


RESEARCH PAPER



Developmental phenotypes of *Arabidopsis* plants expressing phosphovariants of *AtMYB75*

Anna E. Kreyne , Zhenhua Yong, and Brian E. Ellis

Michael Smith Laboratories, Department of Botany, University of British Columbia, Vancouver, Canada

ABSTRACT

The *Arabidopsis* transcription factor Myeloblastosis protein 75 (MYB75, AT1G56650) is a well-established transcriptional activator of genes required for anthocyanin and flavonoid production, and a repressor of lignin and other secondary cell wall biosynthesis genes. MYB75 is itself tightly regulated at the transcriptional, translational and post-translational levels, including protein phosphorylation by *Arabidopsis* MAP kinases. Examination of the behavior of different phosphovariant versions of MYB75 *in vitro* and *in vivo* revealed that overexpression of the MYB75^{T131E} phosphovariant had a particularly marked effect on global changes in gene expression suggesting that phosphorylated MYB75 could be involved in a broader range of functions than previously recognized. Here, we describe a range of distinct developmental phenotypes observed among *Arabidopsis* lines expressing various phosphovariant forms of MYB75. Expression of either MYB75^{T131E} or MYB75^{T131A} phosphovariants, from the endogenous MYB75 promoter, in *Arabidopsis myb75*⁻ mutants (Nossen background), resulted in severely impaired germination rates, and developmental arrest at early seedling stages. *Arabidopsis* plants overexpressing MYB75^{T131E} from a strong constitutive Cauliflower mosaic virus (CaMV35S) promoter displayed slower development, with delayed bolting, flowering and onset of senescence. Conversely, MYB75^{T131A}-overexpressing lines flowered and set seed earlier than either Col-0 WT controls or other MYB75-overexpressors (MYB75^{WT} and MYB75^{T131E}). Histochemical analysis of mature stems also revealed ectopic vessel development in plants overexpressing MYB75; this phenotype was particularly prominent in the MYB75^{T131E} phosphovariant. These data suggest that MYB75 plays a significant role in plant development, and that this aspect of MYB75 function is influenced by its phosphorylation status.

ARTICLE HISTORY

Received 23 August 2020
Revised 8 October 2020
Accepted 9 October 2020

KEYWORDS

Arabidopsis thaliana;
Myeloblastosis protein 75;
mitogen activated protein kinase; phosphorylation;
germination; flowering time;
bolting time; vascular development

Introduction

Transcriptional regulators are a vital part of the cellular circuitry that controls gene expression, and ultimately the biochemical, developmental and physiological changes that occur in living organisms. Many transcription factors characterized over the years have been initially identified as regulators of a specific biological trait for which distinct phenotypes could be easily identified. However, an increasing body of evidence shows extensive interconnectivity between different signaling pathways, and multifunctionality of transcriptional regulators. A prominent example of such multifunctionality is HY5, initially identified as a suppressor of hypocotyl elongation during de-etiolation, but now known to be a global regulator of light-responsive genes and a driver of photomorphogenesis.^{3–5} Other examples include *Enhancer of Glabra 3* (EGL3, AT1G63650) and *Glabra 3* (GL3, AT5G41315), which regulate anthocyanin biosynthesis in concert with MYB75,^{6–8} and, independently of this function also play a role in epidermal cell fate determination.^{9–14} The involvement of MYB75 in multiple, seemingly unrelated biological processes can be in part attributed to its ability to form complexes with different transcriptional regulators. Formation of the ternary complex MYB75-EGL3/GL3/TT8-TTG1 is required for anthocyanin biosynthesis,⁷ while association of MYB75 and KNAT7 (AT1G62990) is needed to suppress expression of lignin and other cell wall biosynthesis

genes.^{15,16} Thus, the ability of MYB75 to control the expression of specific genes depends on the presence of appropriate binding partners, which is ultimately dictated by the cellular milieu in which the protein is found.

We earlier demonstrated that MYB75 can physically interact with a large number of *Arabidopsis* MAP kinases, suggesting that this transcription factor may be involved in multiple MPK signaling cascades.¹ While total anthocyanin production was similar in *Arabidopsis* plants overexpressing MYB75^{T131E} and MYB75^{T131A} phosphovariants, MYB75^{T131E} was found to be a more potent driver of global gene expression changes in *Arabidopsis*, compared to MYB75^{T131A}, and MYB75^{T131E} protein displayed more rapid turnover under certain conditions.¹ These findings suggest that phosphorylation at Threonine 131 influences the ability of MYB75 to drive expression of downstream genes and, through control of protein turnover, could play a role in spatial-temporal distribution of the protein, thus determining the cellular milieu where MYB75 is found.¹

When analyzing the properties of the *Arabidopsis* lines expressing various MYB75 phosphovariants we consistently observed developmental and physiological changes in certain plant lines which affected their viability, suggesting MYB75 function has a profound influence on plant development. In this report we describe some of the phenotypes we observed among *Arabidopsis* lines expressing different phosphovariants

of *MYB75*; phenotypes which do not appear to be directly related to *MYB75*'s role in regulating anthocyanin biosynthesis, the function canonically associated with this protein.

Materials and methods

Site-directed mutagenesis

Phosphovariant (phosphomimic and phosphonull) versions of *MYB75* were created by replacing each threonine found in the putative MPK target site with either alanine or glutamic acid, using site-directed mutagenesis as described in previous work.¹

Plant material and transgenic lines

Arabidopsis seeds (Col-WT and Nossen WT) were purchased from Lehle seeds, 1102 South Industrial Blvd. Suite D, Round Rock, Texas 78681, United States, Phone: 1-(512) 388-3945 (512) 388-3974 Website: <http://www.arabidopsis.com>.

All transgenic *Arabidopsis* lines used in this study were generated in our laboratory. To create transgenic lines where each *MYB75* phosphovariant (*MYB75*^{T126A}, *MYB75*^{T126E}, *MYB75*^{T131A}, *MYB75*^{T131E}, *MYB75*^{T126/131A} and *MYB75*^{T126/131E}) was expressed from the endogenous *MYB75* promoter, cDNA version of each *MYB75* phosphovariant was cloned into the gateway entry vector pENTR2B, behind an endogenous *MYB75* promoter, encompassing 2742bp of genomic sequence upstream of the *MYB75* translational start site. The *MYB75pr:MYB75* cDNA constructs were transferred into the binary GATEWAY® vector pEARLEY-GATE301, by recombination based cloning, using LR Clonase II (Thermo Fisher Scientific). The resulting constructs were subsequently used to transform *myb75*⁻ (Nossen) mutant plants, using *Agrobacterium*-mediated floral transformation as described previously.¹ Successfully transformed T1 plants were isolated on Murashige and Skoog (½MS) agar plates containing 50 µg/mL kanamycin as the selection agent.

Arabidopsis lines overexpressing different phosphovariants of *MYB75* (full gene version, of *MYB75*^{WT}, *MYB75*^{T131A} and *MYB75*^{T131E}), with an N-terminal 3x hemagglutinin (HA)-epitope tag, under the control of a constitutive Cauliflower mosaic virus (CaMV35S) promoter, were generated using GATEWAY® recombination-based cloning, as described previously.¹ Successfully transformed T1 plants were isolated on Murashige and Skoog (½MS) agar plates containing 30 µg/mL hygromycin as the selection agent.

Plant growth conditions

Arabidopsis seeds were surface sterilized, plated on Petri dishes containing ½ MS medium with 0.8% plant agar, and vernalized for 3 days at 4°C. After vernalization, the plates were placed under light fluence of ~30 µmol m⁻² s⁻¹ and a 16 h light/8 h dark photoperiod, at 21°C and grown under these conditions until they were transferred to soil. Between 7 and 12 days after germination, seedlings were planted in soil and transferred to a growth chamber with a light fluence of ~100-120 µmol m⁻² s⁻¹, a photoperiod of 16 h light/8 h dark, and temperature of 21°C.

To evaluate the ability of each *MYB75* phosphovariant to drive anthocyanin production and to assess *MYB75* promoter functionality, in *MYB75pr:MYB75* cDNA lines, 6-week-old transgenic plants were exposed to continuous light for 48 h (fluence of ~100-120 µmol m⁻² s⁻¹).

Anthocyanin quantification

Anthocyanin quantification was performed as previously described,¹⁷ using mature rosette leaves from 6-week-old *Arabidopsis* plants, expressing different *MYB75* phosphovariants from the endogenous *MYB75* promoter, in a *myb75*⁻ mutant background (Nossen ecotype). Fresh, healthy plant tissue (1–2 g) was collected into microcentrifuge tubes and fresh weight was recorded for each sample, which was immediately frozen in liquid nitrogen and ground to a powder. The lyophilized tissue was immediately re-suspended in 1 mL extraction solution (49.5% methanol, 49.5% distilled water, 1% acetic acid), the samples were incubated at 45°C for 4 h, centrifuged at 25000 g and the cleared supernatant was transferred to a cuvette. Absorbance was measured at λ = 530 nm and λ = 650 nm, and total anthocyanin quantity was determined using the equation Q_{anthocyanin} = [A530 - (A650x0.25)]/mass(g). Each sample consisted of tissue from 3–4 plants belonging to an individual line. The number of samples used for each genotype were as follows: four samples for each Nossen WT and *myb75*⁻ controls; six samples for *MYB75*^{T126A} genotype (one sample for each line); six samples for *MYB75*^{T131A} genotype (one sample for each line); three samples for *MYB75*^{T126/131A} genotype (one sample for each line).

Detection of recombinant *MYB75* expression

RNA extraction from *Arabidopsis* plants (mature rosette leaves and stem sections) was performed using TRIzol® reagent (Thermo Fischer Scientific), according to manufacturer's protocol. RNA quality was evaluated by running 5 µL of RNA on 2% agarose gel, and the RNA concentration was determined spectrophotometrically using NanoDrop® (Thermo Fischer Scientific). Reverse transcription was performed using SuperscriptII (Invitrogen) to obtain total cDNA.

In transgenic *Arabidopsis* lines where each phosphovariant was expressed from the endogenous *MYB75* promoter (*myb75*⁻ mutant background), the primers used to detect *MYB75* transcripts were F-5' TCCTAGAGGAAAGCCAAGAGG and R-5'-CTAATCAAATTTACAGTCTCTCCATCG, while Actin1 (AT3G18780) reference gene was detected with the forward F-5' CCACCTGAAAGGA AGTACAGTG and reverse R-5' GTGAACGATTCTGGACCTG primers. Semi-quantitative RT-PCR was performed using MangoMix™ (Bioline), by running the PCR reaction for 25 cycles. The resulting bands were resolved by gel electrophoresis (1% agarose gel) and relative band intensity was quantified using ImageJ software: the relative amount of *MYB75* cDNA in each sample was obtained by evaluating *MYB75* band intensity, normalized to Actin1 signal. As for anthocyanin quantification, each sample consisted of tissue from 3–4 plants belonging to an individual line. The number of samples used for each genotype were as follows:

four samples for each Nossen WT and *myb75*⁻ controls; five samples for *MYB75*^{T126A} genotype (one sample for each line); six samples for *MYB75*^{T131A} genotype (one sample for each line); three samples for *MYB75*^{T126/131A} genotype (one sample for each line).

Expression of recombinant 3xHA:MYB75 in *Arabidopsis* plants overexpressing different phosphovariants of MYB75 from the constitutive 35S promoter, (*35Spr:3xHA:MYB75 gene*) was detected using the forward primer F-5' CAGTGCAGCGCTGTTATCACAAAG, which binds to the HA-tag portion of the construct, and the reverse primer R-5'-CTAATCAAATTTACAGTCTCTCCATCG, which binds to a region of MYB75 coding sequence. PCR reaction was run for 35 cycles, using the MangoMix™ (Bioline), in conjunction with the primers listed above and the PCR products were analyzed by gel electrophoresis (1% agarose gel).

Scoring germination

Germination rate in *MYB75pr:MYB75 cDNA* lines was scored in homozygous T3 plants, including six independent *MYB75pr:MYB75*^{T131A} *cDNA* and four *MYB75pr:MYB75*^{T131E} *cDNA* lines, along with *myb75*⁻ mutant and Nossen WT controls. Seeds were surface sterilized, plated on ½ MS-agar plates and vernalized for 3 days at 4°C, then placed under light fluence of ~30 μmol m⁻² s⁻¹ and 16 h light/8 h dark photoperiod, at 21°C. Ten days after exposure to light, the germination percentage was scored for each plate by counting the number of unresponsive seeds and the number of germinated plants. Plants which were counted as germinated included any seed that showed signs of an emerging seedling. Student T-test was performed to evaluate statistical significance of differences in the average germination rates between different genotypes.

Flowering time of *Arabidopsis* plants overexpressor different recombinant MYB75 phosphovariants

Heterozygous T2 *Arabidopsis* plants carrying *35Spr:3xHA:MYB75 gene* constructs were used in this assay, including three lines for *35Spr:3xHA:MYB75*^{WT} (L11, L12, L14), and *35Spr:3xHA:MYB75*^{T131A}; (L1, L22, L23), and four lines for *35Spr:3xHA:MYB75*^{T131E}; (L2, L4, L5, L9). Seeds of transgenic lines, along with Col-0 WT were germinated simultaneously on Petri dishes containing ½MS agar medium, supplemented with 30 μg/mL hygromycin (except for Col-0 WT controls). Seedlings were grown on petri plates for ten days, at which point they were transferred to soil in a growth chamber (see plant growth conditions). Individuals representative of each line were photographed on the same day, when plants were 5 weeks old and again at 7 weeks, to capture differences in timing of flowering and other developmental transitions, between different phosphovariant genotypes.

Histochemical analysis of stem cross-sections

The lower 5 cm of the stems of 7- and 8-week-old *Arabidopsis* plants were hand-sectioned. Lignin staining was performed by submerging stem sections in phloroglucinol (Sigma) solution (2% w/v phloroglucinol in 95% ethanol) and adding

concentrated HCl dropwise. After 5 min incubation in phloroglucinol-HCl, the sections were washed three times in distilled water, mounted on slides (in distilled water) and imaged immediately. Additional stem sections were stained with toluidine blue by submerging the sections in 0.02% toluidine blue solution for 1–2 min and rinsing in distilled water three times. The toluidine-blue stained sections were then mounted on slides in distilled water and imaged immediately. All imaging was performed using a Leica Mecatron Precision DMR compound microscope, with a mounted digital camera, Canon EOS Rebel T5.

Detection of recombinant 3xHA-tagged MYB75 protein in *Arabidopsis* stems

The lower ~5 cm of *Arabidopsis* stems were collected from 7-week-old plants expressing *3xHA-tagged MYB75*. The stems were ground to a fine powder and the lyophilized material was immediately resuspended in 2xSDS loading buffer. SDS PAGE and Western blot detection of MYB75 was performed as described previously¹ using a 1:1000 dilution of a primary anti-HA antibody (high affinity rat monoclonal, clone 3F10, Roche), and a 1:5000 dilution of a secondary antibody conjugated with Horseradish peroxidase (HRP) (goat anti-rat IgG-HRP, Santa Cruz Biotech Sc-2032). Chemiluminescence signal detection was performed using ECL Western blot reagent (Pierce™ ECL plus) and recorded on photosensitive film (Bioflex Blue Lite EC, Mandel Scientific).

Results

Complementation of anthocyanin biosynthesis in *Arabidopsis myb75*⁻ null mutants, by different MYB75 phosphovariants

Our initial approach to understanding how phosphorylation might affect MYB75 function, was to create MYB75 phosphovariant lines by transforming *Arabidopsis myb75*⁻ null mutants (Nossen background) with each MYB75 phosphovariant *cDNA*, under the control of the endogenous MYB75 promoter. In this complementation experiment the ability of each MYB75 phosphovariant to drive anthocyanin production was tested simultaneously with promoter functionality, by stressing the adult transgenic plants with continuous light (a known driver of MYB75 expression), for 48 h before measuring total anthocyanin content in mature rosette leaves. Complementation of the anthocyanin deficiency typical of *myb75*⁻ null mutant plants, by each phosphovariant, was evaluated by comparing total anthocyanin levels in transgenic lines to the levels found in Nossen wild type plants, grown under the same conditions. Analysis of T2 plants generated with each phosphovariant revealed that *MYB75*^{T126A} -expressing plants grown under light stress were able to accumulate average anthocyanin levels, comparable to those seen in Nossen wild type plants. In contrast, plants carrying *MYB75*^{T131A} and *MYB75*^{T126/131A} phosphovariants showed only marginal levels of anthocyanin production, which did not differ significantly from those seen in the parental *myb75*⁻ mutant phenotype, deficient in anthocyanin production (Figure 1a). Although these data would

suggest that certain phosphovariant forms of MYB75 fail to drive anthocyanin production, when we examined anthocyanin levels in individual lines, as well as recombinant MYB75 gene expression, it became apparent that lines which failed to produce anthocyanins had only negligible expression of recombinant MYB75 (Figure 1b–d). These data suggest that phosphorylation status at T126 does not affect the ability of MYB75 to drive anthocyanin expression, since all MYB75^{T126A} lines which expressed recombinant MYB75 were able to complement anthocyanin deficiency in the *myb75*[−] mutant phenotype. Importantly, all lines where T131 was mutated, (including MYB75^{T131A} and MYB75^{T126/131A}) were displaying very low levels of recombinant MYB75 expression (Figure 1).

Impaired germination in *Arabidopsis* plants expressing MYB75^{T131A} and MYB75^{T131E} from the endogenous MYB75 promoter

An initial screen of large numbers (approximately 100 in total) of T1 plants expressing either MYB75^{T131A} or MYB75^{T131E} from the endogenous MYB75 promoter established that expression of MYB75 phosphovariants in which T131 was mutated to either alanine (T131A) or glutamic acid (T131E), were failing to complement the low anthocyanin phenotype of the *myb75*[−] parent plants because the transgenic lines displayed very low levels of MYB75 transgene expression. One possible explanation for this is that strong expression of either phosphovariant is detrimental to early developmental stages, such as embryogenesis or germination, in these transgenic lines, resulting in artificial selection of plants that express only low levels of recombinant MYB75. Having noted that MYB75*pr*:MYB75^{T131A} and MYB75*pr*:MYB75^{T131E} plants showed low germination rates, we wanted to quantify this phenomenon, in order to test our hypothesis that MYB75 expression and phosphorylation status can have an impact on early development. For this purpose we used seeds from homozygous T3 parent plants, carrying MYB75*pr*:MYB75 *cDNA* (MYB75^{T131A} and MYB75^{T131E} phosphovariants), in a *myb75*[−] mutant background (Nossen ecotype), including six independent MYB75*pr*:MYB75^{T131A} and four independent MYB75*pr*:MYB75^{T131E} lines. Both Nossen WT and *myb75*[−] mutants showed germination rates close to 100%. In contrast, lines carrying either MYB75*pr*:MYB75^{T131A} or MYB75*pr*:MYB75^{T131E} transgene displayed severely impaired germination (26.8% and 62%, respectively; Figure 2). It is challenging to assess this phenotype in the absence of MYB75*pr*:MYB75^{WT} transgenic control, nevertheless when the two phosphovariants are compared to each other, it is apparent that germination is significantly lower in MYB75^{T131A}-expressing plants than in MYB75^{T131E} lines, suggesting that phosphonull MYB75^{T131A} has a greater negative effect on germination than the phosphomimic MYB75^{T131E} version. These low germination rates support the idea that our failure to isolate transgenic lines expressing MYB75^{T131A} and MYB75^{T131E} was caused by artificial selection for plants that express lower levels of these recombinant MYB75 phosphovariants. In addition to low germination rates, many plants from these transgenic lines displayed arrested development at early stages and did not survive past the seedling

stage (data not shown). This phenomenon was very challenging to quantify, as there was no clear phenotype, timing or percentage of developmental arrest in these seedlings; nevertheless, our observations suggested that these transgene variants could be contributing to developmental abnormalities at the embryonic and early seedling stages, and prompted further examination of developmental phenotypes in plants expressing different MYB75 phosphovariants.

Overall our approach, of expressing MYB75 from the endogenous promoter did not yield results which could help us understand how phosphorylation affects MYB75 function, since not all phosphovariant lines could be isolated. For this reason, we shifted our approach to using overexpressor lines, where the full-length MYB75 gene is driven by a strong constitutive 35S promoter, as described in our previous work.¹ Furthermore, we narrowed our focus to the single phosphorylation site T131, as *in vitro* phosphorylation data indicated that this is the primary site for phosphorylation by MPK3, MPK4, MPK6 and MPK11.¹

Flowering time and senescence are delayed in *Arabidopsis* plants over-expressing MYB75^{T131E}

Arabidopsis plants overexpressing different MYB75 phosphovariants (35Spr:3xHA:MYB75^{WT}, 35Spr:3xHA:MYB75^{T131A}, and 35Spr:3xHA:MYB75^{T131E}) were grown side by side with Col-0 WT, in the same growth chamber under regular photoperiod, 16 h light/8 h dark (see materials and methods). Wild type Col-0 plants began to bolt and flower after four-five weeks of growth. Transgenic 35Spr:MYB75^{WT} plants showed differences in flowering time between different lines; L11 displayed flowering time comparable to Col-0 WT plants, while L12 and L14 produced an inflorescence stem and flowers before Col-0 WT of the same age (Figure 3a). This apparent acceleration in development persisted after bolting, as 35Spr:MYB75^{WT} L12 and L14 displayed abundant formation of lateral branches and flowers, at a stage when Col-0 WT and 35Spr:MYB75^{WT} L11 plants had only developed the main inflorescence (Figure 3a). Similar developmental acceleration was observed in 35Spr:MYB75^{T131A} L1, L22 and L23. Early bolting, flowering and development of numerous lateral branches was consistently observed in all 35Spr:MYB75^{T131A} plants, with no observable developmental differences between lines of this genotype. All 35Spr:MYB75^{T131E} plants displayed a delay in bolting and flowering compared to Col-0 WT (Figure 3a). The developmental delay observed in 35Spr:MYB75^{T131E} lines was also evident in later stages of development. At 7 weeks of age Col-0 WT as well 35Spr:MYB75^{WT} and 35Spr:MYB75^{T131A} lines ceased to flower, had formed mature siliques, and were beginning to show signs of senescence manifested as yellowing of the rosette leaves, and desiccating siliques. On the other hand, all 35Spr:MYB75^{T131E} lines displayed abundant flowers at 7 weeks, with only a few young siliques forming at this stage, and healthy green tissue throughout the plant, with no signs of senescence (Figure 3c). Despite the variability between individual lines in 35Spr:MYB75^{WT} plants, a clear trend emerged between the phosphovariant plant lines: development was accelerated in plants over-expressing MYB75^{T131A} and retarded in those over-expressing MYB75^{T131E} (Figure 3b).

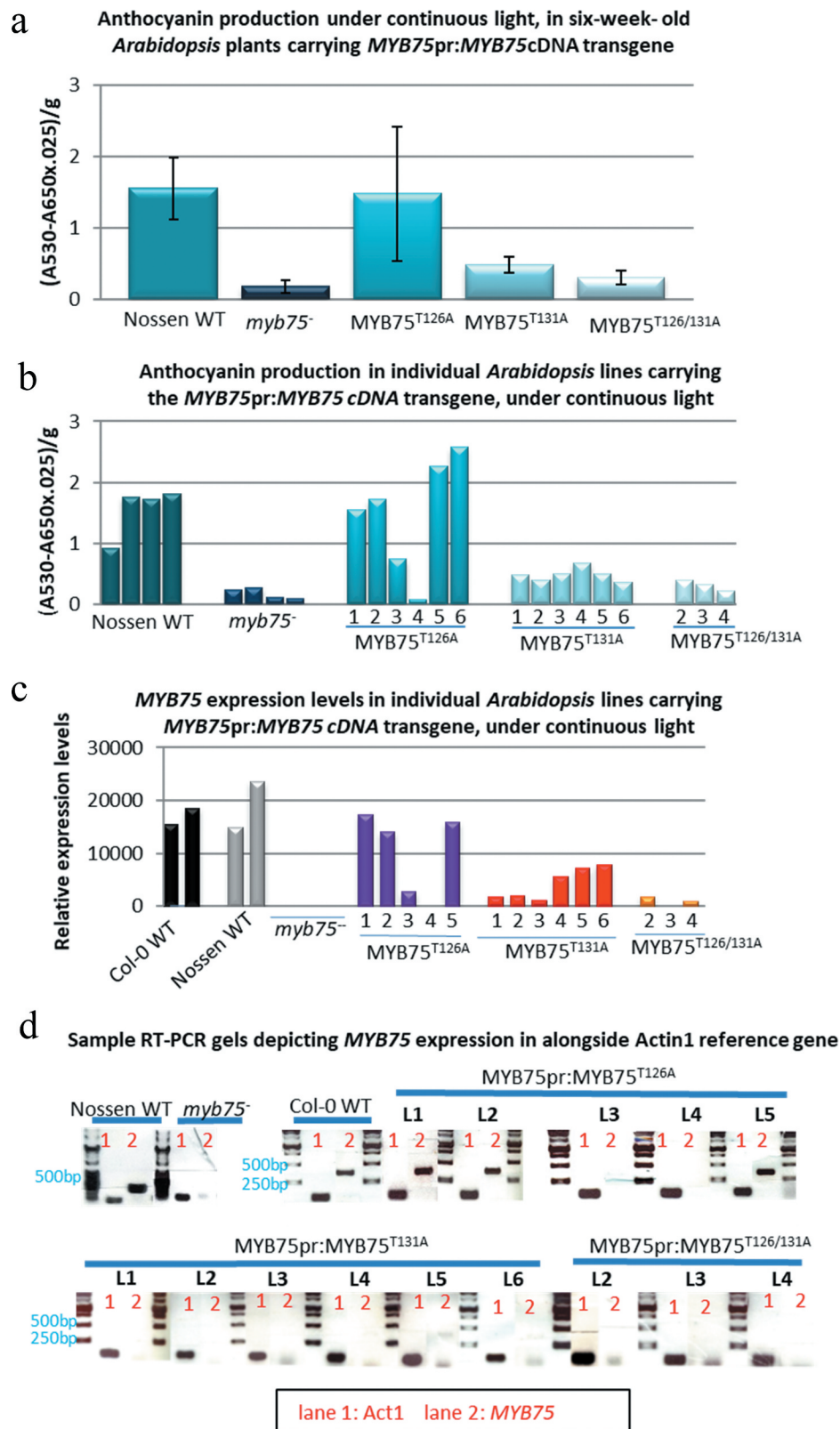


Figure 1. Complementation of anthocyanin biosynthesis in *Arabidopsis myb75*⁻ null mutants (Nossen), by different *MYB75* phosphovariants, driven by the endogenous *MYB75* promoter. The ability of each phosphovariant to complement anthocyanin production in *myb75*⁻ mutants was evaluated in 6-week-old heterozygous T2 plants, exposed to continuous light, for over 48 h. Total anthocyanin (average for each genotype) was quantified as described in materials and methods, error bars represent \pm SD. (a) Anthocyanin production was recovered in *MYB75*pr:*MYB75*^{T126A} genotype, but not in *MYB75*pr:*MYB75*^{T131A} or *MYB75*pr:*MYB75*^{T126/131A} mutants. (b) Examination of anthocyanin levels in individual lines revealed that only lines 1,2,5 and 6 from *MYB75*pr:*MYB75*^{T126A} genotype produced anthocyanin levels comparable to Nossen WT. (c) Gene expression analysis, by semi-quantitative RT PCR revealed that transgenic lines which showed anthocyanin complementation were expressing recombinant *MYB75* at levels comparable to wild type plants, while lines that failed to produce WT levels of anthocyanin had negligible levels of recombinant *MYB75* expression. (d) Sample gels of RT-PCR results depicting expression of *MYB75* in different *MYB75*pr:*MYB75* lines, along with Nossen WT, *myb75*⁻, and Col-0 WT controls. Lane1-Actin1 control, Lane2-*MYB75*.

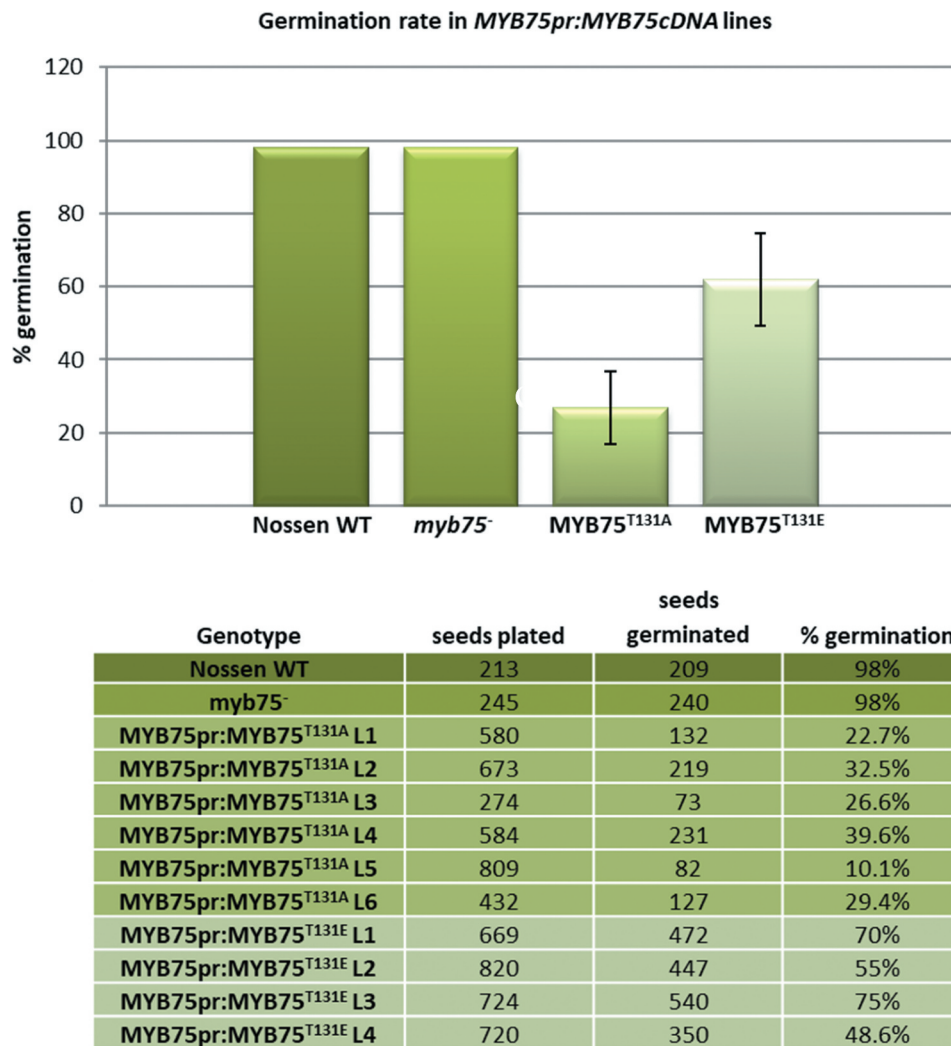


Figure 2. Impaired germination rates in *MYB75pr:MYB75*^{T131A} and *MYB75pr:MYB75*^{T131E} transgenic plants. Seeds from homozygous T3 plants were plated on ½ MS agar medium along with *myb75*⁻ and Nossen WT controls. After vernalization, the plates were placed under light fluence of ~30 μmol m⁻² s⁻¹ and a 16 h light/8 h dark photoperiod, at 21°C. Germination rate was scored ten days after transferring the plates into light. Percentage germination was determined for each line by counting the total number of seeds and the total number of germinated plants. Percentages of germinated plants were averaged for each genotype, error bars represent ±SD. Germination rates were 26.8% (SD = 9.99) for *MYB75pr:MYB75*^{T131A} plants and 62% (SD = 12.62) for *MYB75pr:MYB75*^{T131E}. These data indicate that *MYB75pr:MYB75*^{T131A} plants have a significantly lower germination rate than either Nossen WT, *myb75*⁻ or *MYB75pr:MYB75*^{T131E} transgenic lines. T test <0.05.

Ectopic vessel development in stems of mature *Arabidopsis* plants over-expressing different MYB75 phosphovariants

In addition to regulating anthocyanin production within MYB/bHLH/WDR complexes, MYB75 has also been shown to interact with KNAT7, a member of the Knotted *Arabidopsis Thaliana* Protein family.¹⁵ KNAT7 activity negatively affects lignin deposition and secondary cell wall thickness in interfascicular fibers^{16,19} and appears to act synergistically with MYB75 in inflorescence stems and the seed coat by down-regulating lignin biosynthesis.¹⁶ Because of this involvement of MYB75 in cell wall development, we wanted to determine if phosphorylation status of MYB75 at T131 had an impact on vascular architecture of *Arabidopsis* stems. Stem sectioning experiments were performed on 7-week-old and 8-week-old *35Spr:MYB75* plants, in which we examined the distribution of vascular and supporting tissues in the stem cross-sections.

At 8-weeks-old, the developmentally delayed *35Spr:MYB75*^{T131E} lines had formed mature siliques and were beginning to show signs of senescence, while Col-0 WT, *35Spr:MYB75*^{WT} and *35Spr:MYB75*^{T131A} plants had reached full senescence: rosettes leaves and siliques were beginning to desiccate, but the stems were still green. All plants were watered in the usual cycle, to ensure that the stems did not dry out, which could lead to vascular collapse. The lower portions of the main inflorescence stems (approximately 5 cm from the base) were hand-sectioned, and the stem sections were stained with phloroglucinol-HCl. As expected, wild type *Arabidopsis* stems showed a normal tissue organization, with vessel elements appearing as clusters of cells with dark reddish-brown-staining cell walls, restricted to the vascular bundles (Figure 4a). On the other hand, transgenic plants overexpressing MYB75 showed what appear to be vessel-like cells in the interfascicular region, with dark reddish-brown staining walls, and a larger cell diameter compared to the surrounding fibers (Figure 4b-d). In *35Spr:*

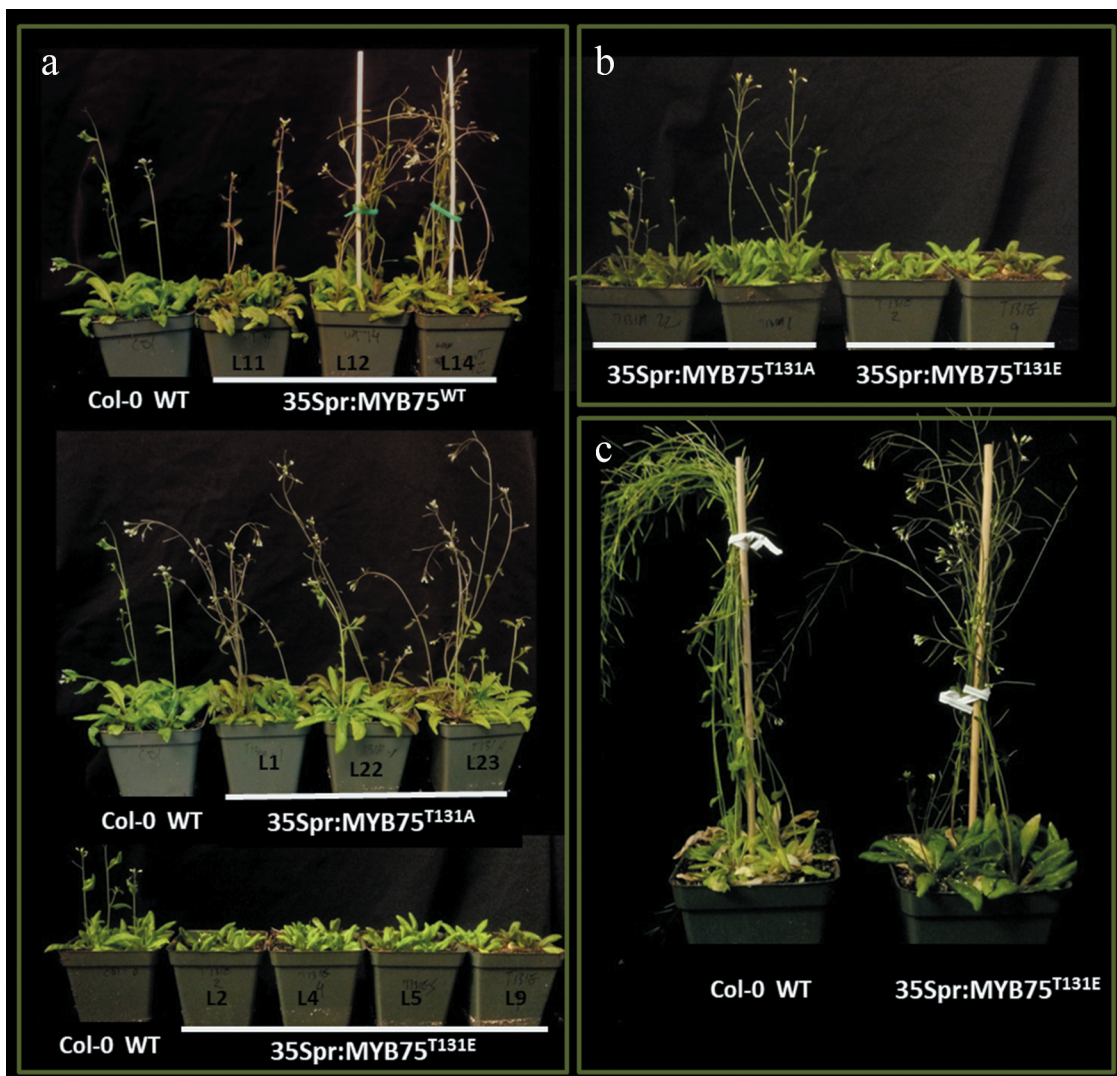


Figure 3. Developmental differences in bolting and flowering time as well as onset of senescence in plants over-expressing different phosphovariants of MYB75 (WT, T131A and T131E) from a constitutive CAMV35S promoter, in the Col-0 WT background. (a) Among $35Spr:MYB75^{WT}$ lines, L 11 bolted and flowered at a rate comparable to Col-0 WT (untransformed control), while L12 and L14 displayed early bolting and flowering compared to Col-0 WT controls. All $35Spr:MYB75^{T131A}$ lines bolted and flowered early compared to Col-0 WT. Conversely, $35Spr:MYB75^{T131E}$ plants showed delayed bolting and flowering. (b) The T131A mutation led to earlier bolting and flowering, while T131E mutation appears to slow down the transition from vegetative to reproductive growth. (c) Furthermore, $35Spr:MYB75^{T131E}$ lines displayed delayed maturation and onset of senescence compared to all recombinant genotypes analyzed and Col-0 WT controls.

$MYB75^{WT}$ stems the phenotype was relatively mild, with only a few isolated dark cell files in the interfascicular region, made up of cells with a slightly larger diameter than the surrounding fibers (Figure 4b). In $35Spr:MYB75^{T131A}$ plants, these darker staining cells were more prominent, with a diameter comparable to vessel elements found in the vascular bundles; however, only a few such cells, or very small cell clusters, could be seen in the interfascicular region (Figure 4c). In contrast, in plants over-expressing the phosphomimic $MYB75^{T131E}$, abundant development of vessel elements could be observed in the interfascicular region, with large cell clusters, stretching between the vascular bundles. In some cases, these prominent clusters of large, dark-staining cells spanned the entire length of the interfascicular arc (Figure 4d).

These stem development phenotypes showed some variability within the population of each phosphovariant genotype; some of the transgenic stems examined did not show any

abnormal tissue distribution at all. However, about 50% of the stems sectioned in each genotype ($35Spr:MYB75^{WT}$, $35Spr:MYB75^{T131A}$ and $35Spr:MYB75^{T131E}$) showed at least some ectopic vessel production, with ~25-30% of the stems examined for each transgenic line displaying more extreme phenotypes, with prominent vessel cell-files in the interfascicular region of $MYB75^{WT}$ and $MYB75^{T131A}$ plants (Figure 4b,c) and extensive vessel clusters, spanning the entire interfascicular arc in $MYB75^{T131E}$ stems (Figure 4d). These results indicate that over-expression of MYB75 can lead to ectopic differentiation in the interfascicular region of cells that appear to be vessels, and that over-expression of the $MYB75^{T131E}$ phosphovariant is more effective at driving this process.

At 8 weeks of age, *Arabidopsis* plants can undergo secondary growth in their lower stems.^{21,22} Signs of this secondary growth were observed in all genotypes, including Col-0 WT, with the appearance of cells around the periphery of the stem, with dark staining cell walls, reminiscent of vessel elements

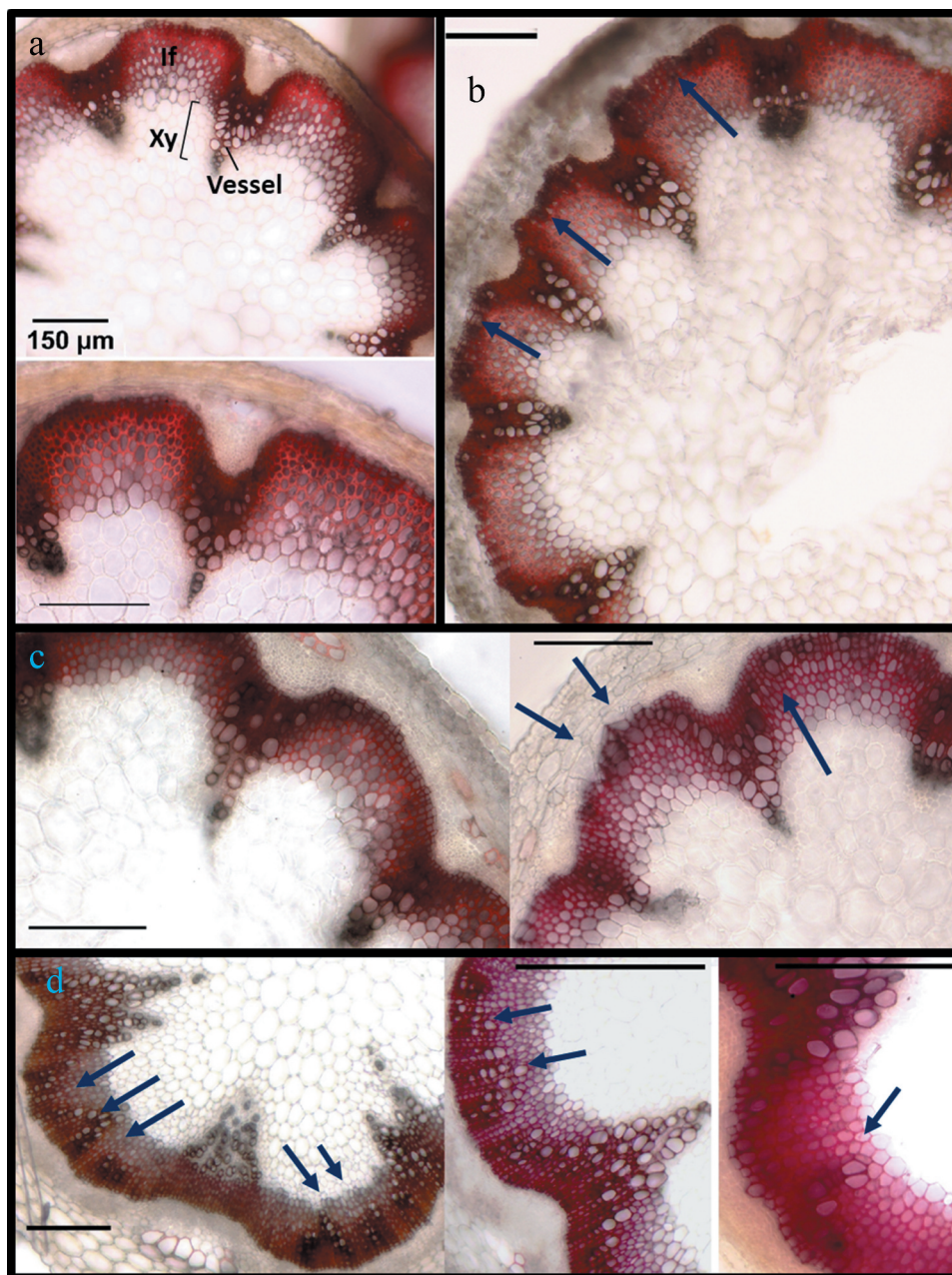


Figure 4. Ectopic vessel development in stems of 8-week-old *Arabidopsis* plants over-expressing different phosphovariants of MYB75. Stems from 8-week-old plants were harvested and the bottom 5 cm of each stem was hand sectioned and stained in phloroglucinol-HCl. (a) Stem sections from Col-0 WT plants display normal distribution of vessels, within vascular bundles, where xylem vessels can be seen, as large perforated cells. In phloroglucinol-HCl stained sections, vessel walls appear dark reddish-brown, while inter fascicular fibers are bright red/fuchsia arcs between the vascular bundles. (b) *35Spr:MYB75^{WT}* lines displayed relatively normal stem tissue organization, with a few large cells resembling vessel elements at the periphery of the interfascicular arc. (c) In *35Spr:MYB75^{T131A}* plants, large isolated cells or short cell files, resembling vessels can be seen in the interfascicular region. (d) In *35Spr:MYB75^{T131E}* plants prominent clusters of vessels could be seen in the interfascicular region.

(Figure 4a). To better distinguish this normal secondary growth, present in all genotypes, from what we believed to be ectopic vessel formation in *MYB75* overexpressing lines, we performed a second round of sectioning using younger plants, (7-weeks-old), in hopes of capturing ectopic vessel development before the onset of secondary growth. In addition to phloroglucinol-HCl staining, we used toluidine blue stain, which offers a better color contrast between vessels and fibers. In toluidine blue-stained sections, inter fascicular fiber walls appear bright turquoise, whereas the xylem vessels are greenish gray.

Histochemical analysis of stems from 7-week-old plants indicates that at this stage in development Col-0 WT plants do not show significant signs of secondary growth (Figure 5a) Furthermore, ectopic vessel differentiation was not observed in *35Spr:MYB75^{WT}* plants (Figure 5a,b), and only a mild phenotype was seen in *35Spr:MYB75^{T131A}* plants (Figure 5a). On the other hand, *35Spr:MYB75^{T131E}* plants showed prominent ectopic vessels developing as bundles or cell files in the interfascicular arcs (Figure 5a,b). In toluidine blue-stained cross-sections, the vascular bundles appear greenish gray, in contrast to the bright blue-green inter fascicular fibers (Figure 5b). Cell

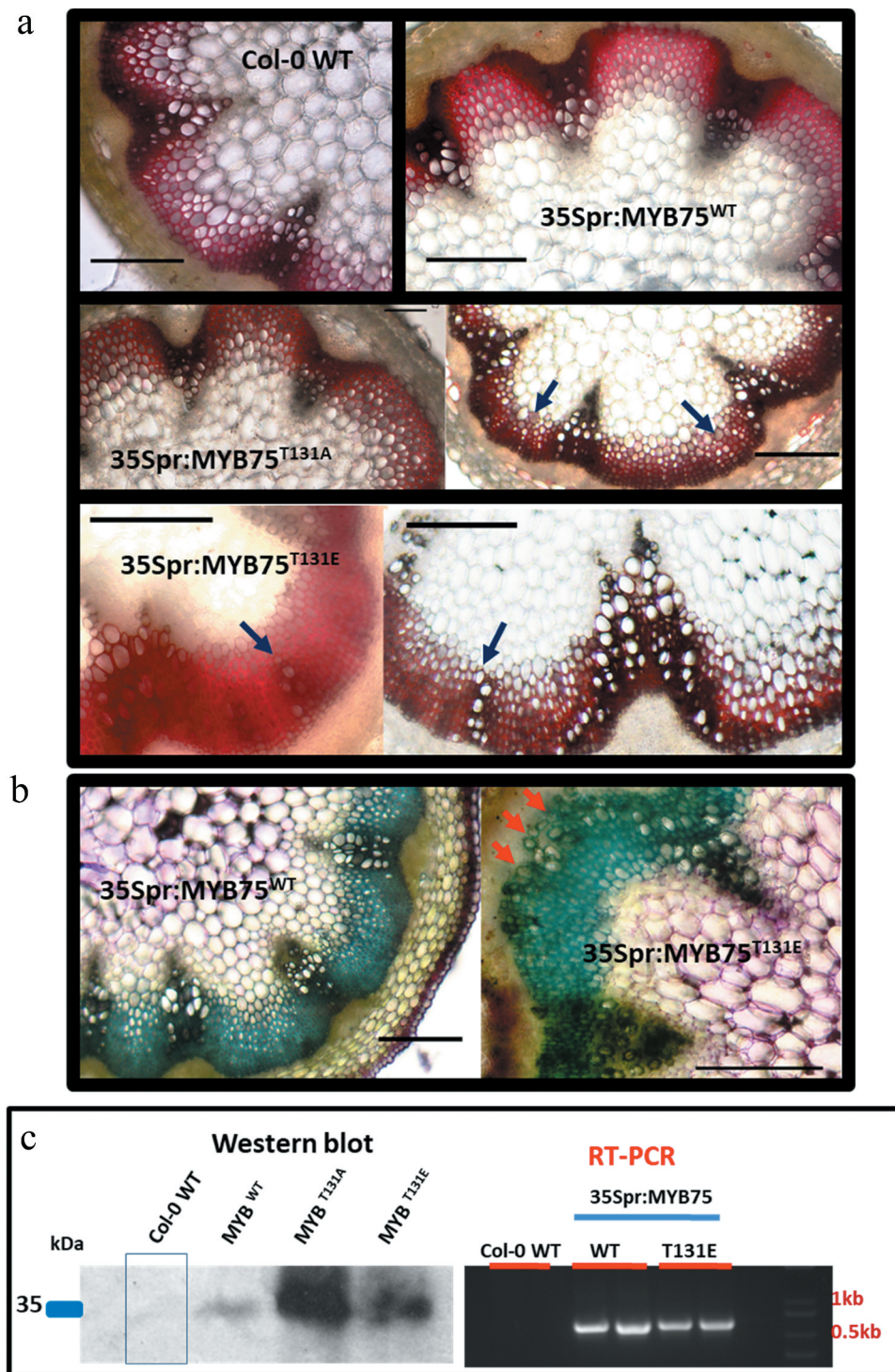


Figure 5. Ectopic vessel development in stems of 7-week-old *Arabidopsis* plants over-expressing different phosphovariants of MYB75. Stems were harvested from 7-week-old plants and the bottom 5 cm of each stem was hand sectioned and stained in phloroglucinol-HCl or toluidine blue. Neither (a), Col-0 WT nor 35Spr:MYB75^{WT} plants showed any signs of ectopic vessels in stem sections at this stage, while 35Spr:MYB75^{T131A} plants displayed a mild phenotype, with isolated cells or small clusters of cells resembling vessel elements in the interfascicular arc. Conversely 35Spr:MYB75^{T131E} display prominent cell files of vessels in the interfascicular region. (b) The presence of vessel elements in the interfascicular region of 35Spr:MYB75^{T131E} plants was confirmed using toluidine blue stain; prominent clusters of vessel elements appear as large olive green staining cells in the interfascicular arc of 35Spr:MYB75^{T131E} plants, but not observed in 35Spr:MYB75^{WT} lines. (c) Western blot and gene expression analysis confirms that recombinant MYB75 is expressed in the stems at this stage.

clusters with greenish gray walls can be seen in the interfascicular arcs of 35Spr:MYB75^{T131E} stems, but not in the 35Spr:MYB75^{WT} plants (Figure 5b). As in the phloroglucinol-stained sections, these unusual cells have a larger diameter than the surrounding interfascicular fiber cells and are similar in

appearance to vessels found in the vascular bundles. We therefore concluded that these are, in fact, ectopic vessel elements that develop in the interfascicular region of MYB75 over-expressing transgenic plants, most prominently in 35Spr:MYB75^{T131E} lines. Due to the limited availability of

heterozygous T2 plants, about a dozen plants were used for each genotype in each round of sectioning. Nevertheless, within this sample pool *35Spr:MYB75^{T131E}* plants consistently showed more prominent ectopic vessel development than the other genotypes. Western blot analysis and RT-PCR confirmed that the transgene was being expressed and that the corresponding recombinant protein was being produced in the stems of *35Spr:MYB75* plants (Figure 5c).

Discussion

Although flavonoids and anthocyanins are considered 'secondary' metabolites in plants, typically associated with stress response, emerging research suggests that these compounds have a profound impact on development. Anthocyanins and some flavonoids are sunscreens, as such their levels and composition in the epidermis can influence how much visible light reaches the photosynthetic machinery, which can ultimately affect light harvesting, photosynthesis and photomorphogenesis.²³ Flavonols also have an established role in development, due to their ability to modulate auxin flow,²⁴ and thereby influence global gene expression and organ differentiation. The influence of flavonols on development is well exemplified in the analysis of *transparent testa* mutants, which are deficient in different flavonoid biosynthesis enzymes and regulatory proteins. These mutants display a range of phenotypic changes in root and shoot architecture, including changes in the number, position and initiation site of root hairs, and lateral roots, inflorescence height and number, and changes in silique density.²⁵ Experiments employing DBPA (diphenylboric acid 2-aminoethylester) staining unveiled cell-specific distribution patterns of quercetin and kaempferol derivatives concentrated in transition zones and actively growing tissues, of *Arabidopsis* seedlings and mature plants.^{26,27} Flavonols, including quercetin, kaempferol and their glycoside derivatives, can negatively impact auxin flow^{24,26} by directly binding to auxin ABCB1 and ABCB19 transporters.^{18,28} Additionally, flavonols can influence the localization of PIN efflux channels, which mediate directional auxin transport, and drive auxin canalization required for vascular differentiation.²⁹ Crucially, changes in spatial-temporal distribution of specific flavonol molecular species correlated with alterations in auxin canalization and direction of auxin flow,²⁶ a primary driving mechanism for development and organogenesis.

Two transcriptional regulators and interacting partners of MYB75, Teosinte branched 1 cycloidea and PCF transcription factor 3 (TCP3, AT1G53230) and Squamosa Promoter binding protein-like 9 (SPL9, AT2G42200), have an established role in developmental processes connected to flavonoid metabolism and the hormone auxin. TCP3 positively regulates complex formation between MYB75, TT8/GL3/EGL3 and TTTG1, leading to increased anthocyanin biosynthesis, and can also bind MYB11 (AT3G62610), MYB12 (AT2G47460) and MYB111 (AT5G49330), enhancing their ability to drive transcription of early flavonoid biosynthesis genes.^{30,31} In *Arabidopsis* plants over-expressing TCP3, increased levels of flavonols coincide with inhibition of auxin-response as well as reduced abundance and altered localization of PIN1 auxin efflux channels.³¹ TCP3-overexpressing plants showed widespread developmental abnormalities such as changes in seed color

and size, high seedling mortality, altered vessel development, organ shape, root growth, reduced apical dominance and more.³¹ Crucially, these phenotypes were abolished when TCP3-overexpressors were crossed with *tt4* mutants, lacking a functional chalcone synthase enzyme, essential for flavonoid biosynthesis, suggesting flavonoid overaccumulation was the underpinning mechanism of these phenotypes.³¹ In contrast to TCP3, SPL9 interferes with MYB75-bHLH-TTG1 complex formation, and therefore negatively impacts anthocyanin production.²⁰ SPL9 over-expression promotes flavonol and inhibits anthocyanin accumulation: in this manner spatial-temporal distribution of SPL9 modulates the proportion of anthocyanins to flavonols in developing stems. The relative proportions of these classes of compounds in *Arabidopsis* stems can affect certain aspects of development, including time of floral transition, in which SPL proteins have been implicated.^{20,32}

Neither *myb75⁻* null mutant³³ nor the PAP-1D overexpressor³⁴ have ever been reported to display any developmental phenotype analogous to the phosphovariant mutants we have analyzed. The unique aspect of our findings is that the expression of either the *MYB75^{T131A}* or *MYB75^{T131E}* phosphovariant appears to have greater impact on development than the absence of MYB75 (*myb75⁻*) or the overexpression of wild type protein. It remains to be seen whether MYB75 is influencing these various developmental processes directly, or more indirectly, through control of spatial-temporal distribution of specific flavonol biochemical species. Nevertheless, the phenotypes we described above clearly demonstrate that this protein, and its phosphorylation status, play a vital role in development.

Germination

In this study we examined germination rate, in *MYB75pr:MYB75* plants, as this was an easily quantifiable phenotype; however, the high seedling mortality and developmental arrest among these lines point to developmental perturbations beyond simply failure to germinate.

According to gene-expression data in the eFP Browser, *Arabidopsis* plants display the highest levels of MYB75 expression in senescing leaves.^{35,36} On the other hand, a recent analysis of the *Arabidopsis* embryonic transcriptome found that MYB75 expression levels spike at the early torpedo and bent cotyledon stages.³⁷ An earlier study, which employed laser capture microdissection to analyze tissue-specific gene expression in the *Arabidopsis* embryos, revealed a detailed picture of the MYB75 expression pattern: at the heart stage, MYB75 expression levels were elevated in cotyledons, compared to other organs, while at the torpedo stage MYB75 expression was concentrated in the root apical meristem.³⁸ These highly specific spatial-temporal distribution patterns suggest that MYB75 plays a role in embryonic development, and that the low germination rate and high seedling mortality observed in *MYB75pr:MYB75cDNA* lines (T131A and T131E) could be a result of altered MYB75 function or protein distribution.

MYB75 (along with MYB123/TTG2) participates in flavonoid biosynthesis during embryogenesis, where proanthocyanidins are the end product.³⁹ Proanthocyanidins (PA's) accumulate in different parts of the developing seed coat,

including the micropyle, chalaza and the endothelium (an inner layer of cells in the seed coat, enclosing the developing embryo).⁴⁰ Recent research indicates that seed coat development can influence cellularization of the endosperm, which has a direct impact on embryogenesis.⁴¹ The authors suggest that part of this mechanism involves modulation of auxin fluxes in the endosperm by flavonoid accumulation in the endothelium.⁴¹ Although the impact of flavonols on auxin transport is typically not lethal in adult plants, at early stages of development small perturbations in auxin flow can have catastrophic effects.⁴² Since auxin plays a central role in embryogenesis and seedling development, it is possible that perturbations in auxin flow, driven by changes in flavonoid profiles, led to developmental abnormalities in *MYB75* phosphomutant lines, and in some cases arrest at these early developmental stages. Developmental arrest and death at early seedling stages, as well as developmental abnormalities, including fused cotyledons, wavy leaves and irregular vasculature, have been documented in *Arabidopsis* plants overexpressing *TCP3*, and linked to altered flavonol production and auxin flow.³¹ A great deal more research is needed in order to explain our findings, such as determining how each phosphovariant version of *MYB75* protein is distributed in the embryo. It would be important to assess impact of *MYB75* phosphorylation on proanthocyanidin production in the seed coat, and on the expression of downstream genes, associated with these early stages of development. Nevertheless, our finding showing that *MYB75* phosphorylation status has an impact on germination is congruent with previous research which suggests that flavonoids play an important role in early plant development.

Flowering time

The transition from vegetative to flowering stage is a complex process, driven by a vast regulatory network that integrates signals from multiple plant hormones and environmental cues such as light, photoperiod, temperature, and the circadian clock. In order to interpret how each *MYB75* phosphovariant might be affecting this process, we can look at the parallels between our observations and phenotypes described in certain *transparent testa* mutants, deficient in various enzymes and regulatory proteins required for flavonoid biosynthesis. Inflorescence elongation rates were faster in mutants which accumulate flavonols in the root-shoot junction, including *transparent testa 8* (*tt8*, AT4G09820) *transparent testa glabra 1* (*ttg1*, AT5G24520) and *transparent testa glabra 2* (*ttg2*, AT2G37260), and slower in those that did not, namely *transparent testa 4* (*tt4*, AT5G13930), *transparent testa 6* (*tt6*, AT3G51240) and *transparent testa 7* (*tt7*, AT5G07990).²⁶ Lesions in the *tt6* and *tt7* genes resulted in an early bolting phenotype.²⁶

Changes in bolting time among *transparent testa* mutants would suggest that the acceleration and delay in bolting times observed in *MYB75^{T131A}* and *MYB75^{T131E}*-overexpressing plants, respectively, are likely to be at least in part caused by changes in distribution of flavonoid molecular species throughout the plant, at these stages of development. However, besides bolting time, *MYB75* phosphomutant-overexpressing plants displayed changes in developmental timing across all stages of the

mature plant development, including flowering time, and onset of senescence, which suggest more profound systemic changes in the plant's responses to developmental cues. Delayed senescence in *35Spr:MYB75^{T131E}* lines is congruent with our previous findings showing that the expression of photosynthesis-related genes is increased in these plants.¹ Upregulation of these photosynthesis genes is typically associated with photomorphogenesis, and active chloroplast development in green tissues. Conversely these genes are down-regulated during senescence, therefore their upregulation in *35Spr:MYB75^{T131E}* plants, implicates this phosphovariant in suppression of senescence.

Overexpression of *MYB75^{T131E}* was earlier shown to influence a much wider spectrum of genes than *MYB75^{WT}* or *MYB75^{T131A}*, including those genes that can impact global gene expression changes.¹ For instance, Histones H3.1 and H2A.11/H2A.Z, upregulated in *35Spr:MYB75^{T131E}* plants, and *ATNAC3* (*OSR1/ATNAC3*, AT3G29035), a global regulator of senescence was downregulated in these lines.¹ Decreased expression of the *NAC3* (*OSR1*) transcription factor could account for the increased expression of photosynthetic genes exclusively observed in *35Spr:MYB75^{T131E}* plants, since an inverse correlation has been observed between *NAC3* expression levels and chlorophyll content,⁴³ which is dependent on expression of photosynthesis- and chloroplast-associated genes.

Vascular development

As a suppressor of biosynthesis of lignin and other secondary cell wall components, *MYB75* has an established role in vascular development.^{15,16} Since polar auxin flow and canalization can be influenced by flavonols, *MYB75* may also play a role in vessel development through control of flavonoid metabolism. To interpret our observations, we look to similar phenotypes reported in the literature. The *pin1pin2* double mutants (defective in auxin efflux channels), as well as plants treated with the auxin transport inhibitor, NPA, display disorganized vessel positioning, with numerous xylem vessels differentiating in the interfascicular regions, outside their normal vascular bundles.⁴⁴ Canalization and drainage of auxin is an essential part of vascular differentiation and continuity. The absence or blockage of auxin efflux carriers results in failure to canalize auxin, leading to spilling of auxin into neighboring cells, which can result in their reprogramming and differentiation into vessel elements. Therefore, the phenotypes that we observed can potentially be a result of reduced polar auxin flow, driven by increased intracellular flavonol levels. All *35Spr:MYB75* plants had elevated flavonol levels compared to Col-0 WT plants,¹ and these lines also showed some degree of ectopic vessel formation. Ectopic vessel formation was most pronounced, and apparent in earlier stages of development, only in *35Spr:MYB75^{T131E}* lines, possibly because expression of this phosphovariant has a greater positive impact on flavonol production.¹

Conclusions

Over the last few decades, our growing understanding of how plants perceive and respond to their environment has been

steadily eroding the boundary that distinguishes primary from secondary metabolism, as we uncover the crucial role many secondary metabolites play in development. The relationship between MYB75, development and the hormone auxin is intuitive, yet not well defined. However, our observations are not without precedent since mutations in other transcriptional regulators of flavonoid metabolism (*tt8*, *ttg1* and *ttg2*) also lead to developmental changes,²⁶ with phenotypes that share features with our *35Spr:MYB75* lines. Spatial-temporal distribution of MYB75 in its various states of phosphorylation is another key question that needs to be addressed, since different phosphorylation states can affect MYB75 protein stability,^{1,2} and as a result may influence protein distribution. Additional experiments are needed to understand how different phospho-variants of MYB75 are distributed across different tissues and organs throughout development, and how this affects localization of specific flavonoid biochemical species, and in turn auxin canalization and development.

We cannot exclude the possibility that MYB75 has additional transcriptional targets that have not been characterized in the literature, and that the developmental phenotypes we observed result from a combination of changes in auxin transport and differential regulation of gene expression by each phosphovariant. For this reason, it would be important carry out more in-depth studies of global gene expression patterns in these phosphovariant lines across developmental time. Such an undertaking would allow us to look for relationships between genes whose expression was affected differently in each phosphovariant, and the phenotypes we describe here. Additional work is required to fully understand the nature and underlying mechanisms of the developmental perturbations observed in this study. One possible approach is crossing *Arabidopsis* plants expressing different MYB75 phospho-variants to plants expressing cell-specific reporters, such as enhancer trap lines expressing Green Fluorescent Protein (GFP) in developing vascular tissues, which would enable visualization of vascular perturbations from embryonic to mature stages of plant development.⁴⁵

The relationship between MYB75 and photosynthetic gene expression described in our previous work¹ is particularly interesting, since MYB75 is directly connected to light-driven signals through transcriptional regulation by HY5, and MYB75 protein turnover mediated by COP/SPA complexes in the dark.^{46–48} The regulation of anthocyanin production through MYB75 is an important ‘dimmer switch’ that dictates how much light reaches the photosynthetic machinery. A recent study depicting MYB75 phosphorylation by MPK4, showed that far-red light was responsible for MPK3, MPK4 and MPK6 activation,² implicating these MPKs in light signal transduction and photomorphogenesis.⁴⁹ These observations reinforce the idea that MYB75, being a downstream target of these MPKs could be implicated in light signal transduction.

Understanding the interplay between secondary metabolism and development is of vital importance since crops and medicinal plants are often bred for characteristics (such as color, flavor, as well as nutritional and medicinal value) conveyed by what we consider to be secondary metabolites. High disease susceptibility and low environmental resilience among economically important crops have often been the outcome of irresponsible

breeding, aimed at enhancing a single characteristic that is of commercial interest. A better understanding of the relationships between various biochemical and developmental pathways can yield new perspectives that help inform crop breeding programs and eventually lead to more sustainable agricultural practices.

ORCID

Anna E. Kreynes  <http://orcid.org/0000-0002-3521-9032>

References

1. Kreynes AE, Yong Z, Xiao-Min L, Wong DCJ, Castellarin SD, Ellis BE. Biological impacts of phosphomimic AtMYB75. *Planta* 2020;251(60). PMID:32030477. doi:10.1007/s00425-020-03350-0.
2. Li S, Wang W, Gao J, Yin K, Wang R, Wang C, Petersen M, Mundy J, Qiu J-L. MYB75 phosphorylation by MPK4 is required for light-induced anthocyanin accumulation in *Arabidopsis*. *Plant Cell* 2016;28(11):2866–2883. PMID:27811015. doi:10.1105/tpc.16.00130.
3. Chang C-S J, Li Y-H, Chen L-T, Chen W-C, Hsieh W-P, Shin J, Jane W-N, Chou S-J, Hu J-M, Somerville S, et al. LZFI, a HY5-regulated transcriptional factor, functions in *Arabidopsis* de-etiolation. *Plant J* 2008;54(2):205–219. PMID: 18182030. doi:10.1111/j.1365-313X.2008.03401.x.
4. Cluis CP, Mouchel CF, Hardtke CS. The *Arabidopsis* transcription factor HY5 integrates light and hormone signalling pathways. *Plant J* 2004;38:332–347. PMID:15078335. doi:10.1111/j.1365-313X.2004.02052.x.
5. Gangappa SN, Botto JF. The multifaceted role of HY5 in growth and development. *Mol Plant* 2016;9(10):1353–1369. PMID:27435853. doi:10.1016/j.molp.2016.07.002.
6. Baudry A, Heim MA, Dubreucq B, Caboche M, Weissshar B, Lepiniec L. TT2, TT8 and TTG1 synergistically specify the expression of *BANYULS* and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. *Plant J* 2004;39(3):366–380. PMID: 15255866. doi:10.1111/j.1365-313X.2004.02138.x.
7. Gonzalez A, Zhao M, Leavitt JM, Lloyd AM. Regulation of the anthocyanin biosynthesis pathway by the TTG1/bHLH/MYB transcriptional complex in *Arabidopsis* seedlings. *Plant J* 2008;53(5):814–827. PMID:18036197. doi:10.1111/j.1365-313X.2007.03373.x.
8. Zimmermann MI, Heim MA, Weisshaar B, Uhrig JF. Comprehensive identification of *Arabidopsis thaliana* MYB transcription factors interacting with R/Blike bHLH proteins. *Plant J* 2004;40(1):22–34. PMID:15361138. doi:10.1111/j.1365-313X.2004.02183.x.
9. Bernhardt C, Zhao M, Gonzalez A, Lloyd A, Schiefelbein J. The bHLH genes GL3 and EGL3 participate in an intercellular regulatory circuit that controls cell patterning in the *Arabidopsis* root epidermis. *Development* 2005;132(2):291–298. PMID:15590742. doi:10.1242/dev.01565.
10. Schellmann S, Hulskamp M. Epidermal differentiation: trichomes in *Arabidopsis* as a model system. *Int J of Dev Biol* 2005;49(5–6):579–584. PMID:16096966. doi:10.1387/ijdb.051983ss.
11. Serna L. Epidermal cell patterning and differentiation throughout apical-basal axis of the seedling. *J of Exp Bot* 2005;56(418):1983–1989. PMID:15967776. doi:10.1093/jxb/eri213.
12. Serna L, Martin C. Trichomes: different regulatory networks lead to convergent structures. *Trends Plant Sci* 2006;11(6):274–280. PMID:16697247. doi:10.1016/j.tplants.2006.04.008.
13. Song SK, Ryu KH, Kang YH, Song JH, Choo YH, Schiefelbein J, Lee MM. Cell fate in *Arabidopsis* root epidermis is determined by competition between WEREWOLF and CAPRICE. *Plant Physiol* 2011;157(3):1196–1208. PMID:21914815. doi:10.1104/pp.111.185785.
14. Ramsay NA, Glover BJ. MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. *Trends Plant Sci* 2005;10(2):63–70. PMID:15708343. doi:10.1016/j.tplants.2004.12.011.

15. Bhargava A, Mansfield SD, Hall HC, Douglas CJ, Ellis BE. MYB75 functions in the regulation of secondary cell wall formation in *Arabidopsis* inflorescence stem. *Plant Physiol* 2010;154(3):1428–1438. PMID:20807862. doi:10.1104/pp.110.162735.
16. Bhargava A, Ahad A, Wang S, Mansfield D, Haughn GW, Douglas CJ, Ellis BE. The interacting MYB75 and KNAT7 transcription factors modulate secondary cells wall deposition both in stems and seed coat of *Arabidopsis*. *Planta* 2013;237(5):1199–1211. PMID:23328896. doi:10.1007/s00425-012-1821-9.
17. Mehrtens F, Kranz H, Bednarek P, The WB. *Arabidopsis* transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid. *Plant Physiol* 2005;138:1083–1096. PMID:15923334. doi:10.1104/pp.104.058032.
18. Bouchard R, Bailly A, Blakeslee JJ, Oehring SC, Vincenzetti V, Lee OR, Paponov I, Palme K, Mancuso S, Murphy AS, et al. Immunophilin-like TWISTED DWARF1 modulates auxin efflux activities of *Arabidopsis* P-glycoproteins. *JBC* 2006;281(41):30603–30612. PMID:16887800. doi:10.1074/jbc.M604604200.
19. Li E, Bhargava A, Qiang W, Friedmann MC, Forniers N, Savidge RA, Johnson LA, Mansfield SD, Ellis BE, Douglas CJ. The Class II KNOX gene KNAT7 negatively regulates secondary wall formation in *Arabidopsis* and is functionally conserved in *Populus*. *New Phytol* 2012;194(1):102–115. PMID:22236040. doi:10.1111/j.1469-8137.2011.04016.x.
20. Gou JY, Felippes FF, Liu CJ, Weigel D, Wang JW. Negative regulation of anthocyanin biosynthesis in *Arabidopsis* by a miR156-targeted SPL transcription factor. *Plant Cell* 2011;23:1512–1522. PMID:21487097. doi:10.1105/tpc.111.084525.
21. Altamura MM, Possenti M, Matteucci A, Baima S, Ruberti I, Morelli G. Development of the vascular system in the inflorescence stem of *Arabidopsis*. *New Phytol*. 2001;151:381–389. doi:10.1046/j.0028-646x.2001.00188.x.
22. Ragni L, Greb T. Secondary growth as a determinant for plant shape and form. *Seminars in Cell and Dev Biol* 2018;79:58–67. PMID:28864343. doi:10.1016/j.semcd.2017.08.050.
23. Steyn WJ, Wand SJE, Holcroft DM, Jacobs G. Anthocyanin in vegetative tissues: a proposed unified function in photoprotection. *New Phytol*. 2002;155:349–361. doi:10.1046/j.1469-8137.2002.00482.x.
24. Brown DE, Rashotte AM, Murphy AS, Normanly J, Tague BW, Peer WA, Taiz L, Munday GL. Flavonoids act as negative regulators of auxin transport in vivo in *Arabidopsis*. *Plant Physiol* 2001;126(2):524–535. PMID:11402184. doi:10.1104/pp.126.2.524.
25. Buer CS, Djordjevic MA. Architectural phenotypes in the transparent testa mutants of *Arabidopsis thaliana*. *J Exp Bot* 2009;60(3):751–763. PMID:19129166. doi:10.1093/jxb/ern323.
26. Buer CS, Kordbacheh F, Truong TT, Hocart CH, Djordjevic MA. Alterations of flavonoid accumulation patterns in transparent testa mutants disturbs auxin transport, gravity responses, and imparts long-term effects on root and shoot architecture. *Planta* 2013;238(1):171–189. PMID: 23624937. doi:10.1007/s00425-013-1883-3.
27. Peer WA, Brown DE, Tague BE, Munday GK, Taiz L, Murphy AS. Flavonoid accumulation pattern in transparent testa mutants of *Arabidopsis*. *Plant Phys* 2001;126(2):536–548. PMID:11402185. doi:10.1104/pp.126.2.536.
28. Bailly A, Sovero V, Vincenzetti V, Santelia D, Bartnik D, Koenig BW, Mancuso S, Martinoia E, Geisler M. Modulation of P-glycoproteins by auxin transport inhibitors is mediated by interaction with immunophilins. *JBC* 2008;283(31):21817–21826. PMID:18499676. doi:10.1074/jbc.M709655200.
29. Michniewicz M, Zago MK, Abas L, Weijers D, Schweighofer A, Meskiene I, Heisler MG, Ohno C, Zhang J, Huang F, et al. Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. *Cell* 2007;130(6):1044–1056. PMID:17889649. doi:10.1016/j.cell.2007.07.033.
30. Stracke R, Ishihara H, Hup G, Mehrtens F, Niehaus K, Weisshaar B. Differential regulation of closely related R2R3-MYB transcription factors control flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. *Plant J* 2007;50(4):660–677. PMID:17419845. doi:10.1111/j.1365-313X.2007.03078.x.
31. Li S, Zachgo S. TCP3 interacts with R2R3-MYB proteins, promotes flavonoid biosynthesis and negatively regulates the auxin response in *Arabidopsis thaliana*. *Plant J* 2013;76:901–913. PMID:24118612. doi:10.1111/tpj.12348.
32. Wang J-W, Czech B, Weigel D. miR156-regulated SPL transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. *Cell* 2009;138(4):738–749. PMID: 19703399. doi:10.1016/j.cell.2009.06.014.
33. Teng S, Keurentjes J, Bentsink L, Koornneef M, Smeekens S. Sucrose-specific induction of anthocyanin biosynthesis in *Arabidopsis* requires MYB75/PAP1 gene. *Plant Physiol* 2005;139:1840–1852. PMID:16299184. doi:10.1104/pp.105.066688.
34. Borevitz JO, Xia Y, Blount J, Dixon RA, Lamb C. Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *Plant Cell* 2000;12(12):2383–2393. PMID:11148285. doi:10.1105/tpc.12.12.2383.
35. Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Schölkopf B, Weigel D, Lohmann JU. A gene expression map of *Arabidopsis thaliana* development. *Nat Genet* 2005;37(5):501–506. PMID: 15806101. doi:10.1038/ng1543.
36. Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ. An “Electronic Fluorescent Pictograph” browser for exploring and analyzing large-scale biological data sets. *PLOS One* 2007;2(8):e718. PMID:17684564. doi:10.1371/journal.pone.0000718.
37. Hoffmann F, Schon MA, Nodine MD. The embryonic transcriptome of *Arabidopsis thaliana*. *Plant Reprod* 2019;32:77–91. PMID:30610360. doi:10.1007/s00497-018-00357-2.
38. Casson S, Spencer M, Walker K, Lindsey K. Laser capture microdissection for the analysis of gene expression during embryogenesis of *Arabidopsis*. *Plant J* 2005;42(1):111–123. PMID: 15773857. doi:10.1111/j.1365-313X.2005.02355.x.
39. Sharma SB, Dixon RA. Metabolic engineering of proanthocyanidins by ectopic expression of transcription factors in *Arabidopsis thaliana*. *Plant J* 2005;44(1):62–75. PMID:16167896. doi:10.1111/j.1365-313X.2005.02510.x.
40. Xu W, Dubos C, Lepiniec L. Transcriptional control of flavonoid biosynthesis by MYB-bHLH-WDR complexes. *Trends Plant Sci* 2015;20(3):176–185. PMID: 25577424. doi:10.1016/j.tplants.2014.12.001.
41. Doughty J, Aljabri M, Scott RJ. Flavonoids and the regulation of seed size in *Arabidopsis*. *Biochem Soc Trans* 2014;42(2):364–369. PMID:24646245. doi:10.1042/BST20140040.
42. Möller B, Weijers D. Auxin control of embryo patterning. *Cold Spring Harb Perspect Biol* 2009;1(5):a001545. PMID:20066117. doi:10.1101/cshperspect.a001545.
43. Balazadeh S, Kwasiński M, Caldana C, Mehrnia M, Zano MI, Xue GP, Mueller-Roeber B. ORS1, an H2O2-responsive NAC transcription factor, controls senescence in *Arabidopsis thaliana*. *Mol Plant* 2011;4(2):346–360. PMID:21303842. doi:10.1093/mp/ssq080.
44. Ibañez M, Fabregas N, Chory J, Caño-Delgado AI. Brassinosteroid signalling and auxin transport are required to establish the periodic pattern of *Arabidopsis* shoot vascular bundles. *PNAS* 2009;106(32):13630–13635. PMID:19666540. doi:10.1073/pnas.0906416106.
45. Kukurshumova W, Koizumi K, Chatfield SP, Sanchez-Buelna SU, Gangaeva AE, McKenzie R, Berleth T. Tissue-specific GAL4 expression patterns as a resource enabling targeted gene expression, cell type-specific transcript profiling and gene function characterization in the *Arabidopsis* vascular system. *Plant Cell Physiol* 2009;50(1):141–150. PMID:19068493. doi:10.1093/pcp/pcn180.
46. Osterlund MT, Wei N, Deng XW. The roles of photoreceptor systems and the COP1-targeted destabilization of HY5 in light control of *Arabidopsis* seedling development. *Plant Physiol* 2000;124(4):1520–1524. PMID:11115869. doi:10.1104/pp.124.4.1520.
47. Saijo Y, Sullivan JA, Wang H, Yang J, Shen Y, Rubio V, Ma L, Hoecker U, Deng XW. The COP1-SPA1 interaction defines a critical step in phytochrome A-mediated regulation of HY5

- activity. *Genes Dev* 2003;17(21):2642–2647. PMID:14597662. doi:10.1101/gad.1122903.
48. Maier A, Schrader A, Kokkelink L, Flake C, Welter B, Iniesto E, Rubio V, Uhrig JF, Hülskamp M, Hoeker U. Light and the E3 ubiquitin ligase COP1/SPA control the protein stability of MYB transcription factors PAP1 and PAP2 involved in anthocyanin accumulation in *Arabidopsis*. *Plant J* 2013;74(4):638–651. PMID:23425305. doi:10.1111/tpj.12153.
49. Arsovski AA, Galstyan A, Guseman JM, Nemhauser JL. Photomorphogenesis. *Arabidopsis Book*. 2012;10:e0147. doi:10.1199/tab.0147.