The Arabidopsis Transcription Factor MYB112 Promotes Anthocyanin Formation during Salinity and under High Light Stress[1][OPEN]

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MYB transcription factors (TFs) are important regulators of flavonoid biosynthesis in plants. Here, we report MYB112 as a formerly unknown regulator of anthocyanin accumulation in Arabidopsis (*Arabidopsis thaliana*). Expression profiling after chemically induced overexpression of *MYB112* identified 28 up- and 28 down-regulated genes 5 h after inducer treatment, including *MYB7* and *MYB32*, which are both induced. In addition, upon extended induction, MYB112 also positively affects the expression of *PRODUCTION OF ANTHOCYANIN PIGMENT1*, a key TF of anthocyanin biosynthesis, but acts negatively toward *MYB12* and *MYB111*, which both control flavonol biosynthesis. MYB112 binds to an 8-bp DNA fragment containing the core sequence (A/T/G)(A/C)CC(A/T)(A/G/T)(A/C)(T/C). By electrophoretic mobility shift assay and chromatin immunoprecipitation coupled to quantitative polymerase chain reaction, we show that MYB112 binds in vitro and in vivo to *MYB7* and *MYB32* promoters, revealing them as direct downstream target genes. We further show that *MYB112* expression is up-regulated by salinity and high light stress, environmental parameters that both require the MYB112 TF for anthocyanin accumulation under these stresses. In contrast to several other MYB TFs affecting anthocyanin biosynthesis, *MYB112* expression is not controlled by nitrogen limitation or an excess of carbon. Thus, MYB112 constitutes a regulator that promotes anthocyanin accumulation under abiotic stress conditions.

Anthocyanins, a subgroup of plant flavonoids, play multiple roles in higher plants: they function as photoprotective pigments in vegetative tissues to absorb UV and high light irradiation, antioxidants that scavenge reactive oxygen species (ROS), visual signals for the attraction of pollinators or seed dispersers, and agents acting against microbes in defense responses (Winkel-Shirley, 2001; Koes et al., 2005; Lepiniec et al., 2006; Pourcel et al., 2007; Mouradov and Spangenberg, 2014). Anthocyanins accumulate in plant tissues in response to different types of abiotic stresses, including osmotic stress, extreme temperature, nitrogen and phosphorous deficiency, excessive light, and salinity (Christie et al., 1994; Dixon and Paiva, 1995; Winkel-Shirley, 2002; Castellarin et al., 2007; Cominelli et al., 2008; Lillo et al., 2008; Feyissa et al., 2009).

The molecular basis of plant anthocyanin biosynthesis is well known (Koes et al., 2005; Falcone Ferreyra et al., 2012). In addition, transcription factors (TFs) regulating the expression of flavonoid biosynthesis genes have been characterized extensively in Arabidopsis (*Arabidopsis thaliana*) and other plants (Broun, 2005; Ramsay and Glover, 2005; Allan et al., 2008; Hichri et al., 2011; Petroni and Tonelli, 2011; Schwinn et al., 2014). Genes encoding the early flavonoid biosynthetic steps, including *CHALCONE SYNTHASE* (*CHS*), *CHALCONE ISOMERASE* (*CHI*), *FLAVANONE 3-HYDROXYLASE* (*F3H*), and *FLAVONOID 3′-HYDROXYLASE* (*F3′H*), as well as the more anthocyanin-specific genes, such as *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*), *ANTHOCYANIDIN SYNTHASE* (*ANS*), and *FLAVONOID 3-O-GLYCOSYL-TRANSFERASE*, are regulated by TFs of the MYB and basic helix-loop-helix (bHLH) families. TFs of the R2R3-MYB subfamily play a central role in regulating both types of genes. In particular, in Arabidopsis,

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the MYB TFs PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1; also known as MYB75), PAP2 (MYB90), MYB113, and MYB114 have been shown to play important roles in activating genes of the anthocyanin biosynthesis pathway (Borevitz et al., 2000; Gonzalez et al., 2008). These TFs interact with bHLH factors, including GLABRA3 (GL3; bHLH001), ENCHANCER OF GLABRA3 (bHLH002), and TRANSPARENT TESTA8 (TT8; bHLH042; Zhang et al., 2003; Baudry et al., 2006; Gonzalez et al., 2008). The regulatory transcription complex also includes the WD40 protein TRANSPARENT TESTA GLABRA1 (TTG1), a homolog of ANTHOCYANIN11 from *Petunia hybrida*, which is also important for anthocyanin production (de Vetten et al., 1997; Walker et al., 1999).

The resulting MYB/bHLH/WD40 TF complex, consisting of at least these three protein members, activates anthocyanin biosynthetic genes, including *ANS*, *DFR*, *F3′H*, *LEUCOANTHOCYANIN DIOXYGENASE*, *ANTHOCYANIN-3*-O-*GLUCOSYL TRANSFERASE* (*UDP-DEPENDENT GLYCOSYL TRANSFERASE78D2* [*UGT78D2*]), and *ANTHOCYANIN-5*-O-*GLUCOSYL TRANSFERASE* (*UGT75C1*; Tohge et al., 2005; Gonzalez et al., 2008). In addition, Arabidopsis PRODUCTION OF FLAVONOL GLYCOSIDE (PFG) factors from subgroup 7 of the R2R3-MYB family, namely MYB11/PFG2, MYB12/PFG1, and MYB111/PFG3, have been reported to control the flavonol branch of the flavonoid biosynthetic pathway by activating *CHS*, *CHI*, *F3H* and *FLAVONOL SYNTHASE* (*FLS*; Mehrtens et al., 2005; Stracke et al., 2007).

In contrast to the positive regulators of anthocyanin biosynthesis, the R3-type single MYB protein MYB-Like2 (MYBL2) functions as a negative regulator (transcriptional repressor) of this biochemical pathway. MYBL2 seems to form a protein complex with TT8 and TTG1 that functions as a transcriptional repressor of anthocyanin biosynthesis (Dubos et al., 2008; Matsui et al., 2008). Another transcriptional repressor of anthocyanin biosynthesis in Arabidopsis is the single-repeat R3 MYB protein CAPRICE (CPC; Zhu et al., 2009).

The accumulation of anthocyanins in response to environmental inputs is, to a large extent, regulated by TFs that affect the expression of genes encoding both early and late biosynthetic reactions in their production. Particularly well studied has been the effect of nutrient (mostly nitrogen) limitation on anthocyanin accumulation. Several early (*CHS*, *F3H*, and *F3′H*) and late (*DFR* and *ANS*) pathway flavonoid/anthocyanin biosynthesis genes are induced upon depletion of nitrogen or phosphorous (Scheible et al., 2004; Misson et al., 2005; Morcuende et al., 2007; Lillo et al., 2008). Furthermore, many genes encoding enzymes known or predicted to be involved in biosynthesis or modification of flavonoids, including various UGTs, were found to be (highly) induced by mineral nutrient depletion (Lillo et al., 2008). Light quantity and quality also strongly affect anthocyanin biosynthesis (Wade et al., 2001; Takos et al., 2006; Cominelli et al., 2008; Albert et al., 2009; Stracke et al., 2010; Azuma et al., 2012). In Arabidopsis, the bHLH TF PHYTOCHROME-INTERACTING TRANSCRIPTION FACTOR3 acts as a positive regulator of anthocyanin biosynthesis (Shin et al., 2007), and the basic Leu zipper TF ELONGATED HYPOCOTYL5, which plays a key role for light-dependent processes, positively regulates the expression of several genes of flavonoid biosynthesis, including *CHS* and *F3H*, as well as the *MYB12/PFG1* TF (Stracke et al., 2010). High light enhances the expression of *PAP1* and *PAP2* and inhibits the expression of *MYBL2* (Dubos et al., 2008). The regulatory genes *PAP1* and *PAP2* are induced by nitrogen and phosphorous depletion (Lillo et al., 2008). PAP1 has been shown to up-regulate the expression of 20 flavonoid biosynthesis genes (Tohge et al., 2005), and expression of almost all of these genes was also elevated by nitrogen and/or phosphorous limitation (for meta-analysis, see Lillo et al., 2008), indicating that PAP TFs play an important role in the mineral nutrition response of flavonoid/anthocyanin pathway genes. Of note, *PAP1* and *PAP2* are also up-regulated by exposure of plants to light (Cominelli et al., 2008). High light induces the expression of *MYB12/PFG1* and *MYB111/PFG3*, key regulators of the flavonol pathway; this induction correlates with the strong influence that high light has on flavonol biosynthesis (Mehrtens et al., 2005). The GL3/bHLH001 TF interacts with PAP1 and PAP2 to activate the expression of the anthocyanin biosynthetic gene *DFR* (Zimmermann et al., 2004). Notably, expression of *GL3* increases upon nitrogen limitation. Given that the nitrogen depletion-triggered accumulation of anthocyanins that is normally seen in wild-type plants was absent in the *gl3* mutant, it was concluded that GL3 is important for the nutrient deprivation response (Feyissa et al., 2009). In addition, the negative regulator CPC was reported to repress anthocyanin accumulation under nitrogen limitation condition. Similarly, CPC represses anthocyanin accumulation in 35S overexpressors grown under osmotic, salinity, or cold stress; however, no difference was observed between wild-type and *cpc-1* mutant plants (fig. 6 in Zhu et al., 2009). Furthermore, LATERAL ORGAN BOUNDARY DOMAIN37 (LBD37), LBD38, and LBD39, members of the LBD TF family, have been shown to negatively regulate nitrogen depletion-induced anthocyanin formation in Arabidopsis, possibly through repression of *PAP1* and *PAP2* (Rubin et al., 2009).

Salinity stress is yet another environmental factor known to trigger anthocyanin accumulation in a number of plant species, including crops and vegetables, such as tomato (*Solanum lycopersicum*), maize (*Zea mays*), sugarcane (*Saccharum officinarum*), and many others (Kaliamoorthy and Rao, 1994; Piao et al., 2001; Wahid and Ghazanfar, 2006; Keutgen and Pawelzik, 2007; Roychoudhury et al., 2008; Matus et al., 2010). Salinity leads to water deficit and ionic stress, resulting in the destabilization of membranes, inhibition of enzymes, and overproduction of ROS (Zhu, 2001; Munns, 2002; Miller et al., 2010), and the accumulation of anthocyanins has been proposed to contribute to plant tolerance against abiotic stresses (Gould et al., 2002;

Nagata et al., 2003; Gould, 2004; Zeng et al., 2010). In contrast to the regulation of anthocyanin biosynthesis under nutrient deprivation, the signaling cascade and involvement of TFs in salt-triggered accumulation of flavonoids/anthocyanins have rarely been investigated so far. For example, constitutive overexpression of the Arabidopsis glycogen synthase kinase3 (GSK3)/shaggy-like protein kinase1 (AtGSK1) in transgenic plants was shown to induce salt stress responses, including the accumulation of anthocyanins, concomitant with a strong increase of *CHS* expression in the absence of stress, indicating that AtGSK1 is involved in a signaling cascade that controls activation of anthocyanin pathway genes upon exposure of plants to salt stress (Piao et al., 2001). Recently, *MYB44*, a member of subgroup 22 of the R2R3-MYB TF family, was shown to be activated by different abiotic stresses, including dehydration, low temperature, and salinity (Jung et al., 2008). After 24 h of salinity stress but not under control condition, the expression of *CHS*, *F3H*, *DFR*, *PAP1*, and *PAP2* was decreased in transgenic plants overexpressing *MYB44* (Jung et al., 2008). In addition, anthocyanin accumulation was less prominent in *35S*:*MYB44* seedlings than in wild-type plants, particularly after jasmonate treatment (Jung et al., 2010). These data indicate a negative role of MYB44 in stress-induced anthocyanin accumulation. However, a direct regulation of flavonoid/anthocyanin pathway genes by MYB44 has not, as yet, been reported.

Here, we report on *MYB112*, showing its role in anthocyanin biosynthesis in Arabidopsis. In contrast to several other MYB TFs regulating genes associated with anthocyanin biosynthesis, *MYB112* expression is controlled by neither nitrogen limitation nor Suc excess, but rather, it is stimulated by salinity and high light stress. We show enhanced and impaired anthocyanin accumulation in *MYB112* overexpressors and *myb112* mutants, respectively. We further determined the binding site of the MYB112 TF by in vitro binding site selection and discovered two other *MYB* genes (i.e. *MYB7* and *MYB32*) as direct downstream targets. Thus, MYB112 does not directly regulate expression of flavonoid biosynthetic genes but seems to function as a formerly unknown modulator of anthocyanin biosynthesis.

RESULTS

Tissue-Specific Expression of *MYB112*

To investigate *MYB112* (At1g48000) expression at the tissue level, an approximately 1.3-kb-long 5′ regulatory region upstream of the ATG was fused to the *GUS* reporter gene, and the resulting construct, Pro_{MYB112}:*GUS*, was transformed into Arabidopsis. *MYB112* promoter-driven GUS activity was tested in five independent transgenic lines of T2 and T3 generations. Representative expression patterns are shown in Figure 1A. *MYB112* expression was observed in most tissues, both in young seedlings and throughout plant development. At approximately 10 d after sowing, GUS activity was detected in cotyledons and the margins of the first true leaves, where hydathodes are localized (Fig. 1A1). Hydathodes enable water conduction (Candela et al., 1999). After 2 weeks, the following rosette leaves were fully expanded and displayed relatively strong GUS activity, whereas GUS staining was restricted to hydathodes in recently emerged leaves (Fig. 1, A2–A4). GUS expression was also detected in lateral roots of young seedlings (Fig. 1A5). In older plants, GUS activity was observed in both leaves and flowers. Young rosette leaves, like young seedling leaves, exhibited gene expression exclusively in hydathodes (Fig. 1, A6 and A9). In mature rosette leaves, GUS staining was also detected at the base of trichomes and around the midvein (Fig. 1, A7 and A10); however, no GUS activity was observed in the midvein itself (Fig. 1, A7 and A11). The strongest GUS signal was observed in old rosette leaves, showing that *MYB112* is a senescence-associated gene (SAG; Fig. 1A8); this finding is in accordance with previous reports that found elevated *MYB112* transcript abundance in senescing leaves compared with nonsenescent leaves as determined by microarray hybridizations and quantitative real-time reverse transcription (qRT)-PCR (Balazadeh et al., 2008; Breeze et al., 2011). In flowers, GUS staining was mainly localized to anthers and pollen (Fig. 1, A13, A16, and A17). Immature floral tissue showed no GUS activity (Fig. 1, A12 and A15), consistent with virtually undetectable *MYB112* expression on microarrays (eFP browser; http://bar.utoronto.ca/efp). GUS expression was also noted at the stigma and bottom end (later fruit abscission zone) of the gynoecium (Fig. 1A14).

MYB112 Is a Nuclear Protein

To test the subcellular localization of MYB112 protein, we expressed it as a fusion to the N terminus of GFP using the Cauliflower Mosaic Virus *35S* (CaMV) promoter. The *35S*:*MYB112-GFP* construct was transformed into Arabidopsis plants, and roots of transgenic seedlings were analyzed. GFP fluorescence was exclusively present in nuclei (Fig. 1B), consistent with the role of MYB112 as a TF.

Identification of *myb112* Mutants

We obtained two *myb112* mutants with transfer DNA (T-DNA) insertions located in the 5′ untranslated region (*myb112-1*; GK093E05) or the 3′ untranslated region (*myb112-2*; Salk098029; Fig. 2A). Expression analysis by qRT-PCR in seedlings of the two mutants revealed decreased *MYB112* transcript abundance in both homozygous mutants, with a stronger reduction of expression in *myb112-1* than in *myb112-2* (Fig. 2B). This result was confirmed by semi-qRT-PCR, where we tested for the presence of full-length *MYB112* transcript (Fig. 2C). The *myb112* mutants germinated uniformly, and early vegetative growth was indistinguishable between wild-type and mutant plants. However, we observed a slightly

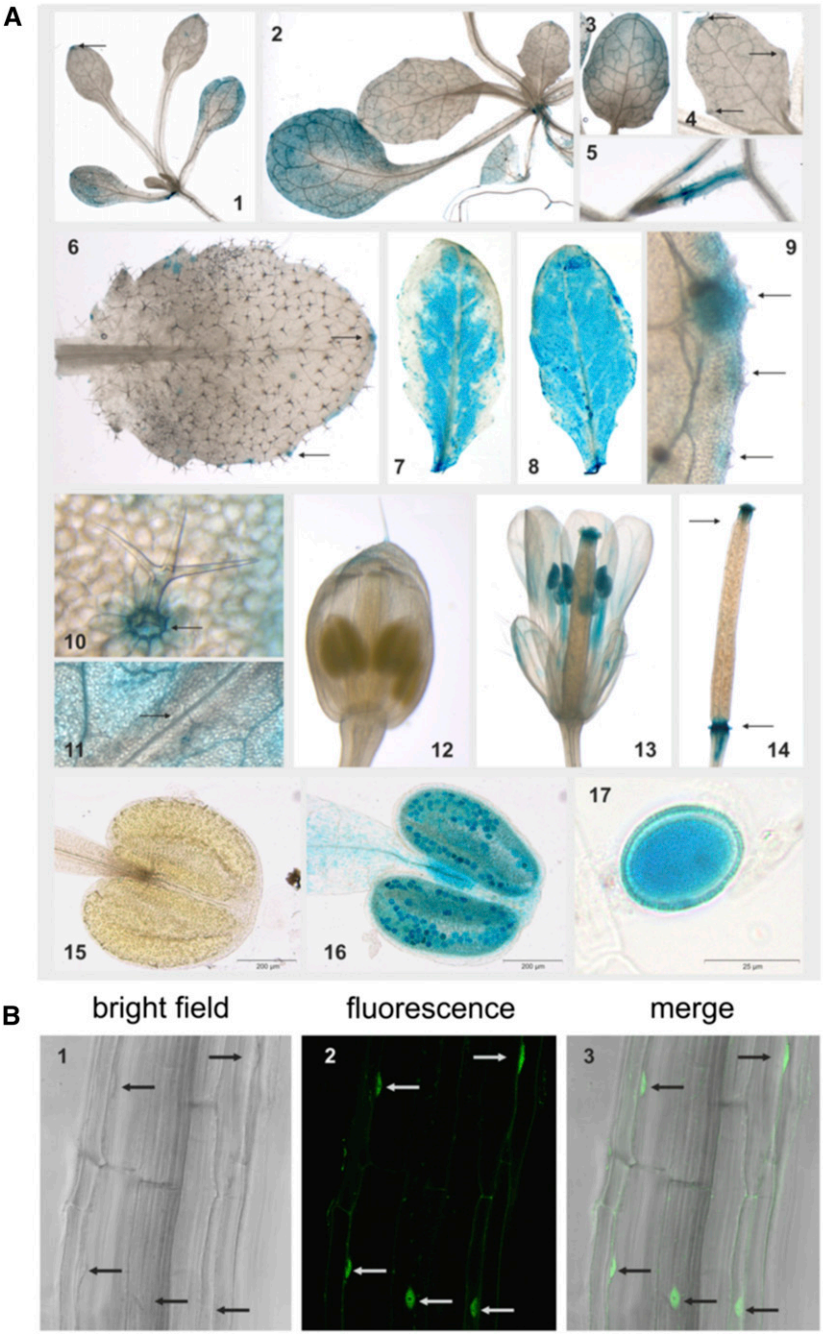

Figure 1. Localization of *MYB112* expression. A, GUS activity in Arabidopsis Pro_{MYB112}:*GUS* seedlings. A1, Ten-day-old seedling. GUS staining is mainly localized to cotyledons. Staining is also visible in the tip and margin regions of leaves numbers 1 and 2 (arrow). A2 to A5, Fourteen-day-old seedlings. A2 and A3, GUS activity in leaves 1 and 2. A4, GUS staining in hydathodes of leaf 6 (arrows). A5, Lateral root. A6 to A14, Three- to six-week-old plants. A6, GUS activity in a young rosette leaf is restricted to hydathodes (arrows). A7, GUS activity in mature rosette leaves is often absent from the leaf edges, with the exception of hydathodes (zoom in A9) and the trichome base (zoom in A10); also, the midvein remains unstained (zoom in A11). A8, Strong GUS staining in a senescent leaf. A12, No detectable GUS expression in a flower bud. A13, GUS activity in a mature flower. A14, GUS staining in stigma and bottom end of gynoecium (arrows). A15, No staining in young anther. A16, Intense GUS staining in mature anther and pollen grains (zoom in A17). Staining was performed for approximately 1 to 2 h. B, Subcellular localization of MYB112-GFP fusion protein in roots of transgenic Arabidopsis. A1, Bright field. A2, GFP fluorescence. A3, Merged. Arrows indicate the presence of MYB112-GFP protein in the nuclei.

later bolting of soil-grown *myb112* mutants compared to wild-type plants under a 12-h/12-h day-night regime, whereas bolting was not seemingly affected in *35S*:*MYB112* overexpressors (not shown).

Estradiol-Inducible Overexpression of *MYB112* Induces Anthocyanin Formation

To analyze the biological role of *MYB112*, we generated estradiol (ESTR)-inducible overexpression (IOE; *MYB112-IOE*) lines using the pER8 vector developed by Zuo et al. (2000) and induced its expression with 10 μM ESTR in seedlings grown in liquid Murashige and Skoog medium (MS) supplemented with 1% (w/v) Suc. As controls, we used dimethyl sulfoxide (DMSO)-treated *MYB112-IOE* lines or ESTR-treated empty vector plants. After 5 d of ESTR treatment, leaves of *MYB112-IOE* seedlings accumulated anthocyanins, whereas mock-treated plants (DMSO; Fig. 3A) as well as ESTR-treated empty vector plants (not shown) did not. At shorter induction times (i.e. 5 h), no anthocyanin accumulation was observed in *MYB112-IOE* lines (not shown), perhaps suggesting that MYB112 is not immediately upstream of

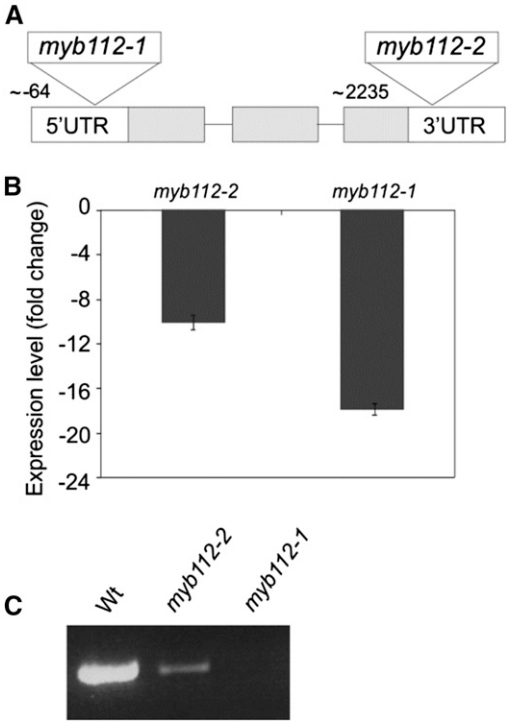

Figure 2. *Myb112* T-DNA insertion mutants. A, Location of T-DNA insertions in the two *myb112* T-DNA insertion mutants. UTR, Untranslated region. B, Fold change decrease in *MYB112* expression in *myb112-1* and *myb112-2* mutant seedlings compared with expression in wild-type (Wt) plants as measured by qRT-PCR. Means ± SD are shown for three biological replicates. C, Decreased level of *MYB112* transcript in *myb112-1* and *myb112-2* mutant seedlings shown by semi-qRT-PCR (26 cycles) with primers annealing to the start and stop regions of the coding segment.

anthocyanin pathway genes. We extracted and measured the anthocyanin level in *MYB112-IOE* seedlings of two lines (IOE1 and IOE2). Interestingly, the two *MYB112-IOE* lines accumulated around 3 times more anthocyanins than the respective mock-treated plants (Fig. 3B). We next analyzed the flavonoid metabolite profiles of *MYB112-IOE* seedlings using liquid chromatography coupled to mass spectrometry (LC-MS). Compared with DMSO-treated plants, ESTR-induced seedlings showed a significant increase in the content of two cyanidin derivatives, namely cyanidin-3-*O*-[2-*O*-(2-*O*-(sinapoyl)-xylosyl)-6-*O*-(*p*-*O*-coumaroyl)-glucoside]-5-*O*-[6-*O*-(malonyl)-glucoside] (A11; Fig. 3C) and cyanidin-3-*O*-[2′′-*O*-(xylosyl)-6′′-*O*-(*p*-*O*-(glucosyl)-*p*-coumaroyl)glucoside]-5-*O*-[6′′′-*O*-(malonyl)-glucoside] (A8; Tohge et al., 2005; Fig. 3D). Other cyanidin derivatives were not reliably detected in the samples. Flavonol glycosides derived from kaempferol (named F1–F3 according to Tohge et al., 2005) were reduced (Fig. 3E), whereas the levels of flavonol glycosides derived from quercetin (named F5 and F6) accumulated in ESTR-treated plants (Fig. 3F) compared with control plants.

We measured the expression of genes encoding enzymes involved in flavonoid biosynthesis as well as regulatory genes in *MYB112-IOE* seedlings treated with ESTR for 5 d (Fig. 3G; Supplemental Data Set S1). Transcript levels of *PHE AMMONIA LYASE* and *CINNAMATE-4-HYDROXYLASE*, genes associated with the pathway of phenylpropanoid biosynthesis, remained unchanged in the ESTR-treated seedlings. *CHS* and *CHI* were slightly down-regulated (approximately 2-fold). Transcript levels of the genes *DFR* and *ANS* (*LEUCOANTHOCYANIDIN DIOXYGENASE*) were significantly increased in seedlings overexpressing *MYB112*. DFR catalyzes the conversion of dihydroquercetin to leucoanthocyanidin, and *ANS* encodes a dioxygenase that operates downstream of DFR and catalyzes the conversion of leucoanthocyanidins to anthocyanidins. In addition to the anthocyanin biosynthetic genes indicated above, several genes proposed to be involved in the production of specific anthocyanin derivatives were up-regulated. These include three glycosyltransferase family genes, namely *UGT79B1* (anthocyanin-3-*O*-glusocide-2′′-*O*-xylosyltransferase [*A3G2′′XT*]; Yonekura-Sakakibara et al., 2012), *UGT84A2* (sinapic acid 1-*O*-glucosyltransferase [*SGT*]), and *UGT75C1* (anthocyanin-5-*O*-glucosyltransferase [*A5GT*]; Tohge et al., 2005), the acyltransferase family gene anthocyanin-5-*O*-glucoside-6′′-*O*-malonyltransferase (*At5MaT*; At3g29590; Luo et al., 2007), and the glutathione *S*-transferase family gene *TT19*. The genes encoding glycosyltransferases and acyltransferases catalyze modification reactions for the formation of the most extensively modified A11 anthocyanin (Tohge et al., 2005). TT19 is required for vacuolar sequestration of anthocyanins (Kitamura et al., 2004). The transcript level of *FLS*, which catalyzes the synthesis of the flavonols quercetin and kaempferol from dihydroquercetin and dihydrokaempferol, respectively, was down-regulated in the ESTR-treated *MYB112-IOE* seedlings. Apart from biosynthetic genes, a set of regulatory genes also displayed altered expression in *MYB112*-overexpressing plants. Expression of *PAP1* as well as *MYB114* was increased in ESTR-treated *MYB112-IOE* plants. It should be mentioned, however, that the *MYB114* gene from the Columbia-0 (Col-0) accession encodes a protein that lacks a transcriptional activation domain because of a stop codon just after the Myb domain (at amino acid 140). The Landsberg *erecta MYB114* allele encodes a full-length gene, and its function in anthocyanin accumulation was confirmed (Gonzalez et al., 2008). Interestingly, although such a truncated MYB may act as a dominant negative regulator (such as MYBL2 and CPC; Dubos et al., 2008; Matsui et al., 2008; Zhu et al., 2009) and counteract the activity of PAP1 in Col-0, up-regulation of both *PAP1* and *MYB114* by MYB112 overexpression triggered an increased expression of anthocyanin biosynthetic genes and an increased level of anthocyanins. *MYB12* and *MYB111*, previously shown to control the expression of genes involved in flavonol biosynthesis, were down-regulated (Fig. 3G; Supplemental Data Set S1). This suggests that *MYB112* acts through the expression of these regulatory genes.

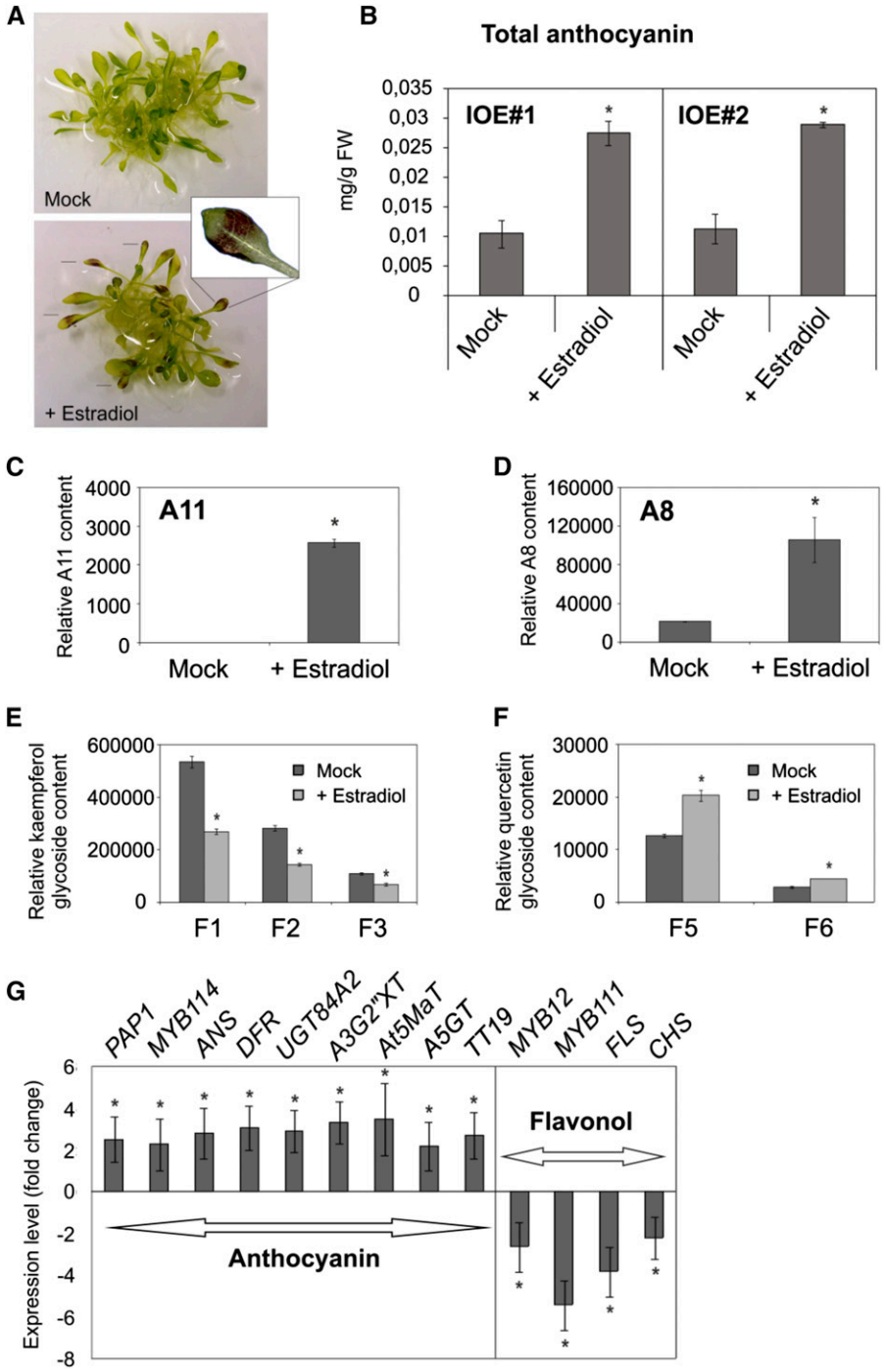

Figure 3. Overexpression of *MYB112* causes changes in flavonoid content. A, *MYB112-IOE* seedlings treated with 10 μM ESTR for 5 d accumulate red pigment in the middle of the leaves spreading to the edges (lines and magnified image). B, Total anthocyanin content was measured spectrophotometrically after extraction with HCl solution. Mean values ± SD of five biological replicates are shown. FW, Frozen weight. LC-MS analysis of A11 (C) and A8 (D) content (Tohge et al., 2005) in mock- and ESTR-treated *MYB112-IOE*. LC-MS analysis of flavonol content F1 (kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside), F2 (kaempferol 3-*O*-glucoside 7-*O*-rhamnoside), and F3 (kaempferol 3-*O*-[2′′-*O*-(rhamnosyl) glucoside] 7-*O*-rhamnoside; E) and F5 (quercetin 3-*O*-glucoside 7-*O*-rhamnoside) and F6 (quercetin 3-*O*-[2′′-*O*-(rhamnosyl) glucoside] 7-*O*-rhamnoside; F). For C to F, mean values ± SD are shown for three biological replicates. *, Statistically significant differences compared with DMSO-treated plants determined by Student's *t* test ($P < 0.05$). G, Transcript levels of selected enzymatic (*ANS*, *DFR*, *TT19*, *A3G2″XT*, *UGT84A2*, *At5MaT*, *A5GT*, *FLS*, and *CHS*) and regulatory (*PAP1*, *MYB114*, *MYB111*, and *MYB12*) genes involved in anthocyanin and flavonol biosynthesis in *MYB112-IOE* seedlings treated with ESTR for 5 d. Data are represented as fold changes compared with the expression in DMSO-treated plants. Only genes showing a change in expression compared with control are shown. Mean values ± SD are shown for three biological replicates. *, Statistically significant differences compared with DMSO-treated plants determined by Student's *t* test ($P < 0.05$).

Silencing of *MYB112* Results in the Down-Regulation of Anthocyanin Pathway Genes

We next analyzed the relative anthocyanin content in the two *myb112* T-DNA insertion mutants using spectrophotometric methods (Fig. 4A). Compared with wild-type seedlings, the total anthocyanin content was reduced by approximately 30% in the *myb112-1* mutant grown for 2 weeks on MS containing 4% (w/v) Suc. No difference in total anthocyanin content was observed between wild-type and *myb112-2* plants, indicating that the level of *MYB112* transcript present in this knockdown mutant (Fig. 2B) is sufficient to sustain normal anthocyanin levels. LC-MS analysis revealed that the changes in the total anthocyanin content in the *myb112-1* mutant are caused by decreased accumulation of A11 (Fig. 4B) and A8 (Fig. 4C) derivatives in these plants. We also detected a slight decrease of flavonol glycoside accumulation in *myb112-1* seedlings compared with the wild type (Fig. 4, D and E). Expression profiling revealed decreased transcript levels of genes

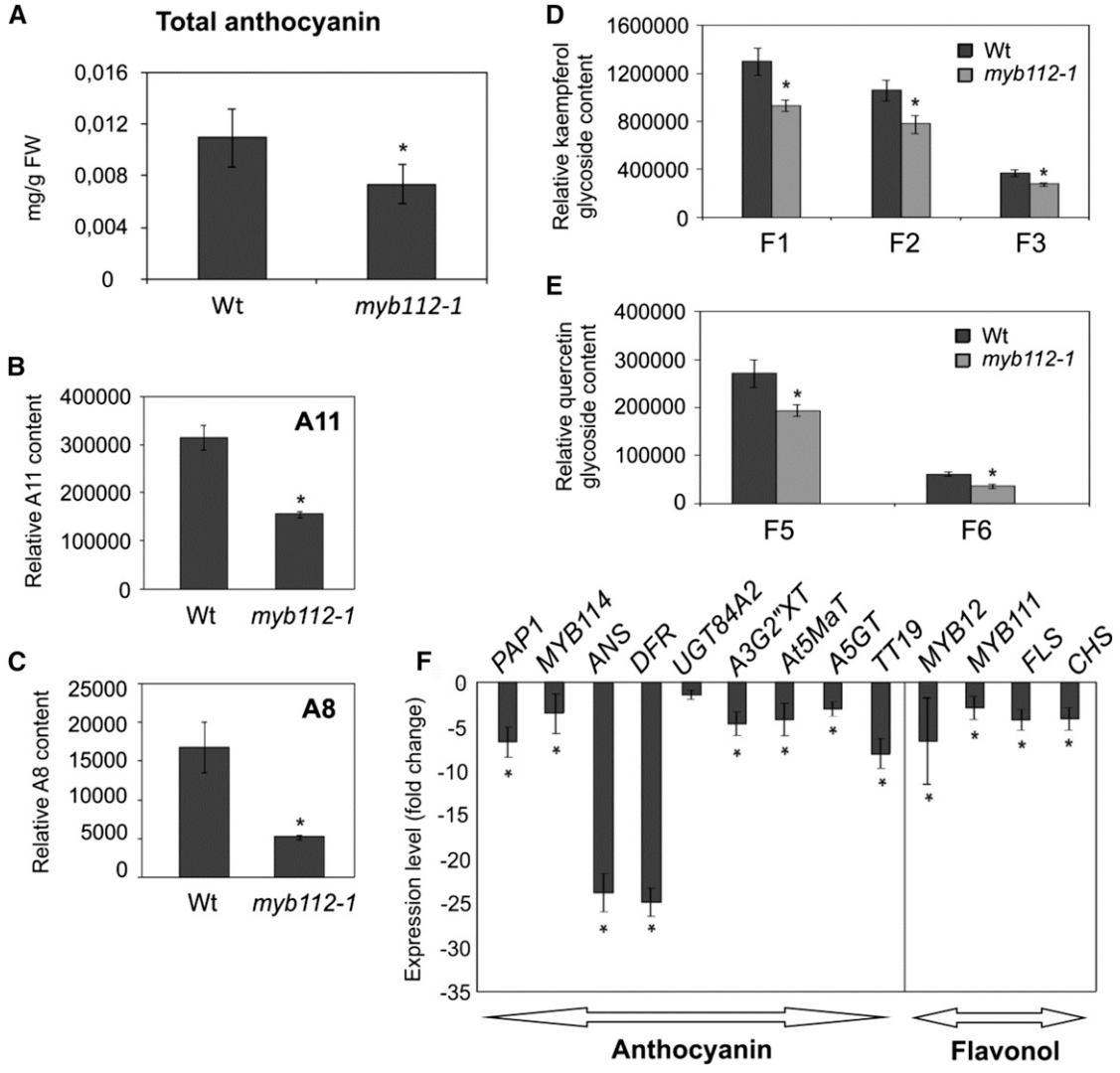

Figure 4. Reduced flavonoid content in *myb112* mutants. Total anthocyanin content measured spectrophotometrically after extraction with HCl solution (A) and LC-MS analysis of A11 (B) and A8 (C) content (Tohge et al., 2005) in *myb112* mutants compared with wild-type (Wt) seedlings grown on 4% (w/v) Suc. FW, Frozen weight. LC-MS analysis of flavonols content F1 (kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside), F2 (kaempferol 3-*O*-glucoside 7-*O*-rhamnoside), and F3 (kaempferol 3-*O*-[2′′-*O*-(rhamnosyl) glucoside] 7-*O*-rhamnoside; D) and F5 (quercetin 3-*O*-glucoside 7-*O*-rhamnoside) and F6 (quercetin 3-*O*-[2′′-*O*-(rhamnosyl) glucoside] 7-*O*-rhamnoside; E) in *myb112* mutants compared with wild-type plants. Mean values ± SDs are shown for five (A) and three (B–E) biological replicates. *, Statistically significant differences compared with wild-type plants determined by Student's *t* test ($P < 0.05$). F, Transcript levels of selected biosynthetic (*ANS*, *DFR*, *A3G2″XT*, *At5MaT*, *A5GT*, *TT19*, *FLS*, and *CHS*) and regulatory (*PAP1*, *MYB114*, *MYB111*, and *MYB12*) genes involved in anthocyanin and flavonol biosynthesis in 2-week-old *myb112-1* seedlings grown on MS and 1% (w/v) Suc. Data are represented as fold change compared with the expression in wild-type plants. Only genes showing a change in expression compared with control are shown. Mean values ± SD are shown for three biological replicates. *, Statistically significant differences compared with the wild type determined by Student's *t* test ($P < 0.05$).

coding for key enzymes of flavonoid biosynthesis (*ANS*, *DFR*, *A3G2′′XT*, *At5MaT*, *A5GT*, *TT19*, and *FLS*) as well as regulatory genes (*PAP1*, *MYB114*, and *MYB12*) in the *myb112-1* mutant (Fig. 4F; Supplemental Data Set S1). An integrated schematic view of the transcriptomic and metabolomic shifts occurring in *MYB112*-modified plants compared with the wild type or DMSO-treated *MYB112-IOE* plants is given in Supplemental Figure S1.

Salt-Induced Expression of *MYB112*

It has been previously shown that Suc is an effective inducer of anthocyanin biosynthesis in Arabidopsis seedlings and that the expression of *PAP1* strongly increases upon Suc treatment (Teng et al., 2005; Solfanelli et al., 2006). To investigate the effect of Suc concentration on *MYB112* expression, we measured its transcript levels in 2-week-old seedlings grown on MS supplemented with either 1% or 4% (w/v) Suc. As shown in

Figure 5A, no substantial change in *MYB112* expression was detected, whereas the transcript level of *PAP1* increased significantly in plants grown on high-Suc medium. Our result is in agreement with previously published global transcriptome data on Suc-responsive genes (Solfanelli et al., 2006; Osuna et al., 2007). Nitrogen deficiency also enhances expression of specific MYB and bHLH TFs and accumulation of end products of the flavonoid pathway (Lea et al., 2007; Rubin et al., 2009). However, *MYB112* transcript abundance in plants grown for 6 d on medium with no nitrogen source was comparable with the transcript level in control plants grown on full (nitrogen-sufficient) medium, whereas in the same experiment, as anticipated, *PAP1* expression increased approximately 8-fold (Fig. 5B). According to microarray data obtained from the GENEVESTIGATOR and eFP databases, expression of *MYB112* increases upon abiotic stress, namely salinity.

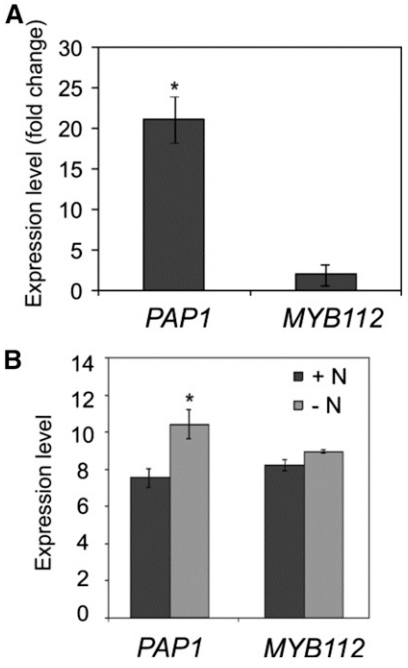

Figure 5. *MYB112* expression is affected by neither Suc nor nitrogen. A, Transcript levels of *PAP1* and *MYB112* determined by qRT-PCR in 2-week-old seedlings grown on MS with 1% (control) or 4% (w/v) Suc. Note the absence of an effect of elevated Suc concentration on *MYB112* expression. The data are represented as fold change compared with the expression in control seedlings. Means of three biological replicates ± SD are shown. *, Statistically significant difference compared with control plants as determined by Student's *t* test ($P < 0.01$). B, Expression level of *PAP1* and *MYB112* in plants grown on medium with or without nitrogen. Plants were grown in a hydroponic system and after 19 d transferred to medium without a nitrogen source. Gene expression was measured 7 d after the transfer. *MYB112* expression is not affected by nitrogen availability, whereas *PAP1* expression increases by approximately 8-fold. Data were extracted from Affymetrix ATH1 hybridizations (Balazadeh et al., 2014) and represent the means ± SDs of three biological replicates. Numbers indicate $\log_2$ values. *, Statistically significant difference compared with control plants as determined by Student's *t* test ($P < 0.05$).

To verify salt-induced expression of *MYB112*, we used the *MYB112* promoter-reporter (GUS) gene fusion lines; we transferred 2-week-old Pro_{MYB112}:*GUS* seedlings to MS containing NaCl (150 mM for 24 h). Histochemical staining revealed enhanced GUS activity in salt-treated seedlings compared with untreated controls (Fig. 6A), showing that the *MYB112* promoter faithfully recapitulated the salt-dependent expression changes observed at the transcript level. Salt-responsive *MYB112* expression was further confirmed by qRT-PCR in seedlings treated with salt (150 mM NaCl) for 4, 8, and 24 h in liquid medium (Fig. 6B). In addition, we grew Arabidopsis Col-0 plants in a hydroponic culture system and subjected them to short- and long-term salt stress (150 mM NaCl) as described previously (Balazadeh et al., 2010a, 2010b). In short-term experiments, leaves of 28-d-old plants were sampled 6 h after stress treatment. In long-term stress experiments, leaves were sampled after 4 d of salt treatment at approximately 20% chlorophyll loss, indicating senescence. Expression of the senescence-specific marker gene *SAG12* (Noh and Amasino, 1999) was not detected in control plants and plants subjected to short-term salt stress, whereas it was induced under long-term salt stress, which induces senescence (for data, see Balazadeh et al., 2010a). Microarray gene expression profiling revealed no change in *MYB112* transcript level after short-term stress; however, after 4 d of salt treatment, the transcript level increased by over 3-fold (Fig. 6C). Similar salinity stress-triggered expression changes have previously been observed for many SAGs (Balazadeh et al., 2010a, 2010b).

MYB112 Regulates Anthocyanin Accumulation under Salt Stress

To test whether MYB112 may be involved in controlling anthocyanin accumulation during salt stress, we transferred 10-d-old seedlings to liquid MS containing 150 mM NaCl. After 3 d, wild-type plants and *MYB112* overexpression plants (i.e. *35S:MYB112* and 35S:*MYB112-GFP* lines) turned red, whereas *myb112-1* mutant plants accumulated visibly less anthocyanin (Fig. 6D). Spectrophotometric analysis of anthocyanin content in these plants revealed a decrease in total anthocyanin content by 20% in the *myb112-1* mutant compared with the wild type. *35S:MYB112* and *35S:MYB112-GFP* plants accumulated 68% and approximately 35% more anthocyanins, respectively, than the wild-type plants (Fig. 6E). Thus, reciprocal effects on anthocyanin accumulation were observed in mutants versus overexpressors of *MYB112*. We therefore concluded that *MYB112* contributes to regulating anthocyanin production in response to salinity stress.

Identification of MYB112 Early Target Genes

To further characterize the MYB112 regulatory network, we used 2-week-old *MYB112-IOE* plants and tested ESTR-dependent *MYB112* expression 1, 3, and

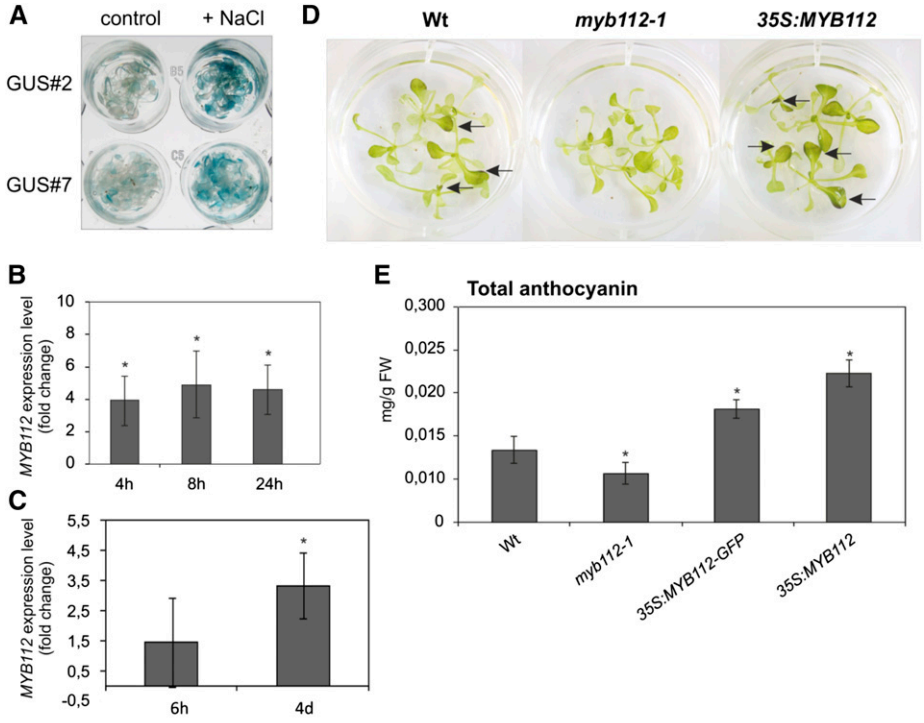

Figure 6. *MYB112* regulates anthocyanin accumulation during salt stress. A, Ten-day-old Arabidopsis Pro_{MYB112}:*GUS* seedlings (lines 2 and 7) were treated for 72 h with 0 mM NaCl (control) or 150 mM NaCl (+NaCl) in liquid MS. Enhanced GUS staining is visible in salt-treated seedlings (right). B, Transcript level of *MYB112* in wild-type seedlings treated with 150 mM NaCl in liquid culture. Incubation times were 4, 8, and 24 h. The data are represented as fold change comparison with untreated control seedlings. Mean values of three biological replicates ± SD are shown. C, Expression profiling using Affymetrix ATH1 arrays shows elevated *MYB112* transcript abundance in plants grown hydroponically for 4 d with 150 mM NaCl compared with control plants. Plants were harvested after 6 h and 4 d of treatment. The experiment was performed in three biological replications. Numbers on the *y* axis indicate fold change values ± SD. D, Appearance of seedlings treated with salt for 3 d. Plants were grown on MS supplemented with 1% (w/v) Suc and after 2 weeks, transferred to liquid medium containing 150 mM NaCl. Note the lack of red pigmentation in *myb112-1* seedlings. E, Total anthocyanin content was measured spectrophotometrically after extraction with HCl solution. Means ± SD are shown for three biological replicates. In B, C, and E, asterisks indicate statistically significant differences compared with wild-type (Wt) plants as determined by Student's *t* test ($P < 0.05$). FW, Frozen weight.

5 h after ESTR treatment. As controls, we used either DMSO-treated *MYB112-IOE* lines or ESTR-treated wild-type control plants. *MYB112* expression increased strongly already within 1 h of ESTR treatment, further increased after 3 h, and reached its maximum 5 h after ESTR application (Supplemental Fig. S2). To find genes responsive to MYB112, 2-week-old *MYB112-IOE* seedlings were transferred to liquid MS containing either 10 μM ESTR or DMSO as control. Seedlings were harvested 3 and 5 h after ESTR induction and after removal of the roots subjected to expression profiling using Affymetrix ATH1 arrays. By including the wild-type control line in our analyses, we could distinguish between genes responding solely to the ESTR treatment and those responding to elevated *MYB112* expression. Two independent experiments were performed and analyzed from the 5-h time point (*MYB112-IOE-5 h*), and one experiment was performed and analyzed from the 3-h time point (*MYB112-IOE-3 h*). Statistical tests using the Limma Bioconductor package (Gentleman et al., 2004) allowed us to identify 56 genes that were significantly differentially expressed (>2-fold) upon 5 h of induction of *MYB112* (Supplemental Table S1), of which 28 were up- and 28 were down-regulated (excluding the ESTR-responsive genes in wild-type plants; Supplemental Data Set S2; Gene Expression Omnibus accession no. GSE36721). Among the up-regulated genes, three encoded TFs, namely *MYB32*, *MYB7*, and *MYB6*. Interestingly, these three R2R3-MYB TFs are closely related and contain an ethylene-responsive factor-associated amphiphilic repression (EAR) motif within their regulatory domains (Matus et al., 2008). Moreover, *MYB32* and *MYB7* were classified to the same subgroup 4 of the R2R3-MYB gene family in Arabidopsis (Stracke et al., 2001). MYB factors of this subgroup were previously shown to be involved in the regulation of pollen development and second cell wall biosynthesis in Arabidopsis (Preston et al., 2004; Zhong and Ye, 2012) and the negative regulation of genes involved in lignin and flavonoid biosynthesis in Arabidopsis

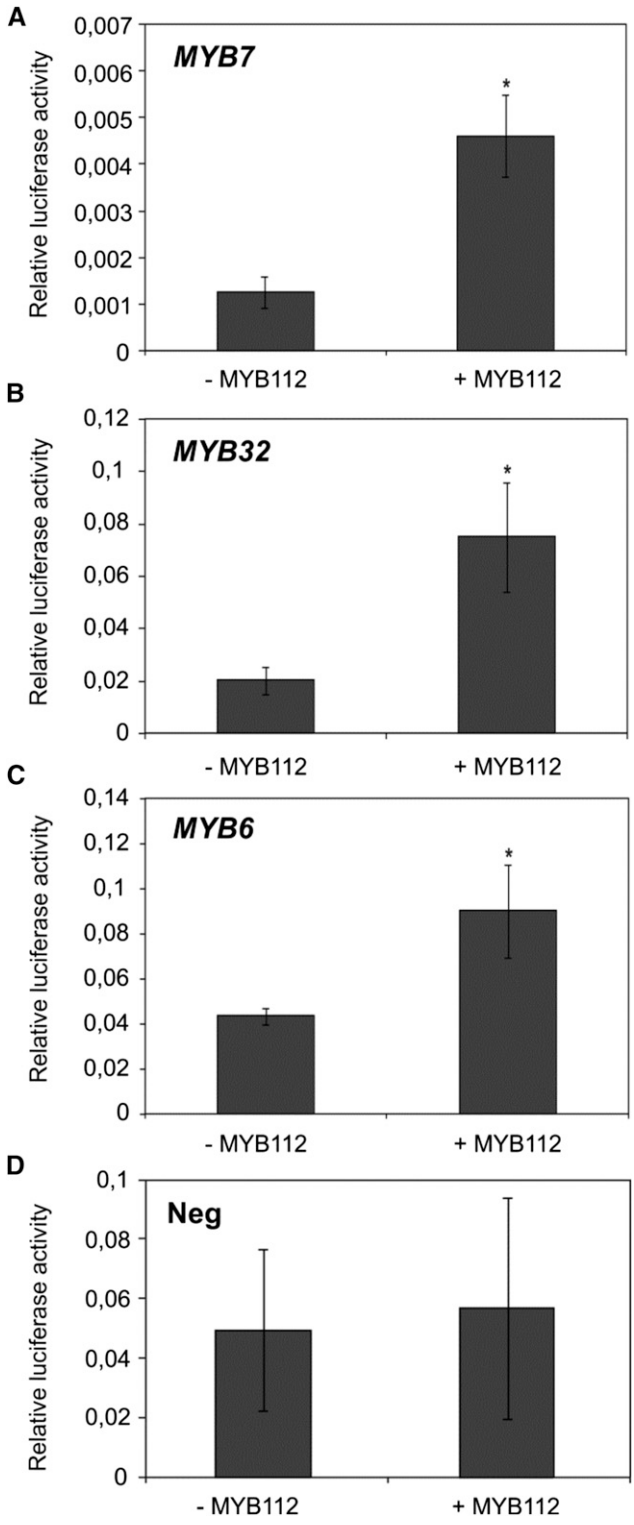

Figure 7. MYB112 transactivates *MYB7*, *MYB32*, and *MYB6* promoters. Transactivation of *MYB7* (A), *MYB32* (B), and *MYB6* (C) expression by MYB112 in Arabidopsis mesophyll cell protoplasts. D, Absence of transactivation of the *CBL-INTERACTING SERINE/THREONINE-PROTEIN KINASE18* (*CIPK18*) promoter (negative control [Neg]). The Pro_{MYB7}:*fLUC*, Pro_{MYB32}:*fLUC*, Pro_{MYB6}:*fLUC*, or Pro_{CIPK18}:*fLUC* constructs harboring the respective promoters (approximately 1.7 kb) upstream of the fLUC open reading frame were cotransformed with the *35S*:*MYB112* plasmid (omitted in control experiments). The *35S*:*rLUC* vector was used for transformation efficiency normalization. Given are means ± SD of four biological replicates. *, Significant differences to control as determined by Student's *t* test ($P < 0.05$).

and maize (Jin et al., 2000; Fornalé et al., 2014). *UGT84A2* is another gene with a function in phenylpropanoid biosynthesis and modification and was up-regulated after 5 h of *MYB112* induction. UGT84A2 is a sinapic acid-*O*-glucosyltransferase (Sinlapadech et al., 2007) that plays a major role in providing sinapoyl-Glc for anthocyanin sinapoylation (therefore, being highly important in the production of the anthocyanin derivative A11; Yonekura-Sakakibara et al., 2012). Moreover, *MYB32*, *MYB7*, and *UGT84A2* were previously shown to be up-regulated during age-dependent senescence in wild-type Arabidopsis (Buchanan-Wollaston et al., 2005; Balazadeh et al., 2008; GENEVESTIGATOR). Other than *MYB32*, *MYB7*, and *UGT84A2*, 11 other up-regulated transcripts (representing one half of the MYB112 up-regulated genes; Supplemental Table S1) were previously reported to be senescence associated (eFP browser; Balazadeh et al., 2008; Breeze et al., 2011; http://www2.warwick.ac.uk/fac/sci/lifesci/research/presta/data/senescence). This result supports the model that MYB112 is a senescence-regulatory TF and that the expression of several known SAGs (including TFs) is regulated by this MYB TF. In the single 3-h Affymetrix experiment, we identified (>4-fold) 15 differentially expressed genes (1 up- and 14 down-regulated genes; Supplemental Data Set S2; Gene Expression Omnibus accession no. GSE36721). We next tested expression of the genes up-regulated after 5 h of induction and the 10 most strongly down-regulated genes after 3 h (which also robustly responded after 5 h) by qRT-PCR; 22 of the 28 up-regulated genes and 9 of the 10 down-regulated genes could be confirmed by qRT-PCR (Supplemental Tables S1 and S2).

MYB112 Triggers the Expression of R2R3-MYB TFs of Subgroup 4

To confirm that MYB112 regulates the expression of genes identified by expression profiling, we assayed the transactivation capacity of MYB112 on the promoters of *MYB32*, *MYB7*, and *MYB6*. These putative downstream target genes were selected from the data set considering their predicted regulatory function in flavonoid biosynthesis. Mesophyll cell protoplasts were prepared from wild-type (Col-0) Arabidopsis leaves and transiently transformed with constructs expressing *MYB112* under the control of the CaMV *35S* promoter. Simultaneously, protoplasts were transfected with vectors carrying the firefly (*Photinus pyralis*) luciferase (fLUC) open reading frame fused to the upstream sequences of the selected MYB112 target genes. For normalization, protoplasts were additionally transformed with a third construct that

expresses *Renilla reniformis* luciferase from the *35S* promoter. Protoplasts lacking the *35S:MYB112* effector construct served as controls. *MYB7* and *MYB32* promoters displayed over 3-fold and *MYB6* promoter displayed approximately 2-fold induction of the reporter gene when *MYB112* was overexpressed in the protoplasts (Fig. 7, A–C). No transactivation was observed for the *CIPK18* promoter included as negative control (Neg in Fig. 7D). In addition, we measured expression of *MYB32*, *MYB7*, and *MYB6* in *35S:MYB112* and *35S:MYB112-GFP* seedlings and *myb112* mutants grown for 2 weeks on MS supplemented with 1% (w/v) Suc. Transcript level of *MYB32* and *MYB7* was elevated by approximately 4- and approximately 3-fold in the *35S:MYB112* and *35S:MYB112-GFP* seedlings, respectively, compared with the wild type. Expression of *MYB6* was increased by approximately 2-fold in the *35S:MYB112* seedlings, but no significant difference was observed in *35S:MYB112-GFP* plants compared with the wild type (Fig. 8A). Notably, expression of *MYB32* and *MYB7* was reduced by approximately 9- and approximately 3-fold in the *myb112-1* and *myb112-2* mutants, respectively, whereas *MYB6* expression was only slightly lowered (Fig. 8B). Taken together, these data confirm a regulatory function of MYB112 toward *MYB32*, *MYB7*, and *MYB6*, which all encode R2R3-MYB TFs of subgroup 4.

Identification of the MYB112 DNA Binding Site

We performed an in vitro binding site selection experiment to identify sequence motifs recognized by MYB112 using the cellulase D (CELD)-TF fusion method (Xue, 2002, 2005). MYB112 was translationally fused to the catalytic domain of a 6× His-tagged CELD from *Neocallimastix patriciarum* and incubated with biotin-labeled random sequence oligonucleotide probes. Oligonucleotides bound by the MYB112-CELD fusion protein were recovered by means of affinity purification of the DNA-MYB112-CELD complex and amplified using PCR (Xue, 2005). Twenty-nine sequences were obtained and analyzed for binding activity. An alignment of the target sequences is shown in Figure 9A. MYB112 binds to an 8-bp DNA fragment containing the core sequence (A/T/G)(A/C)CC(A/T)(A/G/T)(A/C)(T/C). The identified recognition site is present in promoters of a number of genes controlled by MYB112 in ESTR-IOE lines (Supplemental Table S1), including the three *MYB* genes (within the 1-kb upstream regions): *MYB32* (two binding sites), *MYB7* (one binding site), and *MYB6* (three binding sites).

MYB112 Binds to the *MYB7* and *MYB32* Promoters

We next used an electrophoretic mobility shift assay (EMSA) to test in vitro the physical interaction between MYB112 protein and promoter fragments of *MYB6*, *MYB7*, and *MYB32*. Expressed and purified MYB112-CELD protein was incubated with IR dye-labeled 40-bp-long promoter fragments of the selected putative target genes containing the identified MYB112 binding site (Supplemental Material S1). The probes included the single MYB112 binding site of the *MYB7* promoter (fragment *MYB7_1*), either of the two binding sites of the *MYB32* promoter (*MYB32_1* or *MYB32_2*), or two neighboring sites of the three present in the *MYB6* promoter (both included in the *MYB6_1/2* fragment). In all cases, a clear band shift was observed in the presence of MYB112-CELD fusion protein, which disappeared in the presence of unlabeled competitor DNA (Fig. 9B). These results indicate a physical interaction of MYB112 with the *MYB6*, *MYB7*, and *MYB32* promoters. Finally, to verify MYB112 binding to the respective promoters in vivo, we performed chromatin immunoprecipitation (ChIP) coupled to quantitative PCR (qPCR) using plants bearing a *35S:MYB112-GFP* construct (accumulating MYB112-GFP fusion protein in the nucleus; Fig. 1B). We designed primers flanking the MYB112 binding sites of the different promoters (Supplemental Material S1) and measured their abundance using qPCR. As shown in Figure 9C, the *MYB32* (binding site 2) and *MYB7* promoter fragments containing the selected cis-element were enriched relative to control, proving direct binding of MYB112. In contrast, we did not detect significant binding of MYB112 to the *MYB6* and binding site 1 of the *MYB32* promoters. It may, however, be that these

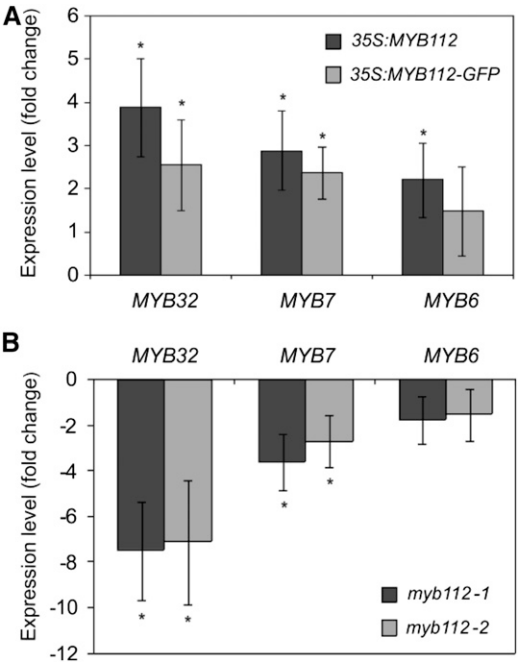

Figure 8. Expression of *MYB32*, *MYB7*, and *MYB6* in *MYB112* overexpression plants and mutants. Transcript levels of *MYB32*, *MYB7*, and *MYB6* in 2-week-old *35S:MYB112* and *35S:MYB112-GFP* plants (A) and *myb112-1* and *myb112-2* mutant seedlings (B) as measured by qRT-PCR. Data are represented as fold change compared with the expression in empty vector control plants or the wild type, respectively. Mean values ± SD are shown for three biological replicates. *, Statistically significant differences compared with controls determined by Student's *t* test ($P < 0.05$).

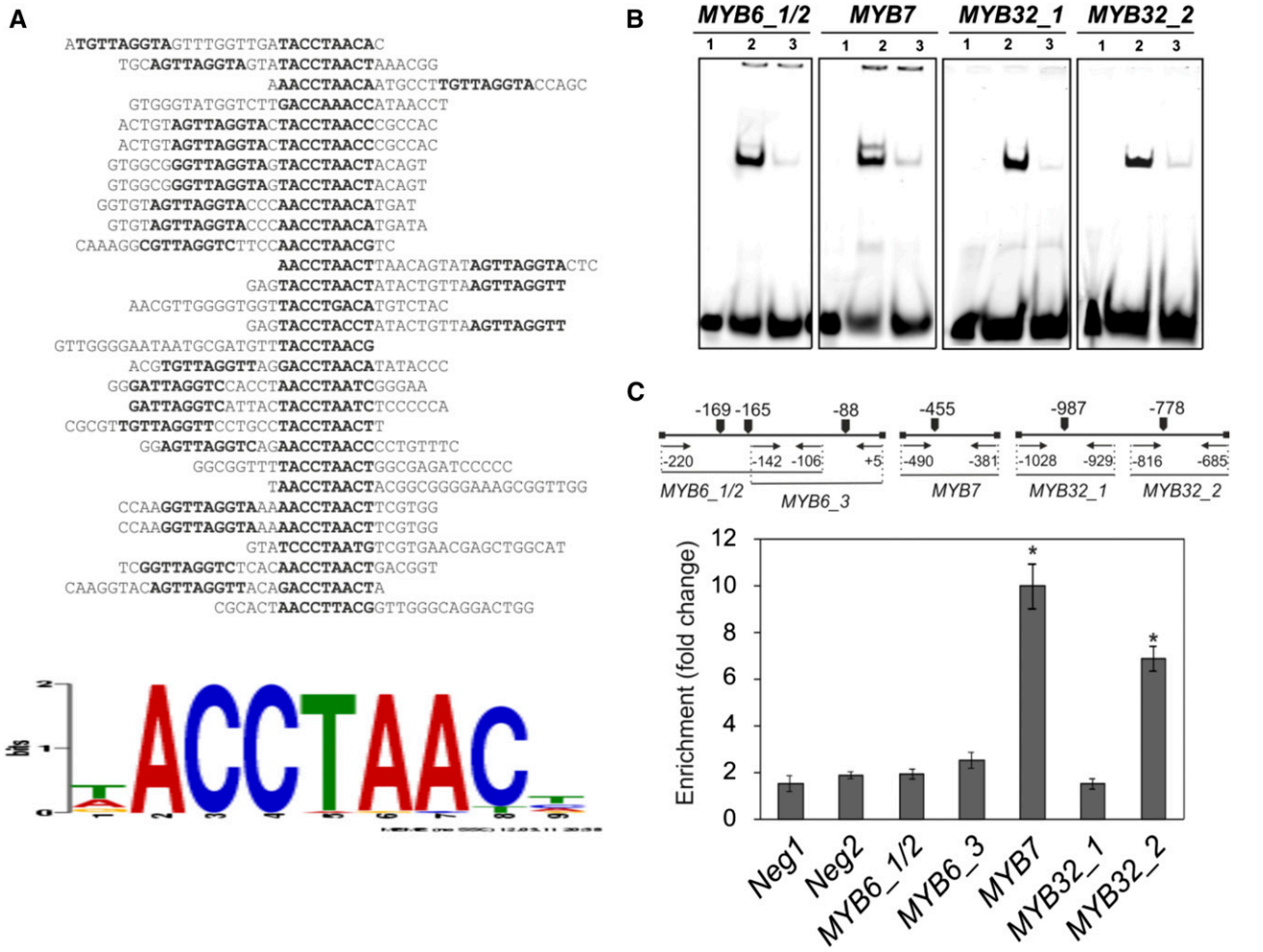

Figure 9. Identification of MYB112 direct target genes. A, MYB112 in vitro binding site selection. A motif common to 29 positive clones was identified using MEME. The motif is present in the promoters of putative direct target genes. B, EMSA. Purified MYB112-CELD-His protein binds specifically to the MYB112 binding sites present in the *MYB6*, *MYB7*, and *MYB32* promoters. In vitro DNA binding reactions were performed with 40-bp double-stranded oligonucleotides, including the MYB112 binding sites of the respective target gene promoters. The DNA fragments were labeled with IR dye. The fragments contained two (*MYB6_1/2*) or one (*MYB7*, *MYB32_1*, and *MYB32_2*) MYB112 binding motif. 1, IR-labeled DNA fragment.; 2, MYB112 protein with labeled DNA fragment (note distinct shift indicating binding); 3, MYB112 protein plus labeled DNA fragment and 200× excess competitor (note the shift disappearance). C, ChIP-qPCR. Whole shoots of 2-week-old Arabidopsis seedlings expressing GFP-tagged MYB112 under the control of *35S* CaMV promoter (*35S:MYB112-GFP*) and wild-type plants were harvested for the ChIP experiment. qPCR primer locations are indicated. Primers annealing to promoter regions of two Arabidopsis genes lacking MYB112 binding sites (i.e. *At2g22180* [Neg1] and *At3g1840* [Neg2]) were used as negative controls. Enrichment of the respective promoter fragments was quantified by qPCR. Data represent means ± SD of three experiments. Enrichment of *MYB7* and *MYB32* promoter fragments is detected. *, Statistically significant differences to controls as determined by Student's *t* test ($P < 0.05$).

two genes are targets of MYB112 in other physiological conditions that we did not test in our experiments. Taken together, our data firmly prove that MYB112 acts as a direct transcriptional regulator of *MYB32* and *MYB7*.

MYB112 Affects Pollen Viability

MYB32, identified here as a direct MYB112 target, as well as the closely related *MYB4* gene are both required for normal pollen development (Preston et al., 2004). The expression of several phenylpropanoid biosynthesis genes is altered in plants with modified expression of the MYB TFs, which likely affects the metabolite flux through the phenylpropanoid pathways. This then affects the formation of the major pollen wall component sporopollenin, which consists of polymerized phenols and fatty acid derivatives, leading to pollen distortion (Preston et al., 2004).

By analyzing *MYB112* promoter activity in Pro_{MYB112}:*GUS* lines, we show that *MYB112* is also expressed in pollen (Fig. 1, A16 and A17). We therefore tested pollen viability in *MYB112* transgenic plants after staining of flowers with Alexander dye (Alexander, 1969). We

found that overexpression of *MYB112* leads to defective pollen; 35% and 70% of pollen was aborted in *35S:MYB112-GFP* and *35S:MYB112* overexpression plants, respectively (Supplemental Fig. S3). The observed decrease in pollen viability in these lines corresponds to the level of *MYB112* overexpression (approximately 5-fold increase in *35S:MYB112-GFP* and approximately 15-fold in *35S:MYB112*; Supplemental Fig. S4).

We then analyzed transcript levels of genes encoding key phenylpropanoid biosynthesis enzymes in the *myb112-1* mutant and the *MYB112-IOE* plants treated with ESTR for 5 d (Supplemental Table S2). Figure 10 shows significant changes in the expression of a number of phenylpropanoid biosynthetic genes in *MYB112* transgenic plants. For example, expression of *CAFFEOYL/COENZYME A 3-O-METHYLTRANSFERASE* (*CCoAOMT*) is increased in plants overexpressing *MYB112* (Fig. 10A), similar to *MYB4* overexpression plants (Jin et al., 2000). *CCoAOMT* is down-regulated in the *myb4* knockout line (Jin et al., 2000) and the *myb112-1* mutant (Fig. 10B). Thus, like in *MYB32*- and *MYB4*-modified plants, aberrant pollen observed in *MYB112* transgenics is likely caused by altered phenylpropanoid biosynthesis.

MYB112 Is a High Light-Induced Gene

Thylakoid-soluble Phosphoprotein of 9 kD (TSP9) is a mobile thylakoid protein that interacts with light-harvesting complex II and the peripheries of both photosystems. It was shown to regulate light harvesting by facilitating the dissociation of light-harvesting proteins from PSII. Upon phosphorylation, TSP9 is partially released from the membrane and therefore, was proposed to play a role in chloroplast signaling (Hansson et al., 2007). Global expression profiling identified 23 genes, including *MYB112*, that were high light dependent in wild-type plants but not in a *tsp9* T-DNA insertion mutant (GK_377A12; Fristedt et al., 2009).

This finding suggests a function of *MYB112* in TSP9-dependent high light signaling. To investigate further the relation between the two genes, we measured total anthocyanin content in two *tsp9* T-DNA insertion lines under high light stress. However, anthocyanin accumulation was not affected in either mutant, suggesting that the level of *MYB112* in these plants was still sufficient to sustain a wild-type phenotype. To verify high light-induced expression of *MYB112*, we used the Pro_{MYB112}:*GUS* reporter lines. Ten-day-old Arabidopsis Pro_{MYB112}:*GUS* seedlings as well as 3-week-old plants were treated for 6 and 20 h, respectively, with high light (500 μmol m^{-2} s^{-1}). In both cases, histochemical staining revealed enhanced GUS activity in high light-treated plants compared with controls kept in normal light condition (100 μmol m^{-2} s^{-1}; Fig. 11, A and B). Spectrophotometric analysis of anthocyanin content in 3-week-old *myb112* mutants grown under high light condition (500 μmol m^{-2} s^{-1}) for 3 d showed decreased pigment content in *myb112-1* plants (Fig. 11C), suggesting that *MYB112* is involved in modulating high light-induced anthocyanin accumulation.

DISCUSSION

We discovered MYB112 as a previously uncharacterized transcriptional regulator affecting flavonoid biosynthesis in Arabidopsis. Based on our experimental data, we suggest that MYB112 acts as a positive regulator of anthocyanin biosynthesis and a negative regulator of flavonol biosynthesis. As we show, MYB112 activates *PAP1* and *MYB114* but inhibits *MYB12* and *MYB111*. PAP1 and MYB114 regulate the expression of genes encoding enzymes involved in anthocyanin formation (for example, *ANS* and *DFR*), and expression of these genes is increased upon *MYB112* induction. However, note that only the Landsberg *erecta MYB114* but not the Col-0 allele encodes a full-length TF (Gonzalez et al., 2008). MYB111 and MYB12 control expression of biosynthetic genes involved in flavonol biosynthesis (for example, *CHS* and *FLS*), and their transcript levels are decreased in *MYB112-IOE* plants after ESTR treatment. We did not obtain evidence for a direct regulation of either the known anthocyanin or flavonol biosynthetic genes or their regulators. Because the expression of *PAP1* and *PAP2* was not affected after short-term induction of *MYB112*, these transcription regulatory genes are unlikely to be direct or early targets of MYB112 and were, therefore, not included in our ChIP or transactivation assays. At this stage of analysis, it thus remains open how the

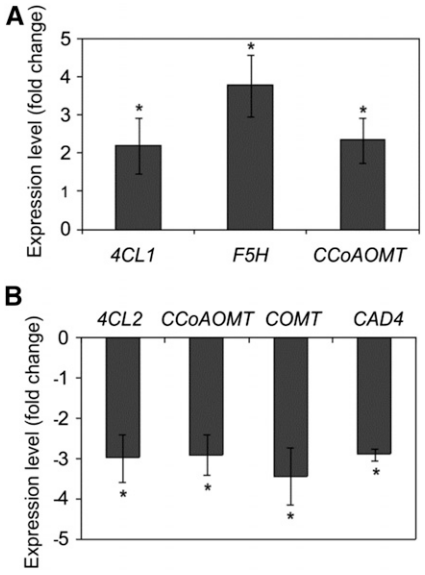

Figure 10. Changes in expression of phenylpropanoid biosynthesis genes in *MYB112* transgenic plants. Expression of phenylpropanoid biosynthetic genes in *MYB112-IOE* seedlings treated with ESTR in liquid MS (1% [w/v] Suc) for 5 d compared with mock (DMSO)-treated plants (A) and *myb112-1* seedlings compared with the wild type (B). Data are represented as fold change compared with the respective control. Only genes showing a change in expression compared with control are shown. Data are means ± SD of three biological replicates. *, Statistically significant differences to control determined by Student's *t* test ($P < 0.05$). *4CL1, 4-COUMARATE COA LIGASE1; F5H, FERULATE 5-HYDROXYLASE; COMT, CAFFEATE O-METHYLTRANSFERASE; CAD, CINNAMOYL-ALCOHOL DEHYDROGENASE.*

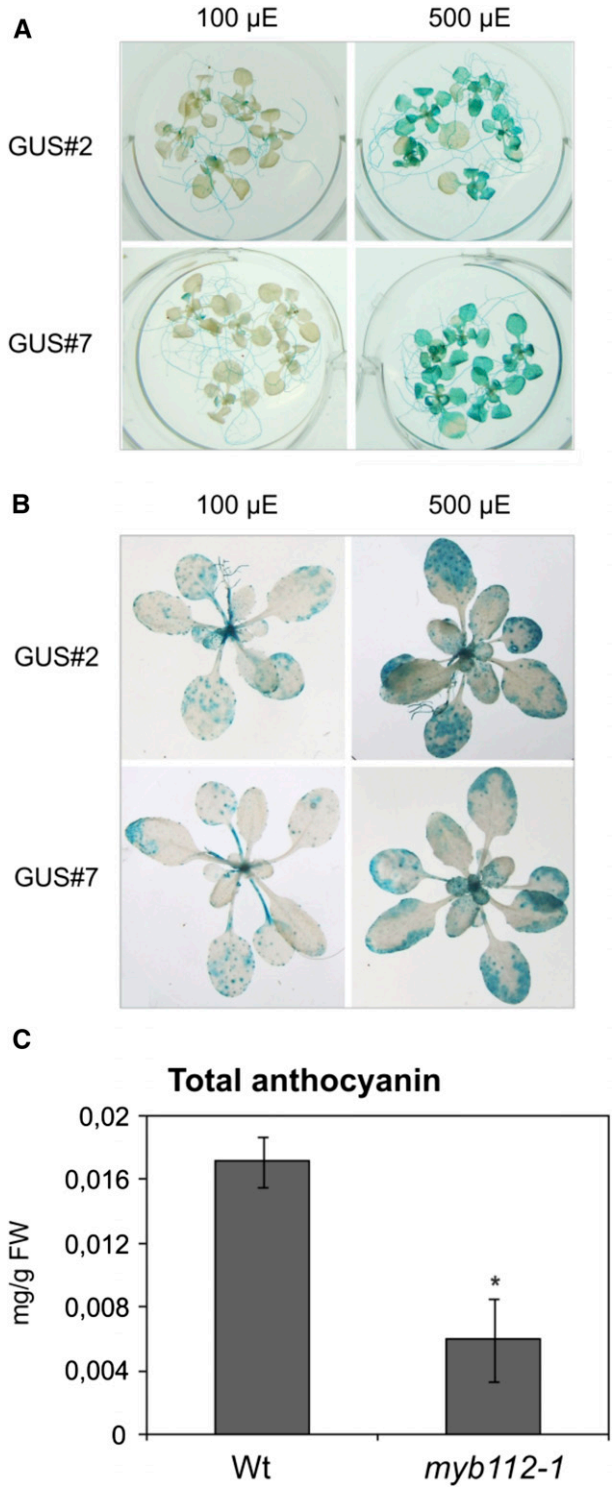

Figure 11. MYB112 regulates anthocyanin accumulation under high light. A, *MYB112* promoter activity in 10-d-old Arabidopsis Pro_{MYB112}:*GUS* seedlings (lines 2 and 7) treated for 6 h with high light (500 μE; right) compared with control seedlings kept at 100 μE (left). B, GUS activity in 3-week-old Pro_{MYB112}:*GUS* plants treated with high light for 20 h (right) compared with control plants kept at 100 μE (left). Staining in A and B is shown for two lines (i.e. GUS#2 and GUS#7). Note the enhanced GUS staining of plants treated with high light. C, Anthocyanin content in *myb112* mutants grown under high light. Plants were grown in long-day condition and after 3 weeks transferred to high light (500 μE) for 3 d. Total anthocyanin content was measured spectrophotometrically after extraction with HCl solution. Means ± SD are shown for five biological replicates. FW, Frozen weight. *, Statistically significant difference to wild-type (Wt) plants as determined by Student's *t* test ($P < 0.05$).

regulation of the flavonoid pathway by MYB112 is achieved. However, one possible scenario is that this regulation by MYB112 occurs through the direct activation of MYB32 and MYB7, which have an EAR repressor motif and a likely role in transcriptional repression of lignin and flavonoid biosynthetic genes. Furthermore, *MYB32* was previously shown to affect expression of flavonoid biosynthesis pathway genes and pollen development. Disruption of *MYB32* by T-DNA insertion leads to the formation of aberrant pollen. A similar phenotype is observed when the closely related *MYB4* gene is mutated or overexpressed. However, in Arabidopsis, flavonoids are not essential for pollen formation or fertility (Burbulis et al.., 1996). It was speculated that changes in the expression of flavonoid and phenylpropanoid biosynthetic genes influence the flux along these pathways, interfering with pollen development by altering structural components of the pollen wall, such as sporopollenin (Preston et al., 2004). Our results reported here reveal that overexpression of *MYB112* also leads to partial pollen abortion. Expression profiling of genes encoding key phenylpropanoid biosynthesis enzymes in the *myb112-1* mutant and the *MYB112-IOE* plants treated with ESTR for 5 d revealed significant changes in the expression of a number of phenylpropanoid biosynthetic genes in *MYB112* transgenic plants. *MYB32*, *MYB7*, and *MYB4* were recently reported to be direct downstream target genes of MYB46 and MYB83. These two TFs recognize the 7-bp consensus sequence ACC(A/T)A(A/C)(T/C) (Kim et al., 2012; Zhong and Ye, 2012), which is highly similar to the MYB112 binding site reported here: (A/T/G)ACC(A/T)(A/G)(A/C)(T/C). MYB46 and MYB83 function redundantly to control the production of all major secondary cell wall components in Arabidopsis fibers and vessels, such as lignin, cellulose, and xylan (Ko et al., 2009; Zhong and Ye, 2012). MYB58 and MYB63 are yet other MYB factors involved in controlling the formation of the secondary cell wall. Contrary to MYB46 and MYB83, they are specifically involved in the regulation of lignin biosynthesis (Zhou et al., 2009). Their overexpression was found to induce ectopic deposition of lignin but not cellulose and xylan, whereas their dominant repression resulted in a reduction of secondary wall thickening. In this network, expression of *MYB46/83* and *MYB58/63* is under the control of the NAC SECONDARY WALL-ASSOCIATED DOMAIN PROTEIN1 (NST1) and its close homologs NST2, VASCULAR-RELATED NAC-DOMAIN6 (VND6), and VND7 (Zhong and Ye, 2009; Zhou et al., 2009; Zhong et al., 2010). Zhong and Ye (2012) suggested that *MYB32*, *MYB7*, and *MYB4* may be involved in fine tuning the regulation of lignin biosynthesis during

secondary wall deposition. Based on microarray experiments, expression of *MYB112* is neither affected by overexpression of the secondary wall NACs (SWNs; Zhong et al., 2010) nor by *MYB46/83* (Ko et al., 2009), and GUS promoter activity assays revealed that *MYB112* is mainly expressed in leaves and pollen, whereas SWNs and *MYB46/83* factors are expressed in vascular tissue. It may, however, be that *MYB112* acts as an SWN-independent regulator of lignin biosynthesis or the biosynthesis of other phenylpropanoids, leading to the observed pollen phenotype (Supplemental Fig. S3).

Further functional characterization of *MYB7* and *MYB32* as well as the identification of direct target genes of both TFs will be necessary to refine the *MYB112* regulatory network.

Expression of *MYB112* increases during senescence as well as upon salt and high light stress, conditions in which plants often receive more light energy than they can use for photochemical reactions. Excess light energy leads to photoinhibition and the formation of ROS. Anthocyanins absorb excess quanta, thereby protecting plant tissue from damaging light levels. High light activation of *MYB112* expression may be regulated through TSP9 signaling. TSP9 is a thylakoid-anchored protein, which under high light, is phosphorylated and partially released from the membrane. In the absence of functional TSP9, high light-induced *MYB112* expression is abolished (Fristedt et al., 2009).

In addition, transcriptome analysis revealed that the salinity stress-triggered up-regulation of *MYB112* expression is strongly reduced (approximately 4- and approximately 20-fold in two independent experiments compared with the wild type) in transgenic plants overexpressing MYB44 (Jung et al., 2008). Notably, the expression of *MYB32* and *MYB7*, the two direct MYB112 target genes identified here, was also lower in *35S:MYB44* than in wild-type plants during salt stress (Jung et al., 2008; E-ATMX-30), indicating that MYB44 negatively modulates *MYB112* expression. In addition, expression of genes associated with anthocyanin biosynthesis, such as *CHS*, *F3H*, *DFR*, *PAP1*, and *PAP2*, is decreased in *35S:MYB44* plants upon salt stress (Jung et al., 2008; E-ATMX-30). Accordingly, *35S:MYB44* seedlings accumulate less anthocyanin than wild-type plants (Jung et al., 2010). Thus, with respect to anthocyanin accumulation, the phenotype of *35S:MYB112* seedlings (elevated anthocyanin) is opposite to that of *35S:MYB44* seedlings (low anthocyanin), whereas the *myb112-1* mutant accumulates reduced levels of anthocyanin, like *35S:MYB44* (Figs. 3, 4, and 6). This finding provides further support of the negative regulation of *MYB112* by MYB44. To verify the model, we created ESTR-inducible *MYB44* overexpression (*MYB44-IOE*) plants, treated transgenic seedlings for 5 h with ESTR in the absence of salt stress, and then induced salt stress (150 mM NaCl) for 2 h (in the presence of ESTR). Using qRT-PCR, we detected a decrease of *MYB112* expression in two of four tested lines (Supplemental Fig. S5), indicating that regulation of *MYB112* expression by MYB44 may be indirect and may require additional factors, modulating this regulation.

Accumulation of anthocyanins has been reported to occur under a number of stresses, and the effects of nutrient (nitrogen or phosphorous) deprivation and light quality and quantity have been subject to the most attention in the past; several TFs have been shown to be involved in this response, including members of the MYB and bHLH families (Lea et al., 2007; Lillo et al., 2008; Stracke et al., 2010). However, although salinity stress is well known to lead to an accumulation of anthocyanin in a number of species, including crops and vegetables, such as tomato, maize, sugarcane, and many others (Kaliamoorthy and Rao, 1994; Piao et al., 2001; Wahid and Ghazanfar, 2006; Keutgen and Pawelzik, 2007; Roychoudhury et al., 2008; Matus et al., 2010), the regulatory mechanisms underlying this phenomena have not been studied intensively to date. Herein, we show that *MYB112* expression is induced by salinity stress, whereas its expression is largely unaffected by nitrogen limitation, and sugar levels in contrast to many of the known transcriptional regulators of flavonoid/anthocyanin biosynthesis (Scheible et al., 2004; Misson et al., 2005; Lea et al., 2007; Morcuende et al., 2007; Lillo et al., 2008). We further illustrate that salinity-induced accumulation of anthocyanin is impaired in *myb112* mutants but stimulated in *35S:MYB112* overexpressors, indicating that MYB112 plays a critical role during salt-induced anthocyanin accumulation. Further studies will, however, be required to reveal the details about the signaling pathways that control *MYB112* expression during salinity and high light stress.

MATERIALS AND METHODS

General

Standard molecular techniques were performed as described (Sambrook and Russell, 2001; Skirycz et al., 2006). Primer sequences are given in Supplemental Table S3. For digital gene expression analyses, the online tools of GENEVESTIGATOR (https://www.genevestigator.com) and eFP browser (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) were used.

Plants

Arabidopsis (*Arabidopsis thaliana*) seedlings were grown on plates in a climate chamber with a 16-h day length provided by fluorescent light at 30 μmol m^{-2} s^{-1} and a temperature of 22°C. After 2 weeks, plants were transferred to soil and grown under controlled conditions in a greenhouse with a 16-h day length (120 μmol m^{-2} s^{-1}), a 21°C /18°C day-night temperature cycle, and a 60%/75% day-night relative humidity cycle. The salt treatment experiments were performed as reported (Balazadeh et al., 2010a; Omidbakhshfard et al., 2012; Allu et al., 2014). For nitrogen depletion experiments, Arabidopsis (Col-0) seeds were surface sterilized. Plants were hydroponically grown on complete Hoagland medium containing 7 mM nitrate and 0.3 mM ammonium (+N medium). A set of plants was transferred to nitrogen-free medium (−N medium) at 19 d after sowing. After 7 d, the first two leaves that emerged after the cotyledons of 26-d-old plants (grown on +N and −N conditions) were harvested and frozen in liquid nitrogen for expression profiling (Balazadeh et al., 2014). The experiment was performed in three biological replicates, each replicate representing a pool of 24 plants.

Constructs

For Pro_{MYB112}*:GUS*, an approximately 1.3-kb 5′ *MYB112* fragment was amplified by PCR from genomic Arabidopsis Col-0 DNA using primers *MYB112:GUS-fwd.* and *MYB112:nested-rev.*, inserted into plasmid pCR2.1 (Invitrogen), reamplified

by PCR using primers *MYB112:GUS-fwd.* and *MYB112:GUS-rev.*, and inserted by *Eco*RI and *Nco*I sites into pCAMBIA1305.1-Hygromycin (CAMBIA). For *35S:MYB112*, *MYB112* open reading frame, amplified by PCR from Arabidopsis complementary DNA (cDNA) using primers *MYB112-OE-fwd.* and *MYB112-OE-rev.*, was inserted into pCR2.1 and then cloned by added *Hin*dIII sites into pGreen0229-35S (Skirycz et al., 2006). For *35S:MYB112-GFP*, *MYB112* coding sequence was amplified by PCR from Arabidopsis cDNA using primers *MYB112-fwd.* and *MYB112-rev.* and inserted into pDONR201 (Invitrogen). Subsequently, the fragment was cloned into the GATEWAY-compatible vector pK7FWG2.0 (Plant Systems Biology, VIB, University of Ghent) by LR reaction. For *MYB112-IOE*, *MYB112* coding region was amplified by PCR from Arabidopsis leaf cDNA using primers *MYB112-IOE-fwd.* and *MYB112-IOE-rev.*, inserted into pCR2.1, and then cloned by *Xho*I and *Spe*I sites into pER8 vector (Zuo et al., 2000). *Agrobacterium tumefaciens* strain GV3101 (pMP90) was used for Arabidopsis (Col-0) transformations. For *Pro:fLUC* fusions, approximately 1.7-kb fragments upstream of the respective coding sequences of the genes *MYB6*, *MYB7*, *MYB32*, and *CIPK18* (negative control) were amplified using PCR from Arabidopsis genomic DNA, ligated into the pENTR/D-TOPO vector (Invitrogen), and subsequently recombined into the GATEWAY-compatible vector pGWL7 (Licausi et al., 2011). For *pTac:MYB112-LCELD6xHis*, *MYB112* sequence was amplified by PCR using primers *MYB112-CELD-fwd.* and *MYB112-CELD-rev.* and cloned into the pTacLCELD6His vector (Xue, 2002, 2005) by *Nhe*I and *Bam*HI restriction sites.

myb112 T-DNA Insertion Lines

T-DNA insertion lines were obtained from the SALK (SALK_098029, *myb112-2*) or GABI-Kat (GK_093E05, *myb112-1*) collections. Homozygous plants were identified by PCR using the following primers: for SALK_098029, T-DNA left border primer LB, gene-specific primers SALK_LP and SALK_RP; and for GK_093E05, T-DNA left border primer LB, gene-specific primers GK_LP and GK_RP. *MYB112* expression in the T-DNA insertion plants was examined by semi-qRT-PCR using primers annealing to the 5′ and 3′ ends of the *MYB112* coding region.

ESTR Induction Experiments

Arabidopsis seedlings transformed with the *MYB112-IOE* construct were grown on solid MS and after 2 weeks, transferred to liquid MS supplemented with 10 μM ESTR for the indicated times. As controls, 0.1% (v/v) DMSO-treated *MYB112-IOE* lines or ESTR-treated empty vector plants were used.

Expression Profiling by qRT-PCR

Total RNA extraction, cDNA synthesis, and qRT-PCR were done as described (Caldana et al., 2007; Balazadeh et al., 2008; Wu et al., 2012). Gene expression was analyzed using the comparative Ct method. Experiments were performed using three biological replicates.

Microarray Experiments

Affymetrix ATH1 hybridizations were performed by ATLAS Biolabs. Expression of *MYB112* was induced by 10 μM ESTR in 2-week-old seedlings grown in liquid MS at continuous light. Identically treated empty vector (pER8)-transformed seedlings and *MYB112-IOE* seedlings treated with DMSO served as controls. Seedlings were harvested after 1, 3, and 5 h, and RNA extracted from shoots was used for expression profiling. Expression data were submitted to the National Center for Biotechnology Information Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo/) under accession number GSE36721.

Transactivation Assays

Protoplasts were isolated from Arabidopsis Col-0 plants as described by Yoo et al. (2007) and transformed with *35S:MYB112* effector construct together with Pro_{MYB32}*:fLUC*, Pro_{MYB7}*:fLUC*, or Pro_{MYB6}*:fLUC* reporter constructs. For signal normalization, protoplasts were simultaneously transfected with *35S:rLUC* plasmid. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) and a GloMax 20/20 Luminometer (Promega). Experiments were performed using four biological replicates.

DNA Binding Site Selection

Binding site selection was performed using the CELD system (Xue, 2005; Balazadeh et al., 2011) with a pTac:MYB112-LCELD6xHis construct using biotin-labeled double-stranded oligonucleotides containing a central 30-nucleotide random sequence. MYB112-selected oligonucleotides were cloned. The cloned oligonucleotides were verified for the presence of MYB112 binding motif by DNA binding activity assays and sequenced.

EMSA

MYB112-LCELD6xHis fusion protein was expressed in *Escherichia coli* strain XL1 Blue and purified using 1-mL nickel columns (GE Healthcare) coupled to an Äkta-Purifier FPLC System (GE Healthcare). EMSAs using 5′-DY682-labeled DNA fragments were performed as reported (Wu et al., 2012).

ChIP-qPCR

To investigate in vivo binding of MYB112 to its DNA binding site in the promoters of *MYB32*, *MYB7*, *MYB6*, and *UGT84A2*, we used ChIP-qPCR using whole shoots from long day-grown, 2-week-old Arabidopsis plants expressing GFP-tagged MYB112 protein from the CaMV *35S* promoter (*35S:MYB112-GFP*). Wild-type plants were used as negative control. For ChIP, we followed a protocol previously described by Kaufmann et al. (2010) using anti-GFP antibody (μbeads; Miltenyi Biotec) to immunoprecipitate protein-DNA complexes. qPCR was used to test binding of MYB112 to its binding site within the selected promoters; the primers flanked the MYB112 binding site. As a negative control, we used primers annealing to promoter regions of two other Arabidopsis genes (At3g18040 and At2g22180) lacking an MYB112 binding site. Primer sequences are given in Supplemental Table S3. We analyzed ChIP-qPCR data relative to input, because these include normalization for both background levels and input chromatin going into the ChIP. The amount of genomic DNA coprecipitated by GFP antibody (ChIP signal) was calculated compared with the total input DNA used for each immunoprecipitation in the following way: cycle threshold (C_T) = C_T(ChIP) − C_T(input). To calculate fold enrichment, normalized ChIP signals were compared between *35S:MYB112-GFP* and wild-type plants, where the ChIP signal is given as the fold increase in signal relative to the background signal. Experiments were performed in three biological replicates.

Spectrophotometric Detection of Anthocyanins

To determinate the content of total anthocyanin, 30 mg of frozen plant tissue was ground in liquid nitrogen, mixed vigorously with 1 mL of extraction buffer (1% [v/v] HCl, 18% [v/v] 1-propanol, and 81% [v/v] water), and incubated at 98°C for 3 min. Samples were then incubated at room temperature in darkness for 10 to 12 h. Afterward, samples were centrifuged at 13,200*g* at room temperature for 20 min. For calculation, the following formula was used: ([optical density$_{535}$ − 0.25 optical density$_{650}$] × total volume of the extract (mL)/[weight of the dry leaf tissue (g) × 1,000]). As a blank, extraction buffer was used.

LC-MS Analysis of Anthocyanins

Anthocyanins and flavanols were extracted and analyzed exactly as described previously (Tohge and Fernie, 2010). Metabolites were evaluated by peak area of parental ion peaks processed using Xcalibur 2.1 software (Thermo Fisher Scientific). Obtained relative peak area was normalized by internal standard (isovitexin; CAS29702-25-8) and fresh weight.

Other Methods

Histochemical GUS assays were performed as described by Plesch et al. (2001). Pollen viability was analyzed using Stereomicroscope MZ 12.5 (Leica) after Alexander staining (Alexander, 1969). Cellular location of MYB112-GFP fusion protein was analyzed by confocal laser fluorescence microscopy (Eclipse E600 Microscope; Nikon).

Statistical Analyses

Unless otherwise specified, statistical analyses were performed using Student's *t* test embedded in the Microsoft Excel software. Only the return of $P < 0.05$ was designated as statistically significant.

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ACTIN2* (At3g18780), *MYB6* (At4g09460), *MYB7* (At2g16720), *MYB32* (At4g34990), *MYB112* (At1g48000), *At5MaT* (At3g29590), *UGT79B1* (*A3G2''XT*; At5g54060), *UGT84A2* (*SGT*; At3g21560), *UGT75C1* (*A5GT*; At4g14090), and *TT19* (*GLUTATHIONE S-TRANSFERASE26* and *GLUTATHIONE S-TRANSFERASE PHI12*; At5g17220). Additional accession numbers are given in Supplemental Table S3 and Supplemental Data Sets S1 and S2.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Integrated transcriptomics and metabolomics data.

Supplemental Figure S2. *MYB112* expression in *MYB112-IOE* plants after 1, 3, and 5 h of induction with ESTR.

Supplemental Figure S3. *MYB112* affects pollen viability.

Supplemental Figure S4. *MYB112* expression level in *MYB112* overexpression lines.

Supplemental Figure S5. Negative regulation of *MYB112* expression by MYB44.

Supplemental Table S1. MYB112-dependent up-regulated genes.

Supplemental Table S2. MYB112-dependent down-regulated genes.

Supplemental Table S3. Oligonucleotide sequences.

Supplemental Data Set S1. Expression of 94 secondary metabolite-associated genes in *MYB112-IOE* plants induced with ESTR for 5 d compared with mock-treated seedlings and in *myb112-1* seedlings compared with the wild type.

Supplemental Data Set S2. ATH1-based gene expression profiling upon short-term *MYB112* induction compared with mock-treated *MYB112-IOE* seedlings.

Supplemental Material S1. MYB112 binding sites in target promoters.

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