoted as conserved motif, ChIP, and DE in Fig. 6, respectively). Although these data sets cover different aspects of gene regulation, it is important to note that the obtained gene regulatory network offers only a partial view of TET regulation, since, as for many Arabidopsis TFs, no binding site information or experimental data are available.

The conservation of cis-regulatory elements has been used to delineate gene regulatory networks in different species (Aerts, 2012), and a recent study in Arabidopsis used a phylogenetic footprinting approach to delineate conserved gene regulatory networks based on known binding sites for 157 TFs (Van de Velde et al., 2014). TF ChIP-Seq and ChIP-chip analyses have been used to identify target genes of different plant regulators, including TFs involved in flowering, circadian rhythm and light signaling, cell cycle, and hormone signaling (Mejia-Guerra et al., 2012). To integrate experimental ChIP TF-binding information, the network of Heyndrickx et al. (2014) was used, in which 27 publicly available TF-ChIP data sets have been reprocessed through an analysis pipeline consisting of quality control, platform-specific signal processing, and peak calling to generate TF-TET gene interactions. Next to the physical binding of a TF in the vicinity of a TET gene, which can serve as a proxy for TET gene regulation, information about the regulation of a TET gene by a specific TF was also taken into account. Therefore, we screened differentially expressed genes that were obtained through transcript profiling after perturbation (knockout or overexpression) of the normal activity of a TF from 15 publicly available studies. Differentially expressed genes from five studies, in which regulatory information about TET genes was reported, were retained, complementing the ChIP binding data for AGL15, BES1, LFY, and FUS3 (see “Materials and Methods”). Most of the TETs were regulated by multiple TFs (i.e. TET3, TET4, TET8, TET9, and TET14). Some duplicated TETs shared common TFs (i.e. TET3 and TET4, TET5 and TET6, and TET8 and TET9; Fig. 6).

To further validate regulatory interactions in the network, such as those inferred through ChIP and conserved TF-binding sites, expression perturbation data from Genevestigator were obtained for nine TFs.
and TF-TET interactions were confirmed by significant differential expression after TF perturbation (Table I). The TFs in the regulatory networks were related to light response (PIF4 and PIF5), circadian clock (TOC1), floral organ identity (AP1, AP2, AP3, SEP3, and PI), flowering initiation (AGL15 and LFY), and defense response (EIN3, MYB4, and SR1) and identify the respective TET genes as novel components of specific developmental or physiological pathways.

**DISCUSSION**

Arabidopsis TET genes are expressed in different organs/tissues and cell types during embryonic and
vegetative development. Intriguingly, the onset of expression coincided with the onset of patterning and cell specification in globular and heart stage embryos (TET1, TET3, TET4, TET5, TET8, TET10, TET13, TET14, and TET15) and in seedlings at the initiation of the stomatal cell lineage (TET2) or at the asymmetric division in the primary root pericycle upon lateral root initiation (TET13), which suggests a role for these TET genes in cell specification. Plant cells are surrounded by a rigid but dynamic cell wall, which prevents them from migration during specification; hence, they acquire their fate by positional information from neighboring cells. The cellular communication involves diffusible or actively transported molecules, such as peptides, small RNAs, TFs, or phytohormones, that upon recognition by competent cells trigger signaling cascades that result in the initiation or repression of specific developmental programs (Sparks et al., 2013). Remarkably, nine TETRASPANINs (TET1, TET3, TET4, TET5, TET8, TET10, TET13, TET14, and TET15) are expressed in embryonic progenitor tissue, and three are expressed early in the cell lineage of the seedling until differentiation (TET2, TET9, and TET13). The expression in the progenitor cells of a cell lineage suggests that they might be required in progenitor cell identity establishment or maintenance or in the lateral inhibition of neighboring cells. Their expression later in the differentiated cells of the same cell lineage suggests a function in the physiology or biochemistry of the mature cells, which might be related to or not to its early function. Hence, the expression of certain TET genes in plants, very early in a cell lineage until the differentiated state, might indicate dynamic interactions in time with partner proteins that are components of different molecular pathways.

Most of the duplicated TET genes have diverged expression patterns during the plant’s life cycle, as was demonstrated in the pTET-reporter lines, and coincided with different regulatory elements in their promoters (Supplemental Table S2). Even though the duplicated TET5 and TET6 genes showed redundant expression in vascular tissue, different regulatory elements related to sugar sensing were identified in their promoters (Supplemental Table S2). Largely overlapping expression patterns were observed in TET genes over the clades in embryos, primary roots, rosette leaves, and pollen and might refer to the conservation of ancestral TET gene functions in specific tissues or cells. Indeed, similar regulatory elements were identified in TET promoters belonging to different clades, such as ABA-responsive, root-specific, and light-related motifs, which add to the putative overlap in function (Supplemental Table S2). Here, it might be more logical to suggest that these paralogous TET genes evolved in their promoter through subfunctionalization (Moore and Purugganan, 2005).

These overlaps might explain the mild or undetectable phenotypes in single mutants in our mutational analysis (Supplemental Table S1) and demonstrate the need for making double or triple mutant combinations over the clades to detect phenotypes. Such a strategy was rewarding in animals, resulting in strong phenotypes in mice and D. melanogaster (Fradkin et al., 2002; Rubinstein et al., 2006).

### Table 1. TET transcript changes upon TF perturbation

Genevestigator microarray studies on genotypes perturbed for the TFs identified in Figure 6 were analyzed for differential TET transcript levels at $P < 0.05$. In double mutant and artificial microRNA backgrounds, only the TFs of interest are listed. Fold change was calculated from the log2 ratio.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DE TF</th>
<th>Fold Change, DE TF</th>
<th>P</th>
<th>DE TF</th>
<th>Fold Change, DE TF</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>agl15-agl18-1/Col-0</strong></td>
<td><strong>AGL15</strong></td>
<td>-3.77</td>
<td>&lt;0.001</td>
<td><strong>TET3</strong></td>
<td>+1.35</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>35S::amiR-mads-2/MIR319a_strong/Col-0</strong></td>
<td><strong>API</strong></td>
<td>-5.24</td>
<td>&lt;0.001</td>
<td><strong>TET4</strong></td>
<td>-1.18</td>
<td>0.051</td>
</tr>
<tr>
<td><strong>35S::LFY-GR/Landsberg erecta</strong></td>
<td><strong>LFY</strong></td>
<td>-1.95</td>
<td>0.004</td>
<td><strong>TET5</strong></td>
<td>-1.04</td>
<td>0.71</td>
</tr>
<tr>
<td><strong>lfy-12/Col-0</strong> (shoot apex, rosette is 50% of final size)</td>
<td><strong>LFY</strong></td>
<td>-3.33</td>
<td>0.007</td>
<td><strong>TET6</strong></td>
<td>+1.19</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>lfy-12/Col-0</strong> (inflorescence, first flower open)</td>
<td><strong>LFY</strong></td>
<td>-1.82</td>
<td>0.006</td>
<td><strong>TET7</strong></td>
<td>+1.66</td>
<td>0.207</td>
</tr>
<tr>
<td><strong>pif4-101pif5-3/Col-0</strong></td>
<td><strong>PIF4, PIF5</strong></td>
<td>-1.80</td>
<td>&lt;0.001</td>
<td><strong>TET8</strong></td>
<td>+1.35</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Alc::TOC1/Col-0</strong></td>
<td><strong>TOC1</strong></td>
<td>+8.59</td>
<td>&lt;0.001</td>
<td><strong>TET9</strong></td>
<td>+1.33</td>
<td>0.039</td>
</tr>
<tr>
<td><strong>camta3-2/Col-0</strong></td>
<td><strong>SR1</strong></td>
<td>-5.61</td>
<td>0.003</td>
<td><strong>TET10</strong></td>
<td>+1.80</td>
<td>0.019</td>
</tr>
<tr>
<td><strong>ein3-1eil1-1/coi1-2</strong></td>
<td><strong>BIN3</strong></td>
<td>-9.53</td>
<td>0.003</td>
<td><strong>TET11</strong></td>
<td>+2.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>35S::TPS1</strong></td>
<td><strong>TPS1</strong></td>
<td>+36.58</td>
<td>&lt;0.001</td>
<td><strong>TET12</strong></td>
<td>+1.18</td>
<td>0.107</td>
</tr>
</tbody>
</table>

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TET5 and TET6 duplicated genes have the same promoter activity pattern in postembryonic vascular tissues and in cells surrounding procambial cells and the phloem, suggesting a putative redundant function in vascular bundle activity, such as in nutrient or photo-assimilate transport, rather than in patterning, because no venation patterning defect was observed in the double tet5tet6 mutant. Combining the single tet5 and tet6 mutants resulted in significantly enhanced growth by cell proliferation in root and shoot, suggesting that TET5 and TET6 restrict growth through a sugar-sensing mechanism, because such cis-regulatory elements were identified in their promoters (Supplemental Table S2). Indeed, Suc promotes cell proliferation by activating the cell cycle (Riou-Khamlichi et al., 2000). Arabidopsis tetraspanins can form homodimers and heterodimers when expressed in yeast (Boavida et al., 2013). The redundant function of TET5 and TET6 suggests that, for proper activity, heterodimers might need to be formed or interaction with a common protein might be required, which needs further testing.

TET13 expression in the pericycle founder cells and the gradual spatial pattern in the LRP resemble auxin accumulation and gradients that regulate lateral root development (Benková et al., 2003). TET13 shows overlapping expression in the pericycle with TET3, TET4, TET5, TET6, TET8, TET9, TET10, and TET12, which might explain the mild phenotype in lateral root development in tet13-1. Significant reduction of stage I LRP density in tet13-1 indicates that lateral root initiation is affected, and TET13 is required to promote lateral root initiation. It nicely correlated with TET13-specific expression in the two pericycle founder cells before the asymmetric divisions that precede lateral root initiation and with its early inducible expression in the xylem pole pericycle at the root basal meristem upon NAA treatment with its early inducible expression in the xylem pole pericycle at the root basal meristem upon NAA treatment. Therefore, TET13 might explain the mild phenotype in lateral root development (Benková et al., 2003).

Meta-analysis of gene responses to certain perturbations gives a general idea about the gene function in biological processes. However, these responses, as measured through differential expression, could be due to indirect or secondary regulation. The regulatory elements residing in the promoter regions are primarily responsible for the gene response to the perturbations, because they are the binding sites for the TFs that regulate gene expression. The identification of upstream TFs that bind to these regulatory elements together with other regulatory data sets complemented the inference of TET gene functions and allowed us to position TET genes in specific regulatory networks describing flowering time, circadian clock, and defense response. Integration of the different expression and regulatory analyses presented in this work suggests a function for TET3 in flowering response under low temperature. Indeed, TET3 is expressed in the SAM-organizing center progenitor cells in embryos that remain in the seedling. Cold and dehydration response elements are enriched in the TET3 promoter that correlate with the up-regulation of TET3 gene expression by ABA, cold, and drought treatment. The PRR5, AGL15, and PIF4 TFs that regulate TET3 (Fig. 6) have a function in flowering response (Adamczyk et al., 2007; Nakamichi et al., 2007; Kumar et al., 2012), and in the TF genetic perturbation backgrounds, AGL15 and LFY expression levels are negatively correlated with TET3 (Table I). Thus, the meta-analysis is hypothesis generating and will be helpful for experimental design in further functional analyses. Our analyses suggest a function for TET8 in the defense response. The TET8 gene is up-regulated upon pathogen treatment, which correlated with enrichment of defense response cis-regulatory elements in its promoter. The SR1/CAMTA3, MYB4, and EIN3 TFs that putatively regulate TET8 (Fig. 6) are involved in the defense response: SR1 suppresses the defense response (Nie et al., 2012), MYB4 is a transcriptional repressor that is induced by the elicitor Flagellin22 (FLG22; Schenke et al., 2011), and EIN3 activates downstream immune-responsive genes in an ethylene-dependent way, such as FLS2, which has EIN3-binding sites at the promoter region (Boutrot et al., 2010). TET8 is up-regulated in the loss-of-function sr1 allele and down-regulated in the ett3-1ell1-1 double mutant, indicating that TET8 expression is antagonistically regulated by SR1 and EIN3 in the immune signaling pathway. TET8 gene expression was significantly induced by the FLG22 and Elongation Factor Tu (EF-Tu) elicitors that mimicked pathogen infection (Supplemental Fig. S4B), which confirmed the perturbation heat map, the cis-regulatory elements in the promoter region, and the TF-TET8 gene regulatory network.

In conclusion, combining in planta expression and mutational analyses with meta-analyses on perturbation responses, cis-regulatory element identification, and the construction of a TF-TET regulatory network provided an exciting novel and integrated approach, revealing already the function of a few plant tetraspanins and enabling the functional prediction of many more TET genes in future work.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The mutant lines tet5-1 (GABI-Kat 290A02), tet5-2 (SALK_148216), tet5-3 (SALK_02009C), tet6-1 (SALK_005482C), tet6-2 (SALK_139305), and tet13-1 (SALK_011012C) were obtained from Arabidopsis (Arabidopsis thaliana) GABI-Kat (http://www.gabi-kat.de/) and the European Arabidopsis Stock Centre (http://www.arabidopsis.org/). The presence of the T-DNA was confirmed by PCR with T-DNA-specific and gene-specific primers (Supplemental Table S3).

Seeds were germinated on Murashige and Skoog medium supplemented with 1% (w/v) Suc and 0.8% (w/v) agarose, pH 5.7. Seeds were surface sterilized...
and stratified at 4°C for two nights and moved to the growth chamber. For pAtET::NLS-GFP/GUS lines, 6-d-old seedlings grown vertically at 21°C under 24-h light conditions (75–100 μmol m⁻² s⁻¹) were used for SAM and root analysis; 14-d-old seedlings corresponding to growth stage 1.04 (Boyes et al., 2001) and grown horizontally at 21°C under 16-h-light/8-h-dark conditions were used for cotyledon and emerging leaf 1, 2, 3, and 4 primordia analysis; 6-week-old plants grown in the soil at 21°C under 16-h-light/8-h-dark conditions were used for the analysis of inflorescences, different stages of flowers, siliques, and embryos. 

Promoter and Open Reading Frame Cloning, Construction of Expression Vectors, and Plant Transformation

Promoter sequences, defined as intergenic regions with sizes ranging between 396 and 3,400 bp, and open reading frame sequences were amplified from the Arabidopsis genome with the primers listed in Supplemental Table S3 and cloned into the entry vectors using BP Clonase (Invitrogen) to generate the entry clone. For complementation of ipt13-1, full-length genomic DNA of pAtET13 was amplified with the primers listed in Supplemental Table S3 and cloned into the entry vectors using BP Clonase (Invitrogen) to generate the entry clone. The expression clones were cloned by LR Clonase (Invitrogen) with the entry clone and the destination vectors pMK5NSFm14G (pAtET17::NLS-GFP/GUS), pK7FW22 (pS55::TET3::GFP), and pB2C47W (pS55::TET13; Karimi et al., 2007). The plasmids were transferred into Agrobacterium tumefaciens pMP90 cells. All constructs were transferred into Arabidopsis ecotype Col-0 or TET13 by floral dip transformation. A total of 25 mg of transgenic seeds of the T1 generation was used for high-density plating on 50 mg L⁻¹ kanamycin (pAtET17::NLS-GFP/GUS and pS55::TET3::GFP) or 7.5 mg L⁻¹ naphthaleneacetic acid (Duchela; complementation of ipt13-1 with pS55::TET13). The resistant seedlings were transferred into soil for the T2 generation seed harvest. 

Histochemical, Histological, and Phenotypic Analyses and Statistical Tests

The X-Gluc assay was performed as described previously (Couchou et al., 2012). Ovules were cleared overnight in an 8:2 (g:mL) mixture of chloroform:acetone:water and mounted on the microscope slides with the same solution (Grini et al., 2002). For the LRP staging and a better visualization of the TET13 expression pattern in the LRP, the samples were treated as described previously with some modifications (Malamy and Benfey, 1997). Both fresh and stained samples were fixed in 70% (v/v) ethanol overnight and transferred into 4% (v/v) formaldehyde and 20% (v/v) methanol and incubated at 62°C for 40 min in 7% (v/v) NaOH and 60% (v/v) ethanol at room temperature for 15 min. The samples were then rehydrated for 10 min in 60%, 40%, 20%, or 10% (v/v) ethanol at room temperature. Finally, the samples were infiltrated in 25% (v/v) glycerol and 5% (v/v) ethanol for 10 min and mounted with 50% (v/v) glycerol. The root meristem size was determined as the number of cells in the cortex cell file from the QC to the first elongated cell (Casamitjana-Martínez et al., 2000). The samples were mounted with 50% (v/v) lactic acid and observed immediately. For the hydroyxuryea, NAA, and NPA treatments, 5-d-old seedlings were transferred onto Murashige and Skoog (MS) medium supplemented for 24 h with 1 μM hydroyxuryea, 1 μM NAA, or 1 μM NPA, respectively. Seedlings treated with hydroyxuryea were counterstained with propidium iodide and observed with a confocal microscope. Seedlings treated with NAA or NPA were also treated for 1 min with Lugol solution to stain the starch granules, mounted with chloral hydrate, and checked immediately with the microscope. For epidermal cell imaging, leaves were fixed in 100% ethanol overnight and mounted with 90% (v/v) lactic acid. The leaf area was measured with the ImageJ software (http://rsbweb.nih.gov/ij/). The epidermal cells on the abaxial side were drawn with a Leica DMLB microscope equipped with a drawing tube and differential interference contrast objectives. The total number of cells per leaf was determined as described previously (De Veylder et al., 2001). All samples were imaged with a binocular Leica microscope or an Olympus DIC-BS51 microscope. The confocal images were taken with an Olympus Fluoview FV1000 microscope or a Zeiss LSM5 Exciter confocal microscope. The fluorescence was detected after a 488-nm (GFP) or 543-nm (propidium iodide) excitation and an emission of 495 to 520 nm for GFP or 590 to 620 nm for propidium iodide. Transverse sectioning was done according to De Smet et al. (2004). Means between samples were compared by a two-tailed Student’s t test; an f test was assessed for the equality between population variances.

Elicitor Treatment

EF-Tu and FLG22 were synthesized and purchased from GenScript (http://www.genscript.com). Col-0 plant samples were grown on MS medium for 16 d and transferred into liquid MS medium supplemented with EF-Tu or FLG22 at the final concentration of 1 μM for 2 h on an orbital shaker.

Quantitative Real-Time PCR Analysis

Total RNA was prepared with the RNeasy kit (Qiagen). One microgram of RNA was used as a template to synthesize complementary DNA with the Script cDNA Synthesis Kit (Bio-Rad). The expression level was analyzed on a LightCycler 480 apparatus (Roche) with SYBR Green, and all reactions were performed in three technical replicates. Expression levels were normalized to reference genes PROTEIN PHOSPHATASE 2A SUBUNIT A3 and UBIQUITIN-CONJUGATING ENZYME21 (Czechowski et al., 2005).

cis-Regulatory Element Analysis

Known TF-binding sites and DNA motifs were mapped on the 2-kb upstream sequence for all genes and for all included species using DNA-pattern allowing no mismatches (Thomas-Chollier et al., 2008). A total of 692 cis-regulatory elements were obtained from AGRIS (Davuluri et al., 2003), PLACE (Higo et al., 1999), and ATHAPAM (Stephan et al., 2004). In addition, 44 positional count matrices were obtained from AthaMap, and for 15 TFs, positional count matrices were obtained from ChIP-seq data (Heyndrickx et al., 2014). Positional count matrices were mapped genome wide using MatrixScan using a cutoff value of P < 1e-05 (Thomas-Chollier et al., 2008). In order to reduce the high false positive rates associated with interfering regulatory interactions based on simple motif mapping, two types of filtering were used. A first approach to enrich for functional interactions consisted of filtering motif matches using cross-species sequence conservation or open chromatin regions. Therefore, motif matches were filtered using conserved noncoding sequences (Van de Velde et al., 2014) and DH sites. The DH sites were downloaded from the National Center for Biotechnology Information Sequence Read Archive database (accession no. SRP090678; Zhang et al., 2012). A second approach started from tightly correlated genes to identify coregulatory motifs or TF-binding sites. For each TET gene, motif-mapping information was combined with a set of coregulated genes from Genestigator. In the perturbations tool, the perturbations under which TET genes were two times up-regulated (P < 0.05) were selected to create new sample sets. Then, in the coexpression tool, the top 200 positively correlated genes under the categories anatomy and perturbations were selected. The overlapping genes were defined as coregulated genes. Only motifs present in the upstream or downstream TET sequence and showing significant enrichment in the coregulation cluster were retained. Motif enrichment was determined using the hypergeometric distribution using false discovery rate correction. Only significantly (P < 0.05) enriched motifs were retained.

Gene Regulatory Network Inference

The gene regulatory networks based on conserved noncoding sequences from Van de Velde et al. (2014) and on TF ChIP data from Heyndrickx et al. (2014) were filtered for TET target genes. For TFs with ChIP data, differentially expressed genes after TF perturbation were also included for AT5G13790 (AGL15; Zheng et al., 2009), AT1G19350 (BES1; Yu et al., 2011), AT5G61850 (LPX-Schneider et al., 2003), and AT5G26790 (SUS3; Yamamoto et al., 2010; Lumb et al., 2012). For other TFs, differentially expressed genes after TF perturbation were obtained through Genestigator.

Gene Ontology Enrichment

Gene Ontology enrichment was performed using the hypergeometric distribution with Bonferroni correction.
Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. TET expression in seedlings and seeds and TET3 protein localization.

Supplemental Figure S2. TET expression in flower organs.

Supplemental Figure S3. tet3-l-1 primary root morphology.

Supplemental Figure S4. tet5 and tet6 single and double mutant phenotypes in other alleles.

Supplemental Figure S5. Heat map of TET responses to different perturbations and TET5 expression levels after elicitor treatment.

Supplemental Table S1. tet mutants collected in this study.

Supplemental Table S2. TET cis-regulatory element information.

Supplemental Table S3. Primers used in the study.

Supplemental Movie S1. TET3-GFP movement on the plasma membrane.

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LITERATURE CITED


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