

Arabidopsis Basic Leucine-Zipper Transcription Factors TGA9 and TGA10 Interact with Floral Glutaredoxins ROXY1 and ROXY2 and Are Redundantly Required for Anther Development^{1[C][W][OA]}

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ROXY1 and ROXY2 are CC-type floral glutaredoxins with redundant functions in *Arabidopsis thaliana* anther development. We show here that plants lacking the basic leucine-zipper transcription factors TGA9 and TGA10 have defects in male gametogenesis that are strikingly similar to those in *roxy1 roxy2* mutants. In *tga9 tga10* mutants, adaxial and abaxial anther lobe development is differentially affected, with early steps in anther development blocked in adaxial lobes and later steps affected in abaxial lobes. Distinct from *roxy1 roxy2*, microspore development in abaxial anther lobes proceeds to a later stage with the production of inviable pollen grains contained within nondehiscent anthers. Histological analysis shows multiple defects in the anther dehiscence program, including abnormal stability and lignification of the middle layer and defects in septum and stomium function. Compatible with these defects, TGA9 and TGA10 are expressed throughout early anther primordia but resolve to the middle and tapetum layers during meiosis of pollen mother cells. Several lines of evidence suggest that ROXY promotion of anther development is mediated in part by TGA9 and TGA10. First, TGA9 and TGA10 expression overlaps with ROXY1/2 during anther development. Second, TGA9/10 and ROXY1/2 operate downstream of SPOROXYTELESS/NOZZLE, where they positively regulate a common set of genes that contribute to tapetal development. Third, TGA9 and TGA10 directly interact with ROXY proteins in yeast and in plant cell nuclei. These findings suggest that activation of TGA9/10 transcription factors by ROXY-mediated modification of cysteine residues promotes anther development, thus broadening our understanding of how redox-regulated TGA factors function in plants.

TGACG (TGA) motif-binding proteins form a distinct subclade in the basic leucine-zipper (bZIP) superfamily of *Arabidopsis thaliana* transcription factors (Jakoby et al., 2002). This subclade contains 10 members: TGA1 to TGA7, PERIANTHIA (PAN), and two uncharacterized members, bZIP21/TGA9 (At1g08320) and bZIP65/TGA10 (At5g06839; Jakoby

et al., 2002). Past studies have identified overlapping roles for TGA transcription factors in plant disease resistance and stress responses (Després et al., 2003; Zhang et al., 2003; Kesarwani et al., 2007; Fode et al., 2008; Mueller et al., 2008), indicating that these factors operate with a high degree of functional redundancy. To date, developmental roles for TGA factors are known only for PAN, a key regulator of floral patterning. Flowers lacking PAN typically contain an extra sepal and petal and one fewer stamen, resulting in a pentamerous arrangement of floral organs (Running and Meyerowitz, 1996; Chuang et al., 1999).

Recent studies have shed light on how posttranscriptional regulation of TGA transcription factors relays changes in intracellular redox status. A pair of Cys residues in the C terminus of TGA1 or TGA4 form an intermolecular disulfide bridge that is reduced upon exposure to salicylic acid (SA; Després et al., 2003). This modification stimulates DNA binding and permits interaction with the BTB-ankryin protein NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1), a transcriptional coactivator (Després et al., 2003; Rochon et al., 2006). PAN similarly partners with BTB-ankryin proteins, BLADE-ON-PETIOLE1 (BOP1) and BOP2, to

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restrict the number of sepal-whorl organs in flowers (Hepworth et al., 2005). One of two regulatory Cys residues in TGA1 is conserved in PAN and required for activity (Li et al., 2009). Modification of this residue is proposed to depend on the activity of ROXY1, a CC-type glutaredoxin that interacts with PAN in plant cell nuclei (Li et al., 2009). *roxy1* mutants initiate fewer petals than the wild type and later petal morphogenesis can be aberrant, indicating that ROXY1 is a regulator of PAN (Xing et al., 2005; Li et al., 2009). ROXY1, together with its closest homolog ROXY2, is also required for anther cell specification and microspore development (Xing and Zachgo, 2008), but TGA partners in this process have yet to be identified.

The Arabidopsis anther is a bilaterally symmetrical four-lobed structure in which pollen develops. The mitotic division of archesporial cells specified in the interior of each lobe generates pollen mother cells surrounded by three morphologically distinct cell layers: the tapetum, middle layer, and endothecium, underlying the epidermis (Sanders et al., 1999). Pollen mother cells divide meiotically to generate tetrads of haploid microspores that separate and mature into pollen grains. The tapetum contributes to microspore development by secreting enzymes necessary for microspore release and by providing nutrients and structural components essential for the production of viable pollen (Scott et al., 2004). Pollen grains are released from the anthers by dehiscence, a process whose terminal step is breakage of the stomium connecting the abaxial and adaxial anther lobes (Sanders et al., 1999; Ma, 2005). Interestingly, abaxial and adaxial anther lobe development is differentially dependent on ROXY activity. Loss of ROXY1/2 blocks sporogenous cell formation only in adaxial anther lobes. In abaxial lobes, anther cell layers are specified but development of pollen mother cells and the tapetum is abnormal, leading to microspore degradation (Xing and Zachgo, 2008).

We show here that mutations in *TGA9* and *TGA10* lead to male sterility and differential defects in abaxial and adaxial anther lobe development similar to *roxy1 roxy2* mutants. Additional defects not seen in *roxy1 roxy2* mutants include abnormal stability of the middle layer and lack of septum and stomium degeneration, resulting in nondehiscent anthers. We show that *TGA9* and *TGA10* are expressed in an overlapping fashion with *ROXY1* and *ROXY2* and regulate an overlapping set of genes with tapetum functions. In addition, ROXY proteins interact directly with *TGA9* and *TGA10* in the nuclei of yeast and plant cells, suggesting that activation of *TGA9* and *TGA10* transcription factors by the modification of redox-sensitive Cys residues is needed for normal anther development. These data reveal a new role for TGA factors in anther patterning and dehiscence, thus broadening our understanding of how redox-regulated TGA factors contribute to plant development.

RESULTS

Mutant Isolation and Characterization

TGA9 and *TGA10* form a distinct subclade in the phylogenetic tree of Arabidopsis TGA bZIP family members (Supplemental Fig. S1), but their functions have yet to be elucidated. Homologs of *TGA9* and *TGA10* in other plant species have developmental roles. In tobacco (*Nicotiana tabacum*), overexpression of Nt*TGA10* causes derepression of lateral branching and defects in plant defense signaling (Schiermeyer et al., 2003). In maize (*Zea mays*), mutation of the *TGA9* homolog *LIGULELESS2* causes defects in the formation of the blade-sheath boundary in leaves and delayed flowering (Walsh et al., 1998; Walsh and Freeling, 1999). To examine the functions of Arabidopsis *TGA9* and *TGA10*, we obtained insertion lines from the Salk T-DNA collection (Fig. 1A). Lines designated *tga9-1* and *tga9-2* contain T-DNA inserts at nucleotides 2,023 and 3,325 of the coding sequence, respectively. No transcripts were produced in these lines (Fig. 1B). Lines designated *tga10-1* and *tga10-2* contain T-DNA inserts at nucleotides 1,826 and 2,284 of the coding sequence, respectively. Partial transcripts were produced in both lines, but the insertion in *tga10-1* disrupts the bZIP domain (Fig. 1, A and C) so as to encode a nonfunctional protein. No single mutants showed an obvious phenotype. Analysis of *TGA9* and *TGA10* transcript by reverse transcription (RT)-PCR revealed an overlapping expression pattern in flowers, suggesting that their activities may overlap in floral development (Fig. 1, D and E). To examine this possibility, we constructed *tga9 tga10* double mutants.

Male-Sterile Phenotype in *tga9 tga10* Double Mutants

Strikingly, four of 72 F2 plants derived from a cross between *tga9-1* and *tga10-1* failed to set seed. Genotyping confirmed that sterility was restricted to double mutants (Fig. 2). Examination of *tga9 tga10* flowers showed no changes in floral patterning, but anther morphology was abnormal (Fig. 3). Stage 12 anthers in the *tga9 tga10* mutant were only partially filled with pollen and failed to dehisce at stage 13 (Fig. 3). Hand pollination of *tga9 tga10* carpels with wild-type pollen yielded a similar number of viable seeds (1.29% aborted seeds; $n = 465$) as a wild-type control cross (1.30% aborted seeds; $n = 461$), confirming that female fertility was normal. Unfortunately, expression of *TGA9* and *TGA10* coding sequences under the control of either a cauliflower mosaic virus 35S or native promoter failed to complement the *tga9 tga10* mutant phenotype. However, an identical male-sterility phenotype was seen in *tga9-1 tga10-2* double mutants (data not shown), providing further evidence that the anther defect in *tga9 tga10* mutants is indeed due to a loss of *TGA9* and *TGA10* function.

To further examine the male-sterility phenotype in *tga9 tga10* mutants, Alexander's stain was used to assess pollen viability (Alexander, 1969). The anthers of late stage 12 flowers in the mutant contained pollen

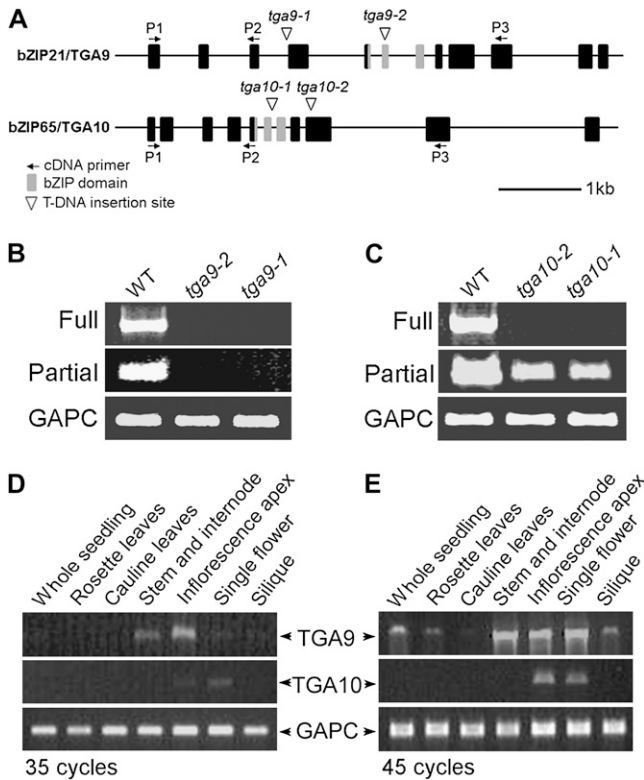


Figure 1. Characterization of *tga9* and *tga10* mutant alleles, and tissue expression of *TGA9* and *TGA10*. A, Scale diagrams of *TGA9* and *TGA10* genomic sequences showing the positions of features as indicated in the key at bottom left. Black boxes indicate exons, and horizontal arrows represent annealing positions of primers used for transcript analysis. B, RT-PCR analysis of *TGA9* transcripts in the wild type (WT) and *tga9* mutants (40 cycles). C, RT-PCR analysis of *TGA10* transcripts in the wild type and *tga10* mutants (40 cycles). Full, Product obtained using P1/P3 primer combination; Partial, product obtained using P1/P2 primer combination. D and E, RT-PCR analysis of *TGA9* and *TGA10* transcripts in wild-type plant tissues (35 and 45 cycles). In B to E, *GAPC* control transcript used 23 cycles.

that were abnormally aggregated but most stained purple-red, suggesting that they were potentially viable (Supplemental Fig. S2, A, B, D, and E). However, pollen grains that were excised from indehiscent anthers failed to germinate on agar medium, revealing them to be unviable (Supplemental Fig. S2, C and F; 0% germination for *tga9 tga10* pollen versus 70.5% germination for wild-type pollen [$n = 200$]). Scanning electron micrographs of mutant pollen showed that grains were small and irregular, lacking the characteristic oblong shape of wild-type pollen grains. The exine structure was also abnormal, with highly compressed columella, indicating defects in pollen wall maturation (Supplemental Fig. S2, G–J).

Differential Effect of *tga9 tga10* Mutation on Abaxial and Adaxial Anther Development

Anther cross-sections were used to examine the detailed morphology of wild-type and *tga9 tga10* an-

thers during development (Fig. 4) Morphological abnormalities in the mutant were apparent from stage 4 onward. In wild-type anthers, primary parietal cells and sporogenous cells were specified in all four anther lobes and underwent cell division to form microspore mother cells surrounded by four morphologically distinct cell layers: the tapetum, middle layer, endothecium, and epidermis (Fig. 4, A and B; Sanders et al., 1999). In the double mutant, sporogenous cells formed reliably only in abaxial anther lobes (Fig. 4H). In adaxial lobes with sporogenous cells, cell divisions were often variable or disorganized, resulting in developmental arrest. Cell division in abaxial lobes followed a pattern similar to the wild type, resulting in microspore mother cells surrounded by tapetum, middle, and endothecium cell layers at stage 5 (Fig. 4, B and I). During stages 6 and 7, pollen mother cells in abaxial lobes underwent meiosis to form tetrads encapsulated by callose (Fig. 4, C, D, J, and K; Supplemental Fig. S3). However, cells in the tapetum layer of the mutant at late stage 6 were expanded, with large vacuoles compared with the wild type (Fig. 4, C and J), suggesting abnormalities in tapetum function.

In the wild type, following the release of microspores at stage 8, the middle layer is crushed and the tapetum compacts as programmed cell death of these layers takes place (Fig. 4, D and E; Sanders et al., 1999).



Figure 2. Sterility phenotype in *tga9 tga10* double mutants. A and B, Comparison of wild-type (WT) and *tga9 tga10* inflorescences. Arrows indicate empty siliques. C, Wild-type and *tga9 tga10* siliques. D and E, Wild-type and *tga9 tga10* dissected siliques. Bars = 1 mm. [See online article for color version of this figure.]

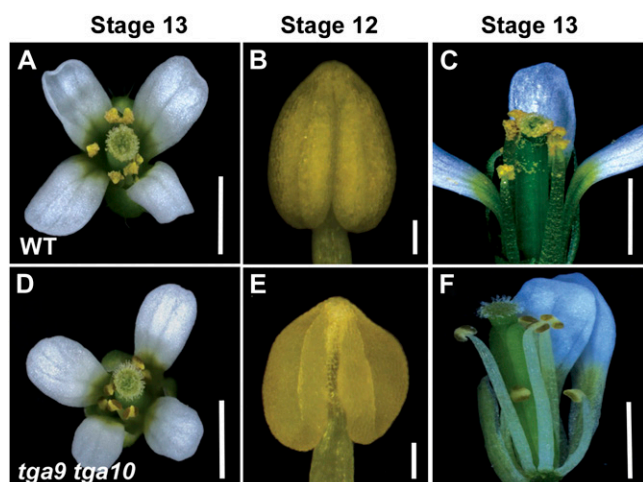


Figure 3. Flower and anther morphology in the wild type and *tga9 tga10* mutants. Floral stages are as indicated. A, Wild-type (WT) flower. B, Wild-type anther, adaxial view. C, Wild-type dissected flower. D, *tga9 tga10* flower. E, *tga9 tga10* anther, adaxial view. F, *tga9 tga10* dissected flower; anthers are nondehiscent. Bars = 1 mm, except 100 μ m in B and E. [See online article for color version of this figure.]

In the mutant, the middle layer was only partly degraded, and by stages 10 and 11, microspore development was visibly impaired (Fig. 4, L and M; Supplemental Fig. S4). Pollen grains in stage 13 anthers of the mutant were clumped together and irregular in appearance (Fig. 4N). Even in anthers with pollen in adaxial lobes, the septum and stomium were nonfunctional, blocking dehiscence (Fig. 4, K and L; Supplemental Fig. S4). Staining of stage 11 anthers with phloroglucinol indicated that the endothecium in both the wild type and mutant is lignified to the same degree, suggesting that reinforcement of this cell layer, essential for dehiscence, is not disrupted (Mitsuda et al., 2005; Mizuno et al., 2007; Yang et al., 2007b). However, the residual middle layer in mutants was highly lignified, which may contribute to nondehiscence (Supplemental Fig. S5).

Similar to *tga9 tga10*, mutations in *roxy1 roxy2* differentially affect adaxial and abaxial lobe development. *ROXY1* and *ROXY2* encode two CC-type glutaredoxins that are expressed in flowers and that interact with several TGA bZIP transcription factors in yeast (Li et al., 2009). Primary parietal and sporogenous cells fail to differentiate in *roxy1 roxy2* adaxial anther lobes, whereas in abaxial anther lobes, microspore development terminates prematurely. Pollen mother cells enter meiosis but eventually aggregate and degrade, giving rise to empty locules (Xing and Zachgo, 2008). Although the *tga9 tga10* block in pollen development is less severe than in *roxy1 roxy2*, the differential effect of these mutations on anther lobe development suggests that ROXY-dependent promotion of anther development may occur in part via the regulation of TGA9 and TGA10, likely at the post-transcriptional level.

TGA9 and TGA10 Expression Overlaps with ROXY in Developing Anthers

ROXY1 and *ROXY2* are expressed in all four anther lobes beginning at stage 3 and in both meiocytes and the tapetum at stage 6 (Li et al., 2009). To examine *TGA9* expression in developing anthers, ecotype Columbia-0 (Col-0) plants expressing a *GUS* reporter gene fused to a 3.9-kb *TGA9* promoter sequence were generated. Whole-mount staining of inflorescences showed activation of *GUS* expression in stage 7 flowers (anther stage 4). Expression peaked in stage 8 flowers (anther stages 5–6) and declined in stage 9 to 11 flowers (anther stages 7–11; Fig. 5, A–E). However, this same promoter driving expression of the *TGA9* coding sequence failed to restore fertility in *tga9 tga10* mutants, suggesting that additional regulatory motifs present in the introns or 3' untranslated region are needed for full expression. Therefore, *in situ* hybridization was used to monitor *TGA9* transcript accumulation in anther cross-sections. This analysis showed activation of *TGA9* in stage 2 anther primordia during archesporial cell specification. During stages 2 to 3 of anther development prior to the emergence of distinct locules, expression was in a horseshoe pattern associated with the lateral and adaxial portion of primordia (Fig. 5, F and G). During stages 4 and 5, expression was throughout sporogenic tissue and surrounding cells layers in adaxial and adaxial locules (Fig. 5, H and I). At stage 6, expression localized to the tapetum and middle layers, gradually fading postmeiosis with degeneration of these cell layers (Fig. 5, J–L). *TGA10* was expressed in a similar pattern, albeit at lower levels (Supplemental Fig. S6). These data show that *ROXY1/2* and *TGA9/10* are coexpressed during stages 3 to 6 of anther development, consistent with functional overlap.

TGA9 and TGA10 Function Genetically Downstream of SPOROCTELESS/NOZZLE and Promote Its Expression in Adaxial Anther Lobes

Initiation of sporogenesis requires *SPOROCTELESS/NOZZLE* (*SPL/NZZ*), a MADS box-like transcription factor regulated by the floral homeotic factor *AGAMOUS* (Ito et al., 2004). *SPL/NZZ* is expressed in archesporial and sporogenous cells and later in pollen mother cells and the tapetum (Schiefthaler et al., 1999; Yang et al., 1999). *In situ* hybridization showed an accumulation of *SPL/NZZ* transcript in the stage 2 anthers of both the wild type and *tga9 tga10* mutants, when archesporial cells are specified (Fig. 6, A and D; Sanders et al., 1999). *SPL/NZZ* transcript was uniform throughout all four anther lobes in the wild type, but in *tga9 tga10* mutants, expression was often less intense in one or both adaxial lobes, consistent with variable specification of sporogenous cells in these lobes (Fig. 6, D–F; stages 2, 4–5, and 6). A similar but more pronounced reduction of *SPL/NZZ* expression in adaxial anther lobes was reported for *roxy1 roxy2* mutants, whose activity lies downstream of *SPL/*

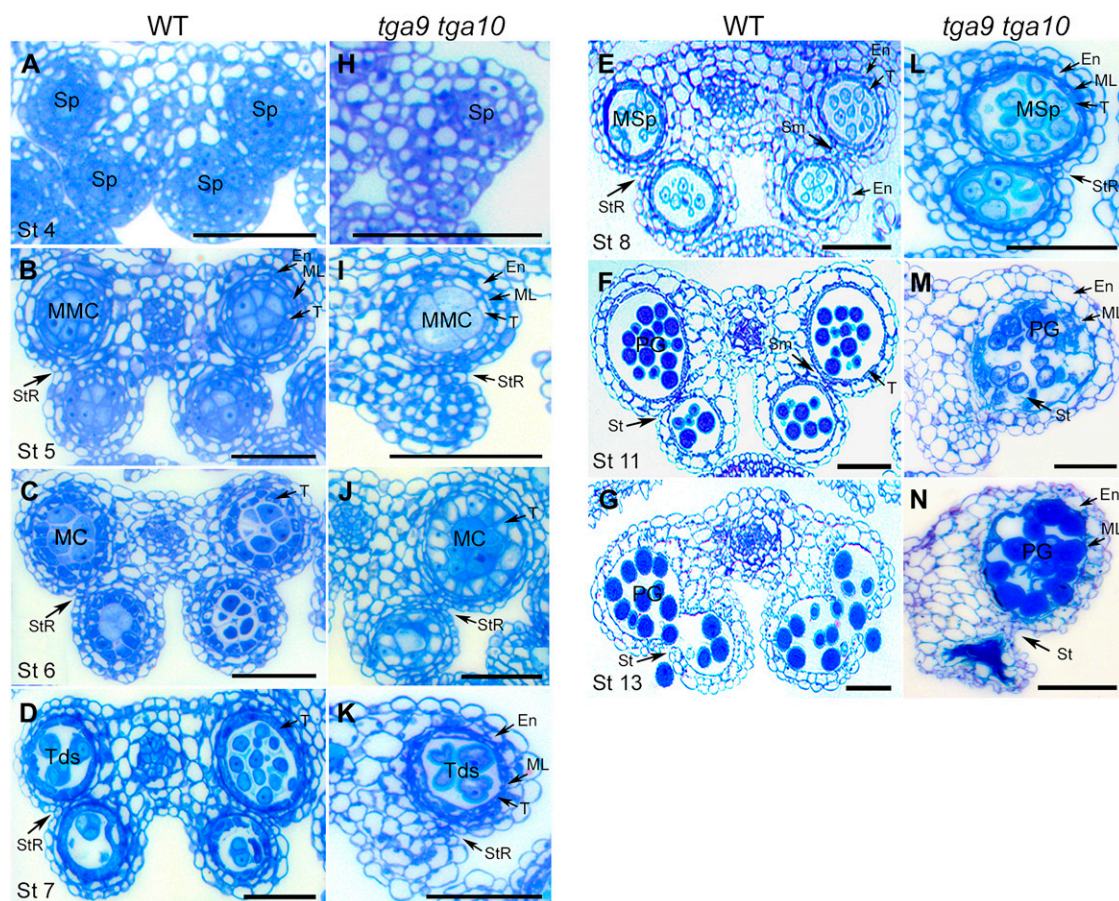


Figure 4. Comparison of wild-type and *tga9 tga10* anther development. Cross-sections of wild-type (WT; A–G) and *tga9 tga10* (H–N) anthers were stained with toluidine blue. A, Stage 4. Sporogenous cells in all four lobes. B, Stage 5. Characteristic layered structure of anther lobes is resolved. C, Stage 6. Meiosis of microspore mother cells. D, Stage 7. Tetrads form, and the middle layer is crushed. E, Stage 8. Developing microspores; tapetum degeneration is under way. F, Stage 11. Developing microspores; septum degeneration begins and endothecium expands. G, Stage 13. Anthers are dehiscent; ruptured stomium. H, Stage 4. Sporogenous cells are often missing in adaxial lobes. I, Stage 5. Sporogenous cells proliferate in abaxial lobes; adaxial lobes are underdeveloped. J, Stage 6. Microspore mother cells in abaxial lobes undergo meiosis, and tapetal cells are abnormally vacuolated; development is variable in adaxial lobes. K, Stage 7. Tetrads appear in abaxial lobes. L, Stage 8. Microspores are released in abaxial lobes; the middle layer fails to degrade. M, Stage 11. Microspore development is delayed; breakdown of the middle layer is incomplete, but endothecium cells are expanded. N, Stage 13. Anthers are indehiscent; pollen is clumped or degraded. En, Endothecium; MC, meiotic cells; ML, middle layer; MMC, microspore mother cells; MSp, microspores; PG, pollen grains; Sm, septum; Sp, sporogenous cells; St, stomium; StR, stomium region; T, tapetum; Tds, tetrads. Bars = 50 μ m. [See online article for color version of this figure.]

NZZ but upstream of DYSFUNCTIONAL TAPETUM1 (DYT1), a key regulator of tapetal differentiation (Zhang et al., 2006; Xing and Zachgo, 2008).

To further examine the position of *TGA9* and *TGA10* in the genetic hierarchy that controls anther development, we crossed *tga9 tga10* mutants with *spl/nzz*, *roxy1 roxy2*, and *dyl1* mutants and analyzed the resulting anther phenotypes. Anthers in the *tga9 tga10 spl-1* triple mutant were similar to those of the *spl-1* parent, which lacks archesporial cells (Fig. 7, A and B; Yang et al., 1999). In *tga9 tga10 roxy1 roxy2* mutants, there was a strong block in adaxial lobe development, typical of the *roxy1 roxy2* parent (Fig. 7, C and D). Interestingly, a few aberrant pollen grains

formed in the abaxial lobes of these quadruple mutants. This partial suppression of the *roxy1 roxy2* phenotype by *tga9 tga10* could indicate that *TGA9/10* are repressors in their inactive form, as shown for *TGA2* (Boyle et al., 2009). Alternatively, the *roxy1 roxy2* phenotype may be milder in Col-0 versus the original Nossen-0 genetic background. These data indicate that *TGA9* and *TGA10* operate genetically downstream of *SPL/NZZ* and *ROXY1/2* but are unlikely to be transcriptional targets of *ROXY1/2*, since their transcript levels are not significantly altered in *roxy1 roxy2* inflorescence apices (Supplemental Fig. S7). In *tga9 tga10 dyl1* triple mutants, microspores failed to develop in either adaxial or abaxial anther lobes, an earlier block

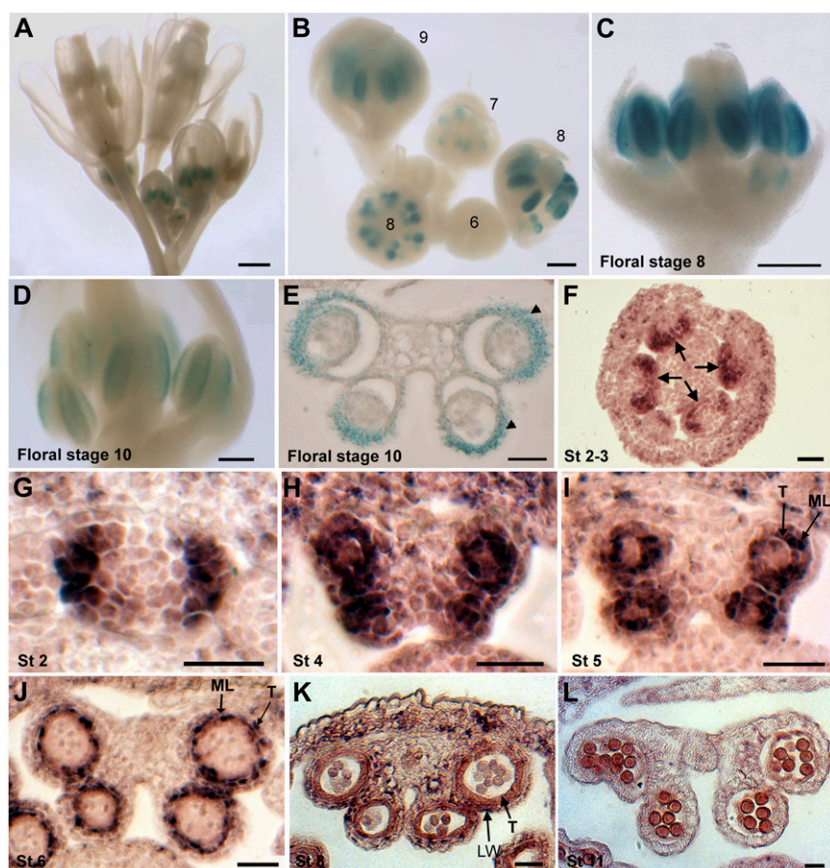


Figure 5. Expression of *TGA9* in wild-type inflorescences and flowers. Expression was monitored using a *TGA9::GUS* reporter gene (A–E) or by in situ hybridization (F–L). A to E, GUS activity is first detected in stage 7 flowers, peaks at stage 8, and declines during stages 9 to 10. E, Stage 10 flower cross section; expression persists in the locule wall (arrowheads). *TGA9* in situ probe hybridized to anther cross-sections. F, Stage 2 to 3 anthers. Expression laterally and along the adaxial face of primordia (arrows) is shown. G, Stage 2. Anther primordia. H, Stage 4. Expression in all cell layers. I and J, Stages 5 to 6. Expression is restricted to the tapetum and middle layer. K and L, Stages 8 to 11. Expression declines. Bars = 100 μm except in A (0.5 mm) and F to L (25 μm). LW, locule wall; ML, middle layer; T, tapetum. [See online article for color version of this figure.]

than for either parent (Fig. 7, E and F). Collectively, these data indicate that *TGA9* and *TGA10* function downstream of *SPL/NZZ* and *ROXY1/2* and upstream or in parallel with *DYT1* in the genetic hierarchy that controls anther development.

***TGA9/10* and *ROXY1/2* Regulate an Overlapping Set of Genes with Functions in Early and Middle Tapetal Development**

Both *ROXY1/2* and *TGA9/10* are coexpressed in the tapetum during stages 4 to 7 of anther development,

and their genetic interactions are consistent with parallel activities downstream of *SPL/NZZ* in anther development. Microarray analysis has identified a set of genes functioning primarily in tapetal development and pollen wall formation whose expression is severely down-regulated in *roxy1 roxy2* inflorescence apices (Xing and Zachgo, 2008). To examine the possibility that some of these genes might also be targets of *TGA9/10* activity, we used RT-PCR to monitor the transcripts of representative genes acting during the early, middle, and late stages of tapetal development in wild-type and *tga9 tga10* inflorescence apices (Fig. 7,

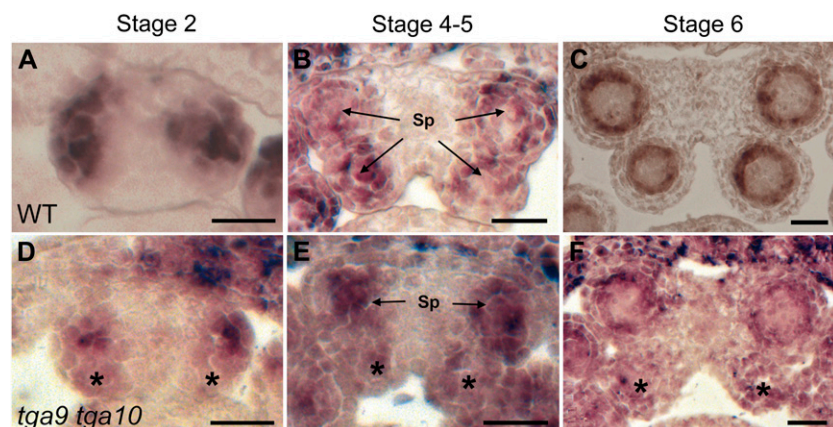


Figure 6. In situ analysis of *SPL/NZZ* expression in wild-type (WT) and *tga9 tga10* anthers. Anther stages are as indicated. A to C, The wild type. *SPL* is uniformly expressed in adaxial versus abaxial anther lobes. D to F, The *tga9 tga10* mutant. *SPL* expression is reduced in adaxial anther lobes (asterisks). Sp, Sporogenous cells. Bars = 25 μm . [See online article for color version of this figure.]

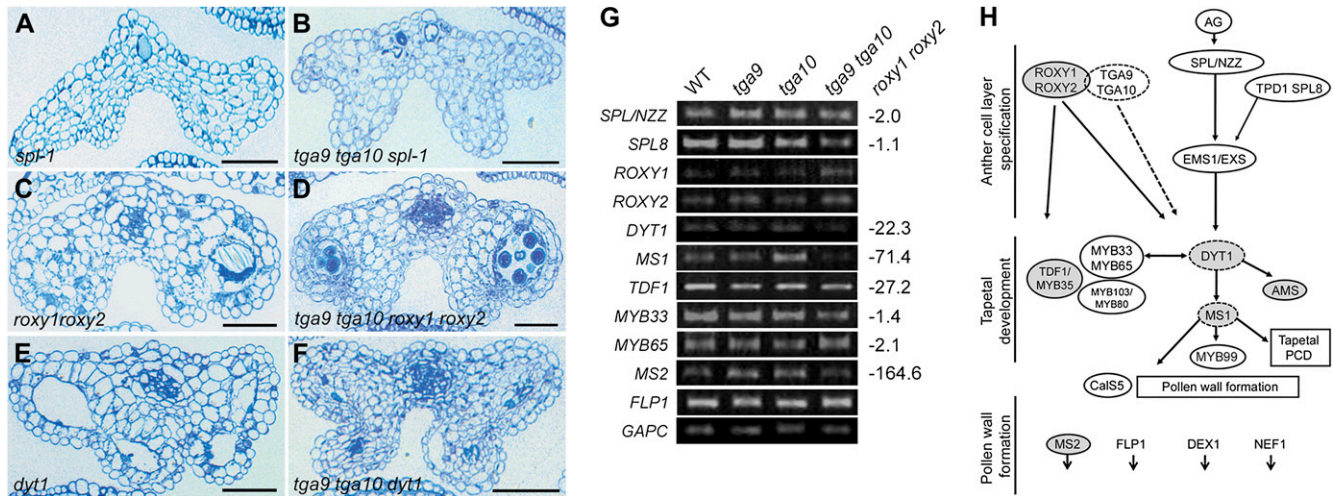


Figure 7. Double mutant analysis and RT-PCR analysis of anther transcripts in the wild type and *tga9 tga10* mutants. A to F, Cross-sections of stage 11 anthers stained with toluidine blue for the indicated genotypes. Bars = 50 μ m. G, RT-PCR analysis of anther transcripts (35 cycles) in wild-type (WT) and mutant inflorescence apices. Numerical values for transcript down-regulation in *roxy1 roxy2* mutants (Xing and Zachgo, 2008) are shown. H, Genetic framework for control of anther development (modified from Wilson and Zhang, 2009) showing overlap for tapetal genes down-regulated in *roxy1 roxy2* and *tga9 tga10* mutants. Shaded ovals, Down-regulated in *roxy1 roxy2*; dashed perimeter, down-regulated in *tga9 tga10*; shaded ovals with dashed perimeter; down-regulated in both. Genes not discussed in the text are as follows: *TAPETAL DETERMINANT1* (*TPD1*; Yang et al., 2003), *EXCESS MICROSPOROCTES1/EXTRA SPOROGENOUS CELLS* (*EMS1/EXS*; Canales et al., 2002; Zhao et al., 2002), *MYB103/MYB80* (Higginson et al., 2003; Zhang et al., 2007), *MYB99* (Ito et al., 2007), *ABORTED MICROSPORES* (*AMS*; Sorensen et al., 2003), *DEFECTIVE IN EXINE FORMATION1* (*DEX1*; Paxson-Sowders et al., 2001), *NO EXINE FORMATION1* (*NEF1*; Ariizumi et al., 2004), and *CALLOSE SYNTHASE5* (*CalS5*; Dong et al., 2005). [See online article for color version of this figure.]

G and H). The expression of genes at the top of the hierarchy required for early archesporial cell formation and specification of anther cell layers, represented by *SPL/NZZ* and *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE8* (*SPL8*; Unte et al., 2003), were not markedly altered in *tga9 tga10* inflorescence apices.

In contrast, a marked reduction was observed in the transcripts of *DYT1* and *MALE-STERILITY1* (*MS1*), tapetal genes operating before and during the meiosis of pollen mother cells (Wilson et al., 2001; Zhang et al., 2006; Ito et al., 2007; Yang et al., 2007a). Similar to *tga9 tga10* mutants, *ms1* mutants show an abnormally vacuolated tapetum and defects in microspore development, resulting in pollen with an altered exine pattern (Ito et al., 2007; Yang et al., 2007a). No obvious changes were observed in the expression of the MYB transcription factors encoded by *MYB35/DEFECTIVE IN TAPETAL DEVELOPMENT AND FUNCTION1* (*TDF1*), or *MYB33* and *MYB65*, of which double mutants show tapetum hypertrophy leading to premeiotic abortion of pollen development (Millar and Gubler, 2005; Zhu et al., 2008). Tapetal genes with post-meiotic functions in pollen coat formation, represented by *MS2* and *FACELESS POLLEN* (*FLP*), were also expressed at wild-type levels in *tga9 tga10* mutant apices (Aarts et al., 1997; Ariizumi et al., 2003). Quantitative RT-PCR in a separate experiment using *roxy1 roxy2* mutant apices as a control confirmed these trends, showing a significant reduction in *DYT1* and *MS1* transcript in

tga9 tga10 mutants (Supplemental Fig. S8; Supplemental Materials and Methods S1).

Therefore, we concluded that *ROXY1/2* and *TGA9/10* function at similar levels in the genetic hierarchy that controls anther development and are likely to coregulate a set of genes expressed in the tapetum before and during meiosis of pollen mother cells, represented by *DYT1* and *MS1*. This result is compatible with the overlapping expression patterns of *ROXY1/2* and *TGA9/10* in the tapetum during stages 4 to 7 of anther development.

TGA9/10 and ROXY1/2 Interact in Yeast and in Planta

Yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assays indicate that *ROXY1* interacts with several TGA transcription factors in yeast and in planta, including PAN, TGA2, TGA3, and TGA7 (Li et al., 2009). We first used a two-hybrid assay to test for a direct interaction between *ROXY1/2* and *TGA9/10*, obtaining a positive result (Fig. 8A). As a further test, we used BiFC to examine the interaction of these proteins in planta. This assay showed reconstitution of yellow fluorescent protein (YFP) fluorescence in the nuclei of transformed tobacco leaf cells, indicating that *ROXY* proteins directly interact with *TGA9* and *TGA10* in plant cells (Fig. 8B). Conversely, *ROXY1/2* (fused to the N terminus of YFP) or *TGA9/10* (fused to the C terminus of YFP) coexpressed with the C or N terminus

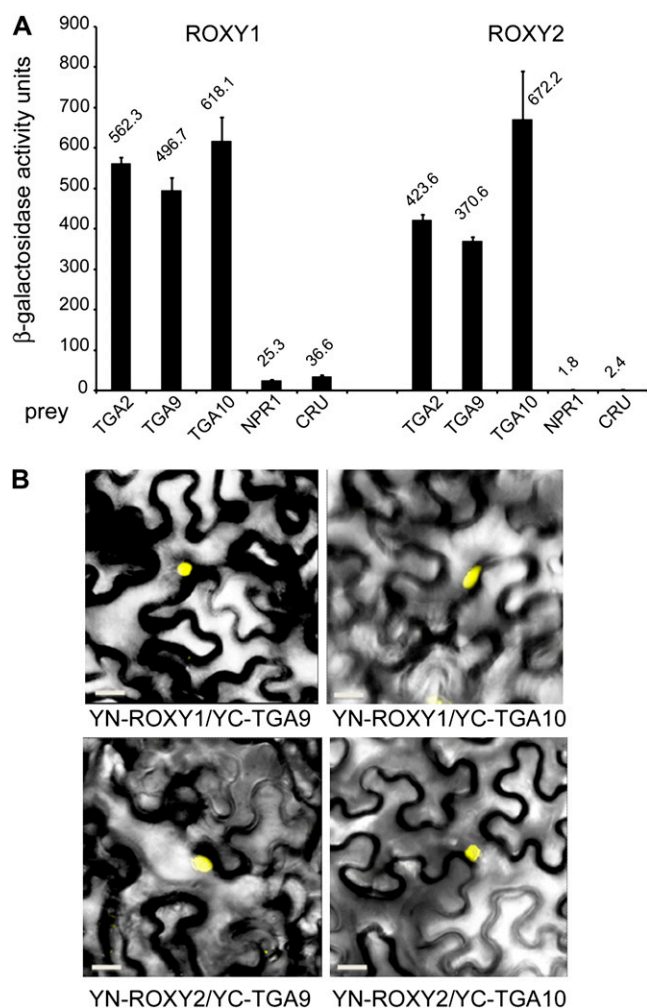


Figure 8. TGA9 and TGA10 directly interact with ROXY1/2. **A**, Yeast two-hybrid assay. ROXY proteins served as bait. NPR1 and CRUCIFERIN were negative controls. Error bars indicate \pm SE (three replicates). **B**, ROXY proteins interact with TGA9/10 in the nuclei of transiently transformed tobacco leaves. Images show reconstitution of YFP fluorescence 3 to 5 d after coexpression of protein pairs. The N terminus of YFP (YN) was fused in-frame upstream of ROXY1 and ROXY2. The C terminus of YFP (YC) was fused in-frame upstream of TGA9 and TGA10. As a negative control, coexpression of YN-ROXY1 and YC alone failed to reconstitute a fluorescent YFP chromophore (data not shown). Bars = 50 μ m. [See online article for color version of this figure.]

of YFP alone, respectively, failed to reconstitute YFP fluorescence (data not shown; Li et al., 2009). Previously, BiFC was used to confirm a nuclear interaction between ROXY1 and PAN, which is important for exerting their function (Li et al., 2009). Our prediction is that ROXY1/2 modification of TGA9/10 in the nucleus promotes their transcription factor activity, since loss-of-function mutations in either set of genes leads to a similar deficiency in adaxial anther lobe development and altered transcriptional profile. Collectively, these results provide new evidence that redox signaling plays

a critical role in anther specification and dehiscence and that TGA factors mediate these signals.

DISCUSSION

We show here that two previously uncharacterized Arabidopsis TGA bZIP family members, TGA9 and TGA10, have overlapping functions in anther development. Loss-of-function *tga9 tga10* mutations differentially disrupt the development of adaxial anther lobes and are required for microspore maturation, pollen viability, degeneration of the middle layer, and dehiscence. A similar differential block in adaxial anther lobe development is seen in *roxy1 roxy2* double mutants, suggesting that TGA9 and TGA10 may be substrates of the CC-type floral glutaredoxins, ROXY1 and ROXY2. In support of this model, we show that ROXY1/2 and TGA9/10 have an overlapping pattern of expression in developing anthers and function downstream of SPL/NZZ, where they positively regulate the expression of a set of genes with tapetal functions. Finally, we show that ROXY1/2 and TGA9/10 directly interact in the nuclei of plant cells.

Differential Requirement for TGA9 and TGA10 in Abaxial and Adaxial Anther Lobes

In contrast to most early-acting gene mutants (e.g. *spl1/nzz*, *tpd1*, and *spl8*), where development is equally dysfunctional in all four anther lobes, mutations in ROXY1/2 and TGA9/10 differentially impair SPL/NZZ expression and the specification and/or proliferation of sporogenous cells in adaxial anther lobes (Schiefthaler et al., 1999; Yang et al., 1999; Unte et al., 2003; Xing and Zachgo, 2008). Compatible with this, TGA9 is expressed during stages 2 to 3 in a horseshoe pattern, laterally (overlapping with SPL) and on the adaxial face of the anther primordium (Fig. 5F). In wild-type anthers, cell development in anther lobes is not synchronous until stage 5, with the adaxial lobes smaller than their abaxial counterparts (Sanders et al., 1999). Specification and proliferation of sporogenous cells in adaxial lobes, therefore, may depend in part on interactions with region-specific factors. In Arabidopsis and rice, abaxial and adaxial polarity determinants, similar to those in leaves, are differentially expressed in the anther and are associated with morphological asymmetry (Sessions et al., 1997; Dinneny et al., 2006; Toriba et al., 2010). At stages 2 to 3, prior to outgrowth of the four anther lobes, abaxial markers *ETTIN/AUXIN RESPONSE FACTOR3* (*ETT/ARF3*) and the YABBY gene *FILAMENTOUS FLOWER* are expressed on the abaxial side of anther primordia in a pattern complementary to adaxial markers such as *PHABULOSA* (*PHB*), encoding a class III homeodomain Leu-zipper transcription factor and the zinc-finger transcription factors encoded by *JAGGED* and *NUBBIN* (Sessions et al., 1997; Dinneny et al., 2006). *PHB* expression in Arabidopsis and rice rearranges to the

notch region at stage 4, presaging the site of stomium development (Dinneny et al., 2006; Toriba et al., 2010). Mutations in *ETT*, whose activity is auxin responsive, and the auxin transport mutants *pin formed1* and *pinoid* also perturb anther structure biased toward the loss of adaxial features (Bennett et al., 1995; Sessions et al., 1997), indicating that adaxial-abaxial patterning relies in part on a gradient of auxin, as in leaves (Pekker et al., 2005; Chitwood et al., 2007). The existence of region-specific factors in the anther, therefore, may lessen the requirement for ROXY-TGA9/10 activity in abaxial anther lobes.

TGA9 and TGA10 Contribute to Tapetal Development

The tapetum is essential for normal microspore and pollen development, as it provides nutrients and secretes enzymes and structural components for the pollen coat. Indeed, mutations in tapetally expressed genes often cause defects in microspore development leading to male sterility (Scott et al., 2004; Ma, 2005). Consistent with this, transcript analysis in *tga9 tga10* mutants identified a significant reduction in *DTY1* and *MS1*, representative genes operating in the tapetum before and during meiosis (Wilson et al., 2001; Zhang et al., 2006; Ito et al., 2007). During this time, *TGA9/10* are strongly expressed in the middle layer and tapetum (Fig. 5, stages 5–6). Unexpectedly, tapetal genes with postmeiotic roles in pollen coat development represented by *MS2* and *FLP1* (Aarts et al., 1997; Ariizumi et al., 2003) were not markedly affected in this assay. *MS2* is down-regulated 164.6-fold in *roxy1 roxy2* mutants, and we expected to see a similar result for *tga9 tga10* but did not. Microspores degenerate completely in *roxy1 roxy2* but continue to develop in *tga9 tga10* mutants, which may account for this difference. Tapetal programmed cell death is initiated at about the time that microspores are released from tetrads (Sanders et al., 1999), during which time *TGA9* and *TGA10* expression in the tapetum declines.

TGA9 and TGA10 Are Required for Anther Dehiscence

Dehiscence is a multistep program that begins with degeneration of the middle layer (stages 7–8) and the tapetum (stages 8–11) and is accompanied by the expansion and reinforcement of the endothecium (stage 11). Degradation of the septum between anther compartments and stomium differentiation are complete by the end of stage 12. At stage 13, breakage of the stomium releases the mature pollen (Sanders et al., 1999, 2005). Our results show that multiple steps of the dehiscence program require *TGA9/10*. First, dissolution of the middle layer is incomplete in the double mutant. Second, thickening and lignification of the endothecium, essential for dehiscence (Steiner-Lange et al., 2003; Mitsuda et al., 2005; Yang et al., 2007b), is similar to the wild type at stage 11, but the middle layer remnant becomes ectopically lignified, which may interfere with dehiscence (Supplemental Fig. S5).

Septums in the mutant are disorganized and fail to degenerate, even when immature pollen grains form in adaxial lobes (Supplemental Fig. S4), and finally, stomium-like cells form at the notch between locules but are nonfunctional (Supplemental Fig. S4).

Relatively few nondehiscent mutants have been described to date (Ma, 2005; Wilson and Zhang, 2009). Mutations that impair jasmonic acid (JA) biosynthesis or signal transduction tend to delay rather than block anther dehiscence (Xie et al., 1998; Sanders et al., 2000; Ishiguro et al., 2001; Mandaokar and Browse, 2009). Jasmonate treatment fails to rescue dehiscence in *tga9 tga10* mutants (data not shown), suggesting that *TGA9/10* function independently or downstream of a jasmonate signal. Auxin similarly regulates the timing of dehiscence, by at least two mechanisms: ARF6 and ARF8 promote dehiscence via JA biosynthesis, and premature lignification of the endothecium occurs in auxin receptor mutants (Nagpal et al., 2005; Cecchetti et al., 2008; Tabata et al., 2010). Mutation of the tapetally expressed *RECEPTOR-LIKE PROTEIN KINASE2* (*RPK2*) gene blocks dehiscence, but its mode of action is unknown (Mizuno et al., 2007). In *rpk2* mutants, the middle layer fails to differentiate from the tapetum, which grows hypertrophically during meiosis and does not fully degenerate. Reinforcement of the endothecium and degeneration of the septum and stomium are also blocked in this mutant, suggesting important functions for the middle layer in dehiscence.

Degeneration of specific cell types during dehiscence relies on programmed cell death (Wu and Cheung, 2000; Sanders et al., 2005), which in senescence and plant defense are dependent on reactive oxygen species as signaling intermediates (Apel and Hirt, 2004; Gechev et al., 2006). Hence, it is unsurprising that the dehiscence program relies on *TGA9/10*, whose activity is likely to be redox regulated. Jasmonates, produced by plants in response to biotic and abiotic stresses, are known to promote the production of reactive oxygen species such as hydrogen peroxide (H_2O_2), which can be stress indicators but also important development signals (Buchanan and Balmer, 2005;

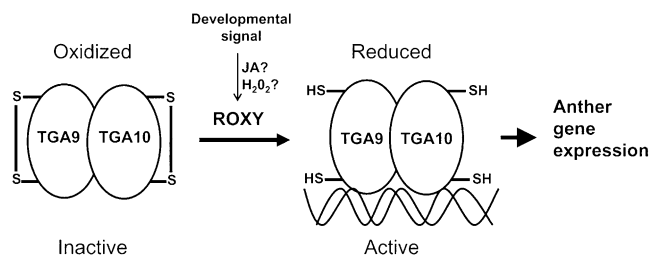


Figure 9. Model for redox control of *TGA9/10* activity by *ROXY1/2*. In their inactive form, one or more oxidized C-terminal Cys residues block *TGA9/10* transcription factor activity (a dithiol bridge is shown) by an unknown mechanism. In response to redox changes triggered by a developmental signal, *ROXY1* and *ROXY2* reduce *TGA9* and *TGA10*, converting them into a transcriptionally active form.

Gapper and Dolan, 2006; Huffaker and Ryan, 2007; Wasternack, 2007). Indeed, the promoter elements to which TGA factors bind respond to various signals, including auxin, JA, and H₂O₂ (Xiang et al., 1996, 1997; Chen and Singh, 1999). Microarray studies show that TGA factors contribute in large part to the plant's transcriptional response to stress, as mimicked by treatment with oxylipins such as 12-oxo-phytodienoic acid and JA (Mueller et al., 2008). In plant defense, the SA-inducible CC-type glutaredoxin ROXY19/GRX480 interacts with TGA factors and plays a role in SA/JA cross talk by repressing JA-responsive *PDF1.2* gene transcription (Ndamukong et al., 2007). Thus, TGA9/10 may respond to redox changes induced by the localized production of auxin, JA, or H₂O₂. Indeed, significant changes in the redox status of protein thiols are seen by two-dimensional gel electrophoresis following treatment of Arabidopsis tissues with JA (Alvarez et al., 2009). These reversible modifications, often in the form of disulfide bridges, can function as redox switches to control protein activity, as shown for TGA1 and TGA4 (Després et al., 2003).

Posttranscriptional Control of TGA9 and TGA10 Activity by Glutaredoxin

In plants, there are now several examples of disulfide bridge formation or protein S-glutathionylation of Cys residues as mechanisms for regulating transcription factor activity (Dietz, 2008; Paulsen and Carroll, 2010). Our results support a model whereby modification of TGA9/10 by ROXY1/2 in response to a developmental signal such as JA biosynthesis promotes transcription factor activity (Fig. 9). Site-directed mutagenesis shows that Cys residues in the conserved C terminus of TGA1 (Cys-260 and Cys-266) and PAN (Cys-340) are crucial for function (Després et al., 2003; Li et al., 2009). Transcriptional activation by TGA1 requires an interaction with NPR1, a BTB-ankryin transcriptional coactivator (Rochon et al., 2006). This interaction relies on the reduction of Cys-266 and Cys-260 to disrupt an intramolecular disulfide bridge, modifications that are induced in SA-treated plants by an unknown glutaredoxin (Després et al., 2003). In contrast, PAN interacts constitutively with BTB-ankryin cofactors BOP1 and BOP2 in yeast and in the nuclei of Arabidopsis leaf mesophyll protoplasts (Hepworth et al., 2005; Xu et al., 2010). A single Cys residue in PAN (Cys-340, equivalent to Cys-266 in TGA1) is crucial for function, making it unclear if PAN is regulated by an intermolecular disulfide bridge or by S-glutathionylation. Genetic analysis shows that the *pan* phenotype (extra petals) is epistatic to the *roxy1 roxy2* phenotype (fewer petals), suggesting that ROXY1 negatively regulates PAN activity (Li et al., 2009). In support of a monothiol mechanism for PAN regulation, complementation studies show that only one of three Cys residues (Cys-49) in the active site of ROXY1 is crucial for its *in vivo* activity (Xing et al., 2005). Alignments show that TGA10 alone has an

equivalent to Cys-340/Cys-260 in PAN/TGA1, respectively, and that both TGA9 and TGA10 have a unique C-terminal Cys residue (Cys-429 and Cys-435, respectively; Supplemental Fig. S9). Our inability at present to complement the *tga9 tga10* mutant phenotype with *TGA9* or *TGA10* cDNAs prevents us from testing if these residues are important for activity.

Functional Redundancy among TGA Family Members

Biochemical studies show that bZIP factors bind to DNA as dimers (Lamb and McKnight, 1991), supporting the notion that TGA9 and TGA10 may function redundantly as homodimers or heterodimers. Moreover, the *tga9 tga10* phenotype is less severe than that of *roxy1 roxy2* in that pollen formation is not completely blocked (Xing and Zachgo, 2008). A likely possibility is that other TGAs expressed in the anther work in conjunction with TGA9 and TGA10 as downstream effectors of ROXY1 and ROXY2. Candidates include TGA2, TGA6, and PAN, which interact with ROXY1 with differing affinities in yeast or in plants (Xiang et al., 1997; Li et al., 2009). The best candidate for redundancy is TGA2, but tight linkage to TGA10 hampers the genetic testing of this model. Given the broad expression pattern of TGA9, PAN, and TGA2 in tissues other than the flower (Fig. 1, D and E; Chuang et al., 1999; Li et al., 2009), it is likely that genetic redundancy continues to mask additional roles for this redox-regulated family of transcription factors in plant development.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Plants were grown in growth chambers on agar plates and/or in soil at 21°C in 24 h of light (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The wild type was the Col-0 ecotype of Arabidopsis (*Arabidopsis thaliana*) unless otherwise stated. T-DNA insertion lines *tga9-1* (SALK_057609), *tga9-2* (SALK_091349), *tga10-1* (SALK_124227), and *tga10-2* (SALK_039672) were obtained from the Arabidopsis Biological Resource Center. The double mutant *tga9-1 tga10-1* was characterized in this study. The position of T-DNA inserts was determined by sequencing, and homozygous mutants were identified by PCR genotyping (<http://signal.salk.edu>). Mutants *roxy1 roxy2*, *spl-1*, and *dyl1* were described previously (Xing and Zachgo, 2008). All mutant combinations were constructed by crossing and confirmed by genotyping. Floral and anther development stages were determined according to Smyth et al. (1990) and Sanders et al. (1999), respectively. Supplemental Tables S1 and S2 list the sequences of most primers used in this study. All other primer sequences are available by request.

Construction of TGA9::GUS Reporter Lines

A TGA9::GUS translational fusion gene was created using the strategy described by Hepworth et al. (2002). A sequence representing the putative promoter of TGA9 (nucleotides -3,895 to +5) was amplified by PCR using Arabidopsis bacterial artificial chromosome T27G7 DNA as the template. Wild-type plants were transformed with pTGA9::GUS by floral dipping (Clough and Bent, 1998) using the *Agrobacterium tumefaciens* strain C58C1 pGV101 pMP90 (Koncz and Schell, 1986). Basta-resistant transformants were selected on soil using the herbicide Finale (AgrEvo).

Pollen Germination and Viability Assays

For the *in vitro* assay of pollen germination, pollen grains from the anthers of late stage 12 flowers were excised into water and transferred to pollen germi-

nation medium presolidified onto a microscope slide (Fan et al., 2001). Slides were incubated at 22°C in a high-humidity chamber (Johnson-Brousseau and McCormick, 2004), and pollen tubes were observed after 24 h by light microscopy. The germination of more than 300 pollen grains was monitored for each genotype. Pollen viability was also assessed with Alexander's stain (Alexander, 1969).

Histological Analysis of Developing Anthers

Floral buds were fixed overnight at 4°C in 2% (v/v) glutaraldehyde as described (Ogawa et al., 2009). Tissues were briefly rinsed in 25 mM NaPO₄ buffer (pH 6.8) and dehydrated with ethanol prior to embedding in LR White resin (London Resin Company). Sections (1 μm) were heat fixed onto glass slides and stained with 0.05% (w/v) toluidine blue prior to imaging. To detect callose, dissected anthers were treated with 0.05% (w/v) aniline blue in 0.067 M phosphate buffer (pH 8.5) and imaged under UV illumination. To detect lignin, paraffin-embedded anther sections were dewaxed and dehydrated prior to successive incubations in 2% phloroglucinol (in 95% ethanol) and 6 N HCl for color development.

GUS Staining and in Situ Hybridization

Staining, sectioning, and embedding of GUS-stained tissues was performed essentially as described by Sieburth and Meyerowitz (1997). Sections (10 μm) were affixed to glass slides and dewaxed with *tert*-butanol prior to imaging. In situ hybridization and antisense probe synthesis was performed as described previously (Hepworth et al., 2005).

RT-PCR

Total RNA was isolated from inflorescence apices using the Trizol method (Invitrogen). Total cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen) using 1 μg of RNA as the template. PCR was subsequently performed using 2 μL of diluted cDNA as the template and Taq as the polymerase (Invitrogen). Primers bZIP21-F2 and bZIP21-R1, and bZIP21-F2 and bZIP21-R2, were used for amplification of *TGA9* transcripts designated full and partial, respectively. Primers bZIP65cDNA-F1 and bZIP65cDNA-R2, and bZIP65cDNA-F1 and bZip65R1-3 were used for amplification *TGA10* transcripts designated full and partial, respectively. *GAPC* encoding glyceraldehyde 3-phosphate dehydrogenase served as a control transcript (Hepworth et al., 2005).

Yeast Two-Hybrid Assay

We used the GAL4-based yeast two-hybrid system described by Kohalmi et al. (1998). To generate bait and prey constructs, the full coding sequences of *TGA9*, *TGA10*, *ROXY1*, and *ROXY2* were amplified by PCR from cloned cDNA template. PCR products were subcloned into pCR-BluntII-TOPO (Invitrogen) and sequenced to ensure fidelity. Recognition sites for the restriction enzymes *SalI* and *NotI* were incorporated into the 5' ends of primers to facilitate the subsequent directional cloning of *ROXY1/2* and *TGA9/10* cDNA inserts into the corresponding sites of bait (pBI-880) and prey (pBI-881) plasmids, respectively. Construction of *TGA2* in pBI-880, yeast transformation, and β-galactosidase assays were described previously (Després et al., 2000).

BiFC Assay

BiFC assays were performed as described by Li et al. (2009). pE-SPYNE and pE-SPYCE (Walter et al., 2004) were used to generate expression vectors using a Gateway cloning strategy (Invitrogen). The N terminus of YFP was cloned in-frame upstream of *ROXY1* and *ROXY2* cDNAs. The C terminus of YFP was fused in-frame to the N termini of *TGA9* and *TGA10* cDNAs. Fusion genes were expressed under the control of a cauliflower mosaic virus 35S promoter. Infiltrated *Nicotiana benthamiana* leaves were examined for reconstitution of YFP fluorescence 3 to 5 d after inoculation using a LSM 510 Meta confocal microscope (Carl Zeiss).

Scanning Electron Microscopy

Samples were prepared for scanning electron microscopy as described previously (Hepworth et al., 2005) and imaged using a VegaII XMU VPSEM apparatus (Tescan).

Sequence data have been deposited in GenBank under accession numbers HQ132743 (*TGA9* cDNA) and HQ132742 (*TGA10* cDNA).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic tree for Arabidopsis TGA transcription factors.

Supplemental Figure S2. Analysis of pollen in wild-type and *tga9 tga10* mutant plants.

Supplemental Figure S3. Callose deposition in wild-type and *tga9 tga10* mutant plants.

Supplemental Figure S4. Comparison of wild-type and *tga9 tga10* late-stage anther lobe development.

Supplemental Figure S5. Endothecium lignification of wild-type and *tga9 tga10* anthers.

Supplemental Figure S6. In situ analysis of *TGA10* expression in developing anthers.

Supplemental Figure S7. RT-PCR analysis of *TGA9* and *TGA10* transcript in wild-type (Nossen-0) and *roxy1 roxy2* apices.

Supplemental Figure S8. Quantitative RT-PCR analysis of tapetum transcripts in wild-type, *tga9 tga10*, and *roxy1 roxy2* apices.

Supplemental Figure S9. Alignment of selected TGA transcription factors.

Supplemental Table S1. List of primers.

Supplemental Table S2. List of primers for RT-PCR transcript analysis of anther development genes.

Supplemental Materials and Methods S1.

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