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Characterization of an activation-tagged mutant uncovers a role of GLABRA2 in anthocyanin biosynthesis in Arabidopsis

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SUMMARY

In Arabidopsis, anthocyanin biosynthesis is controlled by a MYB-bHLH-WD40 (MBW) transcriptional activator complex. The MBW complex activates the transcription of late biosynthesis genes in the flavonoid pathway, leading to the production of anthocyanins. A similar MBW complex regulates epidermal cell fate by activating the transcription of *GLABRA2* (*GL2*), a homeodomain transcription factor required for trichome formation in shoots and non-hair cell formation in roots. Here we provide experimental evidence to show that GL2 also plays a role in regulating anthocyanin biosynthesis in Arabidopsis. From an activation-tagged mutagenized population of Arabidopsis plants, we isolated a dominant, gain-of-function mutant with reduced anthocyanins. Molecular cloning revealed that this phenotype is caused by an elevated expression of *GL2*, thus the mutant was named *gl2-1D*. Consistent with the view that GL2 acts as a negative regulator of anthocyanin biosynthesis, *gl2-1D* seedlings accumulated less whereas *gl2-3* seedlings accumulated more anthocyanins in response to sucrose. Gene expression analysis indicated that expression of late, but not early, biosynthesis genes in the flavonoid pathway was dramatically reduced in *gl2-1D* but elevated in *gl2-3* mutants. Further analysis showed that expression of some MBW component genes involved in the regulation of late biosynthesis genes was reduced in *gl2-1D* but elevated in *gl2-3* mutants, and chromatin immunoprecipitation results indicated that some MBW component genes are targets of GL2. We also showed that GL2 functions as a transcriptional repressor. Taken together, these results indicate that GL2 negatively regulates anthocyanin biosynthesis in Arabidopsis by directly repressing the expression of some MBW component genes.

Keywords: GLABRA2, anthocyanin, flavonoid, transcription factor, *Arabidopsis thaliana*.

INTRODUCTION

In Arabidopsis, several different processes including epidermal cell-fate determination, mucilage biosynthesis and anthocyanin biosynthesis are regulated by similar transcriptional regulatory networks (Schiefelbein, 2003; Petroni and Tonelli, 2011; Lin and Aoyama, 2012; Patra *et al.*, 2013; Schiefelbein *et al.*, 2014). In these networks, an R2R3 MYB transcription factor, a bHLH transcription factor, and the WD40-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1) form a MYB-bHLH-WD40 (MBW) transcriptional activator complex to regulate the expression of downstream target genes (Schiefelbein, 2003; Petroni and Tonelli, 2011; Lin and Aoyama, 2012; Patra *et al.*, 2013; Schiefelbein *et al.*, 2014).

Anthocyanins are produced by a specific branch of the flavonoid pathway (Petroni and Tonelli, 2011). Anthocyanin biosynthesis is the most extensively studied secondary metabolic pathway in plants. More than 10 Arabidopsis biosynthesis genes have been identified to be involved in anthocyanin biosynthesis pathway (Petroni and Tonelli, 2011; Patra *et al.*, 2013). In the initial steps of the flavonoid pathway, enzymes encoded by *PHENYLALANINE AMMONIA LYASE* (*PAL*), *CINNAMIC ACID 4-HYDROXYLASE* (*C4H*)

and *4 COUMARATE CoA LIGASE* (*4CL*) catalyze sequentially the metabolism of phenylalanine to coumaroyl-CoA (Ohl *et al.*, 1990; Lee *et al.*, 1995; Bell-Lelong *et al.*, 1997). Coumaroyl-CoA is then used by enzymes encoded by *CHALCONE SYNTHESIS* (*CHS*) to produce naringenin chalcone (Feinbaum and Ausubel, 1988), which is further isomerized to flavanones by enzymes encoded by *CHALCONE ISOMERASE* (*CHI*) (Shirley *et al.*, 1992). From this step onward, the pathway diverges to different branches to form different classes of flavonoids. In the anthocyanin biosynthesis branch, flavanones are converted to dihydroflavonols by enzymes encoded by *FLAVANONE 3-HYDROXYLASE* (*F3H*) (Pelletier and Shirley, 1996). Dihydroflavonols are converted to flavan-3,4-diols (leucoanthocyanins) by enzymes encoded by *DIHYDROFLAVONOL REDUCTASE* (*DFR*) (Shirley *et al.*, 1992), and these are then converted to anthocyanins by enzymes encoded by *ANTHOCYANIDIN SYNTHASE*/*LEUCOANTHOCYANIDIN DIOXYGENASE* (*ANS*/*LDOX*) and *UDP-FLAVONOID GLUCOSYL TRANSFERASE* (*UFGT*) (Pelletier *et al.*, 1997; Wilmouth *et al.*, 2002; Abrahams *et al.*, 2003; Xie *et al.*, 2003; Yoo *et al.*, 2011).

The expression of early biosynthesis genes (EBGs) in anthocyanin biosynthesis pathway, including *CHS*, *CHI*, and *F3H*, is regulated by three functionally redundant R2R3 MYB transcription factors, MYB11, MYB12 and MYB111 (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007; Li, 2014). The expression of late biosynthesis genes (LBGs) including *F3′H*, *DFR*, *ANS*/*LDOX* and *UFGT*, on the other hand, is regulated by an MBW transcriptional activator complex (Gonzalez *et al.*, 2008; Petroni and Tonelli, 2011; Li, 2014; Xu *et al.*, 2014).

The MBW complex that regulates anthocyanin biosynthesis genes is formed by an R2R3 MYB transcription factor (PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1), PAP2, MYB113 or MYB114), a bHLH transcription factor (TRANSPARENT TESTA 8 (TT8), GLABRA3 (GL3) or ENHANCER OF GLABRA3 (EGL3)), and the WD40-repeat protein TTG1 (Walker *et al.*, 1999; Borevitz *et al.*, 2000; Nesi *et al.*, 2000; Payne *et al.*, 2000; Zhang *et al.*, 2003; Gonzalez *et al.*, 2008). The R2R3 MYB transcription factor PAP1, PAP2, MYB113 and MYB114, and the WD40-repeat protein TTG1 all physically interacted with GL3, EGL3 and TT8 in yeast two-hybrid assays (Zhang *et al.*, 2003; Zimmermann *et al.*, 2004), indicating that these transcription factors can form multiple MBW complexes to regulate the expression of anthocyanin biosynthesis genes.

The components of these MBW complexes, including TTG1, GL3, EGL3 and TT8, are also involved in the regulation of cell-fate determination and/or mucilage biosynthesis in Arabidopsis by forming different MBW complexes with other R2R3 MYBs (Walker *et al.*, 1999; Payne *et al.*, 2000; Bernhardt *et al.*, 2003; Zhang *et al.*, 2003; Lin and Aoyama, 2012). For example, GL3 or EGL3, TTG1, and the R2R3 MYB-type transcription factor GLABRA1 (GL1) form a MBW activator complex to regulate trichome formation (Herman and Marks, 1989; Payne *et al.*, 2000; Esch *et al.*, 2003; Schiefelbein, 2003; Pesch and Hülskamp, 2004, 2009; Ishida *et al.*, 2008); GL3 or EGL3, TTG1, and the R2R3 MYB-type transcription factor WEREWOLF (WER) form a MBW activator complex to regulate root hair and hypocotyl stomata formation (Hülskamp *et al.*, 1994; Hung *et al.*, 1998; Lee and Schiefelbein, 1999; Schellmann *et al.*, 2002; Esch *et al.*, 2003; Schiefelbein, 2003; Pesch and Hülskamp, 2004, 2009; Ishida *et al.*, 2008); whereas EGL3 or TT8, TTG1, and the R2R3 MYB transcription MYB5 or TRANSPARENT TESTA 2 (TT2), form a MBW activator complex to regulate mucilage biosynthesis (Nesi *et al.*, 2001; Western *et al.*, 2001; Zhang *et al.*, 2003; Baudry *et al.*, 2006; Gonzalez *et al.*, 2009; Li *et al.*, 2009; Xu *et al.*, 2014).

As mentioned above, the MBW activator complex regulates anthocyanin biosynthesis by directly activating the expression of the anthocyanin biosynthesis genes. However, the MBW activator complexes that regulate cell-fate determination and mucilage synthesis do so by inducing the expression of a common gene, *GLABRA2* (*GL2*) (Schiefelbein, 2003; Lin and Aoyama, 2012; Schiefelbein *et al.*, 2014), which encodes a homeodomain protein (Rerie *et al.*, 1994; Di Cristina *et al.*, 1996). GL2 positively regulates trichome formation and mucilage synthesis (Rerie *et al.*, 1994). GL2 also positively regulates non-hair cell formation in root, and non-stomata cells formation in hypocotyl, leading to the inhibition of root hair and hypocotyl stomata formation (Di Cristina *et al.*, 1996; Hung *et al.*, 1998).

In addition to activating the expression of *GL2*, the same MBW activator complex also regulates the expression of single-repeat R3 MYB genes (Schiefelbein, 2003; Morohashi *et al.*, 2007; Wang *et al.*, 2008; Zhao *et al.*, 2008; Gan *et al.*, 2011; Schiefelbein *et al.*, 2014; Wang and Chen, 2014). Single-repeat R3 MYB transcription factors, in turn, compete with R2R3 MYB transcription factors for binding the bHLH transcription factors, thus blocking the formation of the MBW activator complexes, and resulting in the inhibition of *GL2* expression (Hülskamp *et al.*, 1994; Schellmann *et al.*, 2002; Esch *et al.*, 2003; Schiefelbein, 2003; Pesch and Hülskamp, 2004, 2009; Ishida *et al.*, 2008; Schiefelbein *et al.*, 2014; Wang and Chen, 2014). In recent years, single-repeat R3 MYB transcription factors have also been found to regulate anthocyanin biosynthesis by directly inhibiting the activity of the MBW complex on biosynthesis genes (Zhu *et al.*, 2009; Nemie-Feyissa *et al.*, 2014). However, it remains unclear if GL2 is involved in the regulation of anthocyanin biosynthesis.

Although the MBW activator complexes regulate cell-fate determination and mucilage synthesis by activating the common gene *GL2*, no common regulators downstream of *GL2* have been identified. However, large-scale gene expression analyses indicate that a large number genes are regulated downstream of *GL2* during epidermal cell-fate determination (Lieckfeldt *et al.*, 2007; Marks *et al.*,

2008; Won *et al.*, 2009; Bruex *et al.*, 2012). A few downstream genes have been identified as direct targets of GL2. *PLDζ1*, an Arabidopsis phospholipase D (PLD) gene was the first identified GL2 target (Ohashi *et al.*, 2003). *In vitro* protein-DNA-binding analyses have identified an L1-box sequence TAAATGTT in the promoter region of *PLDζ1* as a GL2 binding sequence (Ohashi *et al.*, 2003). Two polysaccharide-metabolizing genes, *CELLULOSE SYNTHASE5* (*CESA5*) and *XYLOGLUCAN ENDOTRANSGLUCOSYLASE17* (*XTH17*) have also been identified as direct targets of GL2 (Tominaga-Wada *et al.*, 2009). Yeast one-hybrid analysis showed that GL2 binds to the L1-box sequence TAAATGTA present in the *CESA5* and *XTH17* promoters (Tominaga-Wada *et al.*, 2009). Recently, *MYB23* has been identified as a direct target of GL2, and chromatin immunoprecipitation (ChIP) assay showed that GL2 binds to the L1-box sequence in its promoter (Khosla *et al.*, 2014).

We report here the identification of GL2 as a regulator of anthocyanin biosynthesis. Our results show that GL2 is a transcription repressor, and that GL2 negatively regulates anthocyanin biosynthesis by directly repressing the expression of some MBW activator complex component genes.

RESULTS

The *gl2-1D/gpa1-2* mutants accumulate less anthocyanins

In an attempt to identify genetic interactors of Arabidopsis heterotrimeric G-protein α subunit (GPA1), we generated an activation-tagged mutagenized population of Arabidopsis plants using *gpa1-2*, a null mutant allele of *GPA1* as parental genotype (Ullah *et al.*, 2001). From the mutagenized population, we identified a dominant mutant with reduced anthocyanin accumulation (Figure 1a). Molecular cloning indicates that the *GLABRA2* (*GL2*) gene had been tagged (see next section for details), thus the mutant was named *gl2-1 Dominant* (*gl2-1D*)/*gpa1-2.*

Compared with Ws wild type plants and *gpa1-2* mutants, *gl2-1D*/*gpa1-2* mutants accumulated less anthocyanins in stems, petioles, and rosette leaves (Figure 1a). As exogenous sugars have been shown to cause anthocyanin levels to increase in Arabidopsis seedlings (Tsukaya *et al.*, 1991; Teng *et al.*, 2005), we further confirmed our observation by examining anthocyanin accumulation in the *gl2-1D*/*gpa1-2* mutant seedlings in the presence or absence of 3% sucrose. As shown in Figure 1(b), anthocyanin accumulation in *gl2-1D/gpa1-2* mutants is indistinguishable from that in *gpa1-2* mutants or Ws wild type seedlings in the absence of sucrose. However, in the presence of sucrose, *gl2-1D/gpa1-2* mutants accumulated less anthocyanins.

Quantitative analysis showed that the anthocyanin level in the *gl2-1D/gpa1-2* mutants in the presence of sucrose is about half of that in Ws wild type or *gpa1-2* mutant seedlings, and that the anthocyanin level in *gpa1-2* mutants is indistinguishable from that in Ws wild type seedlings (Figure 1c).

Identification of T-DNA insertion site and recapitulation of the *gl2-1D* phenotypes

By using thermal asymmetric interlaced (TAIL)-PCR, we identified that the T-DNA in the *gl2-1D/gpa1-2* mutant was inserted in chromosome 1 at a position that is 1698 bp upstream of the start codon of *GOLGIN CANDIDATE 5* (*GC5*) gene, and 1886 bp upstream of the start codon of the *GL2* gene, with the four outward-facing *35S* enhancers facing *GL2* (Figure 2a). RT-PCR results indicated that the expression of *GL2*, but not *GC5*, was elevated in the tagged mutant (Figure 2b), indicating that the *GL2* gene was tagged. Thus, the mutant was named *gl2-1D*/*gpa1-2.*

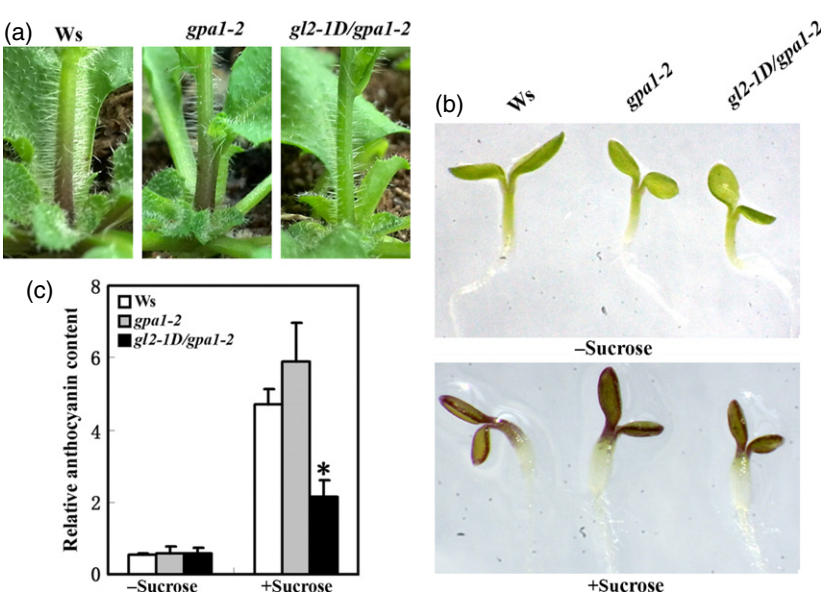

Figure 1. *gl2-1D*/*gpa1-2* mutant is a gain-of-function dominant mutant with reduced anthocyanin accumulation.
(a) Anthocyanin accumulation in Ws (left), *gpa1-2* mutant (middle) and *gl2-1D*/*gpa1-2* mutant (right) plants. Photographs were taken from approximately 1-month-old plants.
(b) Anthocyanin accumulation in Ws (left), *gpa1-2* mutant (middle) and *gl2-1D*/*gpa1-2* mutant (right) seedlings in the absence (top) or presence (bottom) of 3% sucrose. Photographs were taken from 6-day-old seedlings.
(c) Relative anthocyanin contents in Ws, *gpa1-2* mutant and *gl2-1D*/*gpa1-2* mutant seedlings in the presence or absence of 3% sucrose. Data represent mean ± standard deviation (SD) of three biological repeats. *Significantly different from Ws wild type ($P < 0.001$).

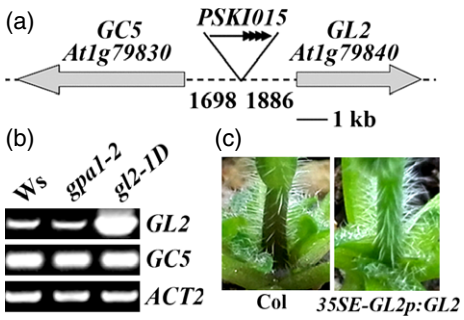

Figure 2. Identification of the activation-tagged T-DNA insertion site in the *gl2-1D*/*gpa1-2* mutant.
(a) Diagram illustrating the T-DNA insertion site in the *gl2-1D*/*gpa1-2* mutant. The orientation of the four *35S* enhancer repeats in the T-DNA situated 1886 bp upstream of the start condon of *GL2* is indicated by arrowheads.
(b) Transcript levels of *GL2* and *GC5* in Ws wild type, *gpa1-2* mutant and *gl2-1D*/*gpa1-2* mutant seedlings. RNA was isolated from 10-day-old seedlings and RT-PCR was used to examine the expression of *GL2* and *GC5*. *ACTIN2* was used as a control.
(c) Anthocyanin accumulation in Col (left) wild type and *35SE-GL2p:GL2* transgenic plants (right). Photographs were taken from approximately 1-month-old plants.

To examine whether the *gpa1-2* mutant background might be related to the phenotypes observed in *gl2-1D*/*gpa1-2* mutants, we crossed the mutant with Col wild type plants. Col rather than Ws was chosen because we wished to obtain a *gl2-1D* mutant in the Col genetic background, so that we could directly compare the difference between *gl2-1D* and *gl2-3*, a *GL2* null mutant in the Col background (Wang *et al.*, 2010). We found that all F1 plants accumulated less anthocyanins, and an ~3:1 (plants accumulating less anthocyanins:plants with normal anthocyanin accumulation) segregation ratio of the phenotypes was observed in the F2 generation, suggesting that the phenotype was caused by a single activation-tagged T-DNA insertion, and is independent of the *gpa1-2* mutant background. One of the *gl2-1D* mutants with longer leaves similar to Col wild type plants and without a T-DNA insertion in *GPA1* gene was selected from the F2 generation, re-confirmed in the F3 to F4 generations, and used for in-depth analysis.

To further confirm the anthocyanin accumulation phenotype is caused by elevated expression of *GL2*, a fragment containing the four outward-facing *35S* enhancers and the 1886 bp upstream sequence of *GL2* (*35SE-GL2p*) was cloned from the *gl2-1D*/*gpa1-2* mutant genomic DNA, and used to drive the expression of *GL2* in Col wild type plants. As shown in Figure 2(c), *35SE-GL2p:GL2* transgenic plants recapitulated the *gl2-1D*/*gpa1-2* mutant phenotypes.

The *gl2-3* mutants accumulate more anthocyanins

The results described above indicate that GL2 is involved in the regulation of anthocyanin synthesis (Figures 1 and 2). To further dissect the role of GL2 in anthocyanin biosynthesis in Arabidopsis, we examined anthocyanin accumulation in *gl2-3* mutants by growing them side-by-side with *gl2-1D* mutants and Col wild type plants in soil pots in a growth chamber under identical growth conditions. As shown in Figure 3(a), *gl2-3* mutants accumulated more, whereas *gl2-1D* mutants accumulated less, anthocyanins in stems, petioles, and rosette leaves. Interestingly, others processes known to be regulated by GL2, including trichome formation, root hair formation, and seed mucilage production, remain largely unchanged in *gl2-1D* mutants when compared with Col wild type plants (Figure S1).

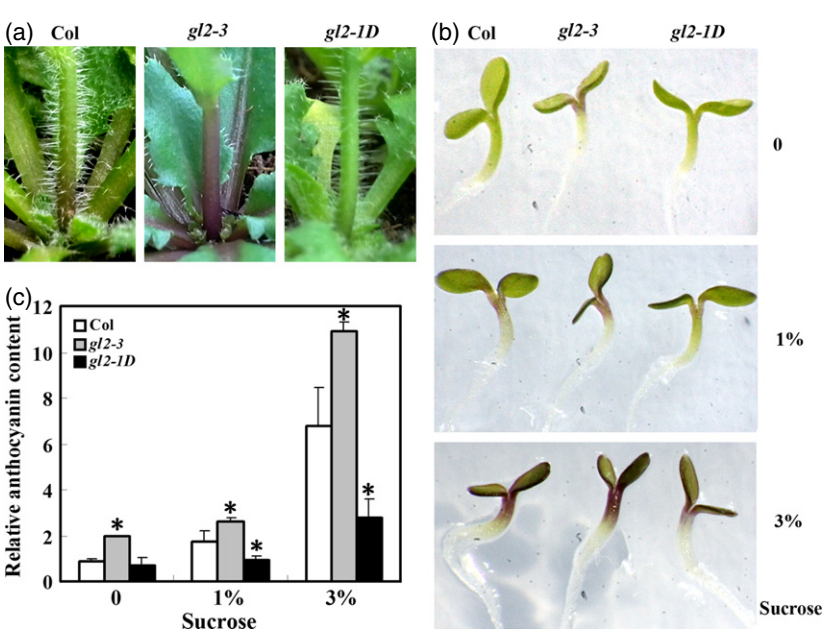

Figure 3. Anthocyanin accumulation in Col wild type, *gl2-3* and *gl2-1D* mutants in response to sucrose.
(a) Anthocyanin accumulation in Col (left), *gl2-3* mutant (middle) and *gl2-1D* mutant (right) plants. Photographs were taken from approximately 1-month-old plants.
(b) Anthocyanin accumulation in Col (left), *gl2-3* mutant (middle) and *gl2-1D* mutant (right) seedlings in the absence (top) and presence of 1% (middle) and 3% sucrose (bottom). Photographs were taken from 6-day-old seedlings.
(c) Relative anthocyanin contents in Col, *gl2-3* mutant and *gl2-1D* mutant seedlings in the absence or presence of sucrose. Data represent the mean ± standard deviation (SD) of three biological repeats. *Significantly different from Col wild type ($P < 0.001$).

When grown on ½MS medium in the absence of sucrose, anthocyanin accumulation in *gl2-1D* mutants was indistinguishable from that in Col wild type seedlings (Figure 3b), a result similar to that observed in *gl2-1D*/*gpa1-2* mutants (Figure 1b). However, *gl2-3* mutants accumulated more anthocyanins (Figure 3b). Quantitative analysis showed that the anthocyanin content in *gl2-3* mutants was about twice that seen in Col wild type seedlings, or in *gl2-1D* mutants (Figure 3c). The presence of sucrose promoted anthocyanin accumulation in all the plants tested, however, *gl2-3* still accumulated more whereas *gl2-1D* accumulated less anthocyanins in the presence of either 1 or 3% sucrose when compared with that in Col wild type seedlings (Figure 3b,c).

Expression of biosynthesis genes in the anthocyanin pathway is reduced in *gl2-1D* and elevated in *gl2-3* mutants

More than 10 biosynthesis genes have been identified to be involved in the anthocyanin biosynthesis pathway. According to their functions in the pathway, these genes have been divided into two different groups: EBGs and LBGs. To investigate how GL2 regulates anthocyanin synthesis, we examined the expression of the EBGs and LBGs in *gl2-1D* and *gl2-3* mutant plants by using quantitative RT-PCR (qRT-PCR). As shown in Figure 4, the expression level of *DFR*, *ANS* and *UF3GT* decreased about 10-fold in *gl2-1D* mutants and increased more than six-fold in *gl2-3* mutants, while only about three-fold increase in *gl2-1D* mutants and less than two-fold decrease in *gl2-3* mutants were observed for the expression of all the other genes examined. These results indicate that GL2 may mainly regulate the expression of LBGs in the anthocyanin biosynthesis pathway.

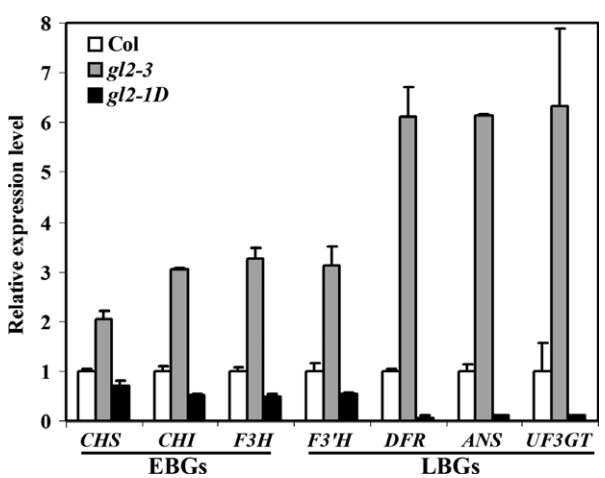

Figure 4. Expression of the biosynthesis genes in the flavonoid pathway in Col wild type, *gl2-3* mutant and *gl2-1D* mutant seedlings.
Quantitative RT-PCR analysis of EBGs and LBGs in the flavonoid pathway. RNA was isolated from 6-day-old seedlings and qRT-PCR was used to examine the expression of the biosynthesis genes in the flavonoid pathway. Expression of *ACTIN2* was used as a reference gene, and expression of each gene in wild type was set as 1. Data represent the mean ± standard deviation (SD) of three replicates. EBGs, early biosynthesis genes; LBGs, late biosynthesis genes.

GL2 directly regulates the expression of some MBW component genes

In Arabidopsis, the biosynthesis genes in the flavonoid pathway are regulated by different transcription factors. The expression of EBGs is controlled by several R2R3 MYB transcription factors, while the expression of LBGs is regulated by the MBW transcriptional activator complex (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007; Petroni and Tonelli, 2011; Li, 2014; Xu *et al.*, 2014). Because we have shown that the expression of the LBGs tested was most strongly affected in both *gl2-3* and *gl2-1D* mutants (Figure 4), we wanted to further examine whether GL2 may regulate the expression of component genes of the MBW complex. We examined the expression of an array of known MBW component genes, including *TTG1*, *PAP1*, *PAP2*, *MYB113*, *MYB114*, *TT8*, *GL3* and *EGL3* by using qRT-PCR. The results showed that the expression level of *PAP1*, *PAP2*, *MYB113*, *MYB114* and *TT8* decreased approximately 2-fold to 15-fold in *gl2-1D* mutants and increased approximately 2-fold to 4-fold in *gl2-3* mutants when compared with that in Col wild type plants (Figure 5a). Conversely, the expression of *TTG1*, *GL3* and *EGL3* remained largely unchanged in *gl2-1D* or *gl2-3* mutants compared with that in Col wild type plants (Figure 5a). These results suggested that GL2 may regulate the expression of *TT8*, *PAP1*, *PAP2*, *MYB113* and *MYB114*, and that the resulting impact on formation of MBW complexes could in turn, affect the expression of late biosynthesis genes in the anthocyanin pathway.

So far, several genes, including *PLDζ1*, *CESA5*, *XTH17* and *MYB23* have been identified as direct targets of GL2 (Ohashi *et al.*, 2003; Tominaga-Wada *et al.*, 2009; Khosla *et al.*, 2014). *In vitro* protein–DNA-binding analysis has identified the L1-box sequence, TAAATGTT, as the GL2 binding sequence in the *PLDζ1* promoter (Ohashi *et al.*, 2003), while yeast one-hybrid analysis has confirmed the ability of GL2 to bind to the L1-box sequence TAAATGTA in the *CESA5* promoter (Tominaga-Wada *et al.*, 2009), and ChIP assay has showed GL2 bind to the L1-box in *MYB23* promoter (Khosla *et al.*, 2014). To determine whether any of the MBW component genes whose expression is altered in the *gl2-3* and *gl2-1D* mutants might be direct targets of GL2, we scanned their promoter regions (2000 bp DNA sequence upstream of the ATG start codon) for the presence of TAAATGTT/A L1-box sequence. We found that there is a TAAATGTT L1-box sequence in the promoter regions of *MYB113* and *TT8*, and a TAAATGTA L1-box sequence in the *PAP2* promoter (Figure 5b). These results suggest that GL2 has the potential to directly regulate the expression of *TT8*, *MYB113* and *PAP2* by binding to the L1-box in the promoter regions of these genes.

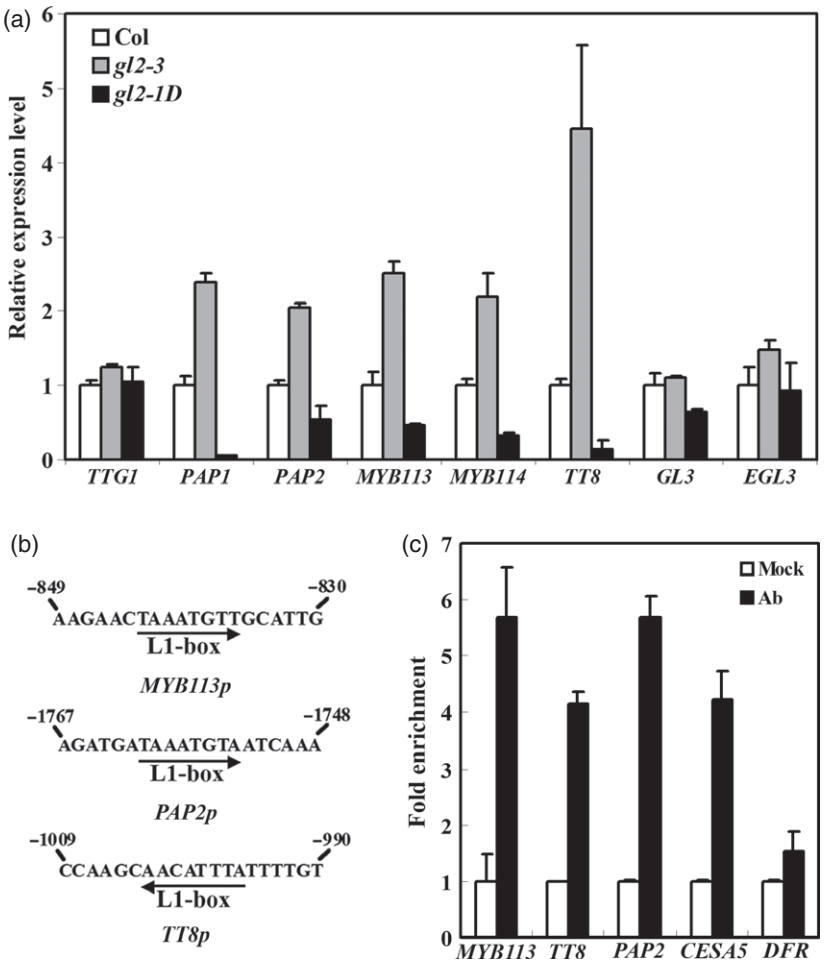

Figure 5. Expression of MBW activator complex component genes in Col wild type, *gl2-3* mutant and *gl2-1D* mutant seedlings.
(a) Quantitative RT-PCR analysis of MBW component genes in Col, *gl2-3* and *gl2-1D* mutant seedlings. RNA was isolated from 6-day-old seedlings, and qRT-PCR was used to examine the expression of the MBW component genes in the flavonoid pathway. Expression of *ACTIN2* was used as a reference gene, and expression of each gene in wild type was set as 1. Data represent the mean ± standard deviation (SD) of three replicates.
(b) TAAATGTT/A L1-box in the promoter regions of some MBW activator complex component genes. L1-box sequences are indicated by arrows, and numbers indicate the nucleotide position relative to the start codon of the gene.
(c) Chromatin immunoprecipitation (ChIP) analysis. ChIP was performed with *35SE-GL2p:GL2* transgenic plants using anti-HA antibodies. Rabbit preimmune serum was used as mock control. Primer sets specific for the L1 box promoter region of the MBW component genes were used in PCR reaction. *CESA5* served as a positive, and *DFR* as a negative control. Data represent the mean ± standard deviation (SD) of three replicates.

To test this hypothesis directly, we used ChIP assays to detect the association of GL2 protein with the L1-box sequences of these genes. Indeed, an about four-fold to six-fold enrichment was detected by using primers spanning the L1-box sequence in the promoter regions of *MYB113*, *PAP2* and *TT8.* As expected, an approximately four-fold enrichment was also obtained for L1-box sequence region in the promoter of *CESA5*, but not for a region in the promoter of *DFR* (Figure 5c).

GL2 represses reporter gene expression in transfected protoplasts

Opposite roles for GL2 in the regulation of different aspects of plant growth and development, as well as in the regulation of its target genes expression, have been reported before. For example, GL2 functions as a positive regulator in the regulation of trichome formation, but as a negative regulator in the regulation of root hair formation (Rerie *et al.*, 1994; Di Cristina *et al.*, 1996; Hung *et al.*, 1998). Both *CESA5* and *XTH17* are direct target genes of GL2, but GL2 negatively regulated the expression of *CESA5* while acting as a positive regulator of *XTH17* (Tominaga-Wada *et al.*, 2009). However, none of the previous studies has directly examined the transcriptional activities of GL2.

To determine if GL2 functions as a transcriptional activator, GL2 was fused to an N-terminal *Saccharomyces cerevisiae* GAL4 DNA-binding domain (GD), and co-transfected with a plasmid containing a GUS reporter gene with GD binding sites (*Gal4:*GUS) into Arabidopsis mesophyll protoplasts. GD alone and a transactivator gene encoding a chimeric protein consisting of the GD fused to the herpes simplex virus VP16 activation domain (GD-VP) were co-transfected as controls. The results showed that co-transfection with GD-VP resulted in strong activation of the reporter gene whereas co-transfection with either GD alone or GD-GL2 had little, if any, effect on the expression of the reporter gene (Figure S2), indicating that GL2 itself may not function as a transcriptional activator.

We then tested whether GL2 functions as a transcriptional repressor in protoplast transfection assays by using a GUS reporter gene with both *LexA* and *Gal4* DNA-binding sites (*LexA-Gal4:*GUS). GD-GL2 was co-transfected with the *LexA-Gal4:*GUS reporter gene and a transactivator gene encoding a chimeric protein consisting of the LexA

DNA-binding domain (LD) fused to the VP16 activation domain (LD-VP). GD alone and GD-OFP1, a transcriptional repressor (Wang *et al.*, 2007; Gan *et al.*, 2011) were co-transfected as controls. As shown in Figure 6, co-transfection of the LD-VP16 transactivator gene and the effector gene encoding only the GD resulted in strong activation of the reporter gene. Co-transfection of LD-VP16 with GD-GL2 or GD-OFP1, on the other hand, resulted in an approximately six-fold decrease in the expression of the reporter gene (Figure 6). These results indicate that GL2 is a transcriptional repressor.

DISCUSSION

The WD40 repeat protein TTG1 and the bHLH transcription factors GL3, EGL3 and TT8 are shared among different MBW complexes that have been shown to be involved in the regulation of anthocyanin biosynthesis (Walker *et al.*, 1999; Nesi *et al.*, 2000; Payne *et al.*, 2000; Zhang *et al.*, 2003). Here we provide evidence that GL2, a downstream target of the MBW complexes that help to regulate the trichome formation, root hair formation and seed mucilage synthesis processes (Schiefelbein, 2003; Lin and Aoyama, 2012; Schiefelbein *et al.*, 2014), and a regulator of seed oil production (Shen *et al.*, 2006; Shi *et al.*, 2012), is also involved in the regulation of anthocyanin biosynthesis.

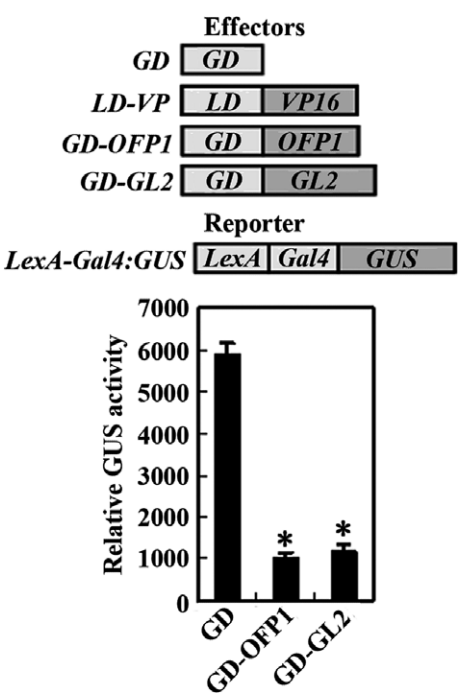

Figure 6. GL2 is a transcriptional repressor.
Protoplasts were isolated from 3 to 4-week-old Arabidopsis rosette leaves. Effectors and reporter (diagrammed on the top of the figure) plasmids were co-transfected into protoplasts. The protoplasts were incubated in darkness for 20–22 h, and then GUS activity was assayed. GD-OFP1 was used as control. Data represent the mean ± standard deviation (SD) of three biological repeats. *Significantly different from GD control ($P < 0.001$).

GL2 is a negative regulator of anthocyanin biosynthesis

GL2 regulates epidermal cell-fate determination and mucilage biosynthesis downstream of a MBW activator complex consisting of an R2R3 MYB transcription factor, a bHLH transcription factor, and the WD40 repeat protein TTG1 (Schiefelbein, 2003; Lin and Aoyama, 2012; Schiefelbein *et al.*, 2014). A similar MBW activator complex regulates anthocyanin biosynthesis (Walker *et al.*, 1999; Zhang *et al.*, 2003; Gonzalez *et al.*, 2008). We found that GL2 acts as a negative regulator of anthocyanin biosynthesis, a conclusion that is supported by several lines of evidence. First, plants with elevated expression of *GL2* have reduced anthocyanin levels. Anthocyanin accumulation in the *gl2-1D*/*gpa1-2* mutant is greatly reduced (Figure 1) and *35SE-GL2p:GL2* transgenic plants recapitulated the *gl2-1D* mutant phenotypes (Figure 2c). Second, the loss-of-function mutant *gl2-3* accumulated more anthocyanins when compared with Col wild type plants (Figure 3). Third, *gl2-3* mutants accumulated more, while *gl2-1D* mutants accumulated less anthocyanins in the present of sucrose (Figure 3). Taken together, these results strongly suggest that GL2 is a negative regulator of anthocyanin biosynthesis. It should be note that anthocyanin accumulation pattern in the *gl2-3* mutants (Figure 3a) is not completely positively correlated with *GL2* expression pattern as described previously (Wang *et al.*, 2010), possibly due to anthocyanin transport, and/or different expression patterns of transcription factor genes involved in the regulation of anthocyanin biosynthesis genes.

Considering that GL2 is activated by MBW complex involved in the regulation of cell-fate determination and mucilage biosynthesis, and some components of the MBW complex including TTG1, GL3 and EGL3 also involve in the regulation of anthocyanin biosynthesis in Arabidopsis (Figure 7), it is likely that GL2 function as a feed back loop to control anthocyanin biosynthesis.

GL2 is a transcriptional repressor

GL2 is homeodomain transcription factor that regulates several aspects of plant growth and development (Rerie *et al.*, 1994; Di Cristina *et al.*, 1996; Hung *et al.*, 1998). Available evidence suggests that expression of many genes may be affected by GL2, but only a few have been demonstrated to be direct targets of GL2 (Ohashi *et al.*, 2003; Lieckfeldt *et al.*, 2007; Marks *et al.*, 2008; Tominaga-Wada *et al.*, 2009; Won *et al.*, 2009; Bruex *et al.*, 2012; Khosla *et al.*, 2014). By using Arabidopsis protoplast transfection assays, we showed that, when recruited to the promoter region of the *LexA-Gal4:*GUS reporter by a fused DNA-binding domain (GD), GL2 repressed the expression of the reporter gene activated by a co-transfected LD-VP

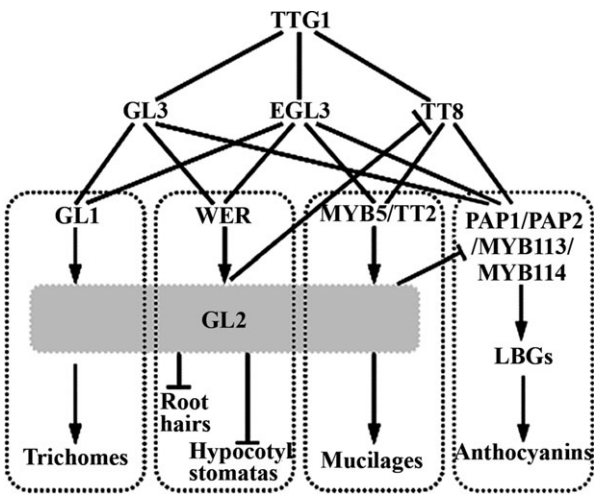

Figure 7. Illustration of the role of GL2 in the regulation of cell fate determination, mucilage and anthocyanin biosynthesis.
A MBW activator complex consisting of the WD40 repeat protein TTG1, a bHLH transcription factor and an R2R3 MYB transcription factor regulates cell fate determination including trichome formation, root hair formation, hypocotyl stomata formation, and mucilage biosynthesis by activating the expression of *GL2*. A similar MBW activator complex regulates anthocyanin biosynthesis by activating the expression of late biosynthesis genes in the flavonoid pathway. Results in this study indicate that GL2 negatively regulates anthocyanin biosynthesis by repressing the expression of some MBW component genes. It should note that GL2 is also involved in seed oil production (Shen *et al.*, 2006; Shi *et al.*, 2012), however, it remains unclear if the MBW activator complex is involved in the process, so the regulation of seeds oil production is not included in this diagram. Lines indicate protein–protein interaction; arrows indicate positive regulation; lines with blocks indicate negative regulation.

activator (Figure 6). The degree of suppression of the reporter by GL2 is similar to that achieved by a known active repressor, AtOFP1 (Wang *et al.*, 2007; Gan *et al.*, 2011). These results indicated that GL2 is a transcriptional repressor.

We also found in the GL2 sequence an LxLxL motif adjacent to the homeodomain, a domain that is conserved in plant homeodomain transcription factor family proteins (www.phytozome.net). The LxLxL motif is conserved in Aux/IAA and ERF transcription repressors, where it is required for their repression functions (Ohta *et al.*, 2001; Tiwari *et al.*, 2004), and the motif is also present in the AtOFP1 protein (Wang *et al.*, 2007). It will be of interest to test whether the LxLxL motif is also required for the repression function of GL2.

Identification of GL2 as a transcriptional repressor may open further avenues of investigation into the biological functions of GL2, i.e. how does GL2 promote trichome formation and mucilage biosynthesis, and how does GL2 regulate its direct targets? Considering the opposing roles of GL2 in the regulation of its known target genes (Ohashi *et al.*, 2003; Tominaga-Wada *et al.*, 2009; Khosla *et al.*, 2014), it is very likely that GL2 could interact with other regulator proteins to activate some of its target genes. Alternatively, an apparent activation may also result from a repression effect of GL2 on some other transcription repressors involved in the regulation of its targets.

Some of the MBW activator complex component genes are direct targets of GL2

Previous studies have shown that GL2 is involved in the regulation of epidermal cell-fate determination and mucilage biosynthesis downstream of a MBW activator complex (Hülskamp *et al.*, 1994; Schellmann *et al.*, 2002; Esch *et al.*, 2003; Schiefelbein, 2003; Pesch and Hülskamp, 2004, 2009; Ishida *et al.*, 2008; Schiefelbein *et al.*, 2014; Wang and Chen, 2014). However, it is still unclear how GL2 regulates those processes. We previously showed that GL2 may play a complex role in trichome formation in Arabidopsis (Wang *et al.*, 2010). Indeed, large-scale gene expression analyses have identified a large number of genes that appears to be regulated downstream of GL2 (Lieckfeldt *et al.*, 2007; Marks *et al.*, 2008; Won *et al.*, 2009; Bruex *et al.*, 2012), but so far only a few have been identified as direct target genes of GL2 (Ohashi *et al.*, 2003; Tominaga-Wada *et al.*, 2009; Khosla *et al.*, 2014). The L1-box sequence TAAATGTT/A has been identified as the GL2 binding sequence in some of these target genes (Ohashi *et al.*, 2003; Tominaga-Wada *et al.*, 2009; Khosla *et al.*, 2014).

In an attempt to investigate the mechanism underlying the regulation of anthocyanin biosynthesis by GL2, we examined the expression of anthocyanin biosynthesis genes in *gl2-3* and *gl2-1D* mutants, and found that the expression of LBGs was altered in a relative higher degree in the mutants (Figure 4). We hypothesized that GL2 may regulate the expression of components of the MBW complex. Indeed, the expression of some of the component genes was down-regulated in the *gl2-1D* mutant, but up-regulated in the *gl2-3* mutant (Figure 5a). The presence of TAAATGTT/A L1-box sequence in the promoter regions of some of the MBW complex component genes (Figure 5b) suggested that they may be direct target genes of GL2. ChIP assay results indicated that this is indeed the case (Figure 5c). However, as the expression of EBGs was also altered (although to lesser degree) in the mutants (Figure 4), we are unable to rule out the possibility that GL2 may also be involved in the regulation of other regulator genes.

In summary, our results suggest that in addition to regulating epidermal cell-fate determination, mucilage biosynthesis (Figure 7), and seed oil production, GL2 regulates anthocyanin biosynthesis in Arabidopsis. We have established that GL2 is a transcriptional repressor, and that GL2 suppresses anthocyanin biosynthesis by directly repressing the expression of some MBW activator complex component genes (Figure 7).

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The *gpa1-2* mutant is in the Ws ecotypic background (Ullah *et al.*, 2001), and *gl2-3* mutant is in the Columbia-0 (Col) ecotypic background (Wang *et al.*, 2010). The *gl2-1D*/*gpa1-2* mutant was identified from an activation-tagged mutagenized population of Arabidopsis plants in *gpa1-2* background. The *gl2-1D* mutant was obtained by crossing the *gl2-1D*/*gpa1-2* mutant with a Col wild type plant, and examining the F2 progeny for the mutant phenotype. Its mutant and homozygous status was confirmed in the F3 to F4 generations.

For plant transformation and protoplast isolation, approximately 15 Arabidopsis seeds were germinated and grown in 2 × 2 inch pots containing a moistened TS-1 white peat bedding substrate (Epagma, www.epagma.org) in a growth chamber at 22°C with16 h/8 h (light/dark) photoperiod at approximately 120 μmol m^{-2} sec^{-1}. About 5-week-old plants with several mature flowers in the main inflorescence were used for plant transformation. Leaves from plants that were about 3–4 weeks old were used for protoplast isolation. For phenotypic analysis in mature plants, plants were obtained either by germinating and growing seeds in soil pots, or transferring seedlings from MS/G plates to soil pots.

To produce seedlings for use in phenotypic analysis, anthocyanin content assays, and DNA and RNA isolation, Arabidopsis seeds were surface-sterilized and sown on MS/G plates consisting of ½MS (Murashige & Skoog) medium with vitamins (PlantMedia, www.plantmedia.com), 0.6% w/v phytoagar (PlantMedia), and 1% w/v sucrose unless indicated otherwise, pH adjusted to 5.7 with 1 N NaOH. The plates were cold-treated at 4°C in darkness for 2 days, and then moved to a growth chamber at 22°C, with a 16 h/8 h (light/dark) photoperiod at approximately 120 μmol m^{-2} sec^{-1}.

Isolation of the *gl2-1D*/*gpa1-2* mutant and TAIL-PCR

The *gpa1-2* mutants were mutagenized by transforming with the activation tagging vector *pSKI015* (Weigel *et al.*, 2000). The *gl2-1D/gpa1-2* dominant mutant was isolated from approximately 10 000 plant mutant population.

Genomic DNA was isolated from 10-day-old *gl2-1D/gpa1-2* mutant seedlings by using a DNeasy Plant Mini Kit (Qiagen, www.qiagen.com) according to the manufacturer's instructions.

TAIL-PCR was performed as described by Liu *et al.* (1995), except that bands of PCR product from a second round of TAIL-PCR cycling were recovered and purified for sequencing. The T-DNA border-specific primers used in the first and second round TAIL-PCR cycling are 5′-GGGCTAGTATCTACGACACACCGAG-3′ and 5′-TGACAGTGACGACAAATCGTTGGGC-3′, respectively. The arbitrary degenerate (AD) primer pools, AD1, AD2, AD3 and AD6 (Liu *et al.*, 1995) were used per round of TAIL-PCR cycling.

Plasmid constructs

To generate HA- or GD-tagged GL2 constructs, the full-length open reading frame (ORF) of *GL2* was amplified by RT-PCR using RNA isolated from 10-day-old Col wild type seedlings, and the PCR products were cloned in frame with an N-terminal HA or GD tag into the *pUC19* vector under the control of the double *35S* promoter of *CaMV*, and terminated by the 3′ UTR (untranslated region) derived from nopaline synthetase gene (Tiwari *et al.*, 2001; Wang *et al.*, 2005).

To generate the *35SE-GL2p:GL2* construct, an approximately 3200 bp *35SE-GL2p* fragment was amplified by PCR using genomic DNA isolated from 10-day-old *gl2-1D/gpa1-2* mutant seedlings. The primers used are T-DNA specific primer 5′-CAAGAATTCGGCCGCT CTAGAACTAGTG-3′, and *GL2*-specific primer 5′-CAACCATGGGC CAGCTACAGCATTGGC-3′. The PCR fragment was then used to replace the *35S* promoter in the *35S:HA-GL2* construct. For plant transformation, the *35SE-GL2p:GL2* construct in *pUC19* was digested with *Eco*RI, then sub-cloned into the binary vector *pPZP211* (Hajdukiewicz *et al.*, 1994).

The transactivator LD-VP, reporter *LexA-Gal4:*GUS, GD, and GD-OFP1 constructs have been described previously (Tiwari *et al.*, 2001, 2004; Wang *et al.*, 2007).

Plant transformation and transgenic plants selection

Plants that were about 5 weeks old with a few mature flowers on the main stems were transformed with the *pPZP35SE-GL2p:GL2* construct in *Agrobacterium tumefaciens* by the floral dip method (Clough and Bent, 1998). T1 seeds were selected on ½ MS/G plates containing 50 μg ml^{-1} kanamycin and 50 μg ml^{-1} carbenicillin. About 10-day-old transgenic plant seedlings were transferred to soil pots. Phenotypes of transgenic plants were visible in T1 plants, and confirmed in subsequent generations. Elevated expression of *GL2* in transgenic plants was confirmed by RT-PCR.

Plasmid DNA preparation, protoplast isolation, transfection and GUS activity assay

Effector and reporter plasmids were prepared using the EndoFree Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions. Protoplast isolation, transfection and GUS activity assays were performed as described previously (Tiwari *et al.*, 2001; Wang *et al.*, 2005, 2008; Wang and Chen, 2008; Wang *et al.*, 2014; Zhou *et al.*, 2014). Briefly, protoplasts were isolated from rosette leaves collected from 3 to 4-week-old Col wild type plants. Effector plasmids encoding GD alone, or the full-length protein of GL2 or OFP1 fused in frame with GD, were co-transfected with transactivator LD-VP16 and reporter *LexA-Gal4:*GUS into protoplasts. The protoplasts were incubated at room temperature for 20–22 h under darkness before GUS activities were measured using a Synergy™ HT microplate reader (BioTEK, www.biotek.com).

RNA isolation, RT-PCR and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from of 6- or 10-day-old Arabidopsis seedlings (~100 mg) using an RNeasy Plant Mini Kit (Qiagen), and treated with RNase-Free DNase (Qiagen) to remove DNA contamination according to the manufacturer's instructions.

cDNA was synthesized using 1 μg total RNA by oligo(dT)20-primed reverse transcription using the Omniscript RT Kit (Qiagen). The total reaction volume was 20 and 1 μl of the reaction mixture was subjected to the PCR reaction. *ACTIN2* (*ACT2*) was used as control for RT-PCR and qRT-PCR. RT-PCR cycles were 28 for *DFR* and *ACT2*, 32 for *TT8* and *MYB113*, and 30 for all other genes examined. Primers used for RT-PCR examination of the expression of *GL2* are 5′-CAACATATGAAGTCGATCGATGGCTGC-3′ and 5′-CAACTTAAGTCAGCAATCTTCGATTTGTAGAC-3′, and for *GC5* are 5′-TGAGTCTGATGGTTCTCCTTACG-3′ and 5′-AGGGCATTTTCCAACATTTTTAT-3′. Primers used for RT-PCR and qRT-PCR examination of the expression of *ACT2* and biosynthesis genes in the flavonoid pathway and MBW activator complex component genes have been described previously (Gonzalez *et al.*, 2008; Jeong *et al.*, 2010; Gan *et al.*, 2011; Gou *et al.*, 2011; Peng *et al.*, 2011; Qi *et al.*, 2011; Bonawitz *et al.*, 2012).

Anthocyanin content assays

Anthocyanin content was measured as described previously (Mancinelli, 1984; Laby *et al.*, 2000) with some modifications. Briefly, 6-day-old seedlings were weighed, and anthocyanin pigments were extracted by shaking the seedlings in 75:1:24 (v/v/v) methanol:HCl:H_2O overnight at 4°C. The OD530 and OD657 values for each sample were measured using a Synergy™ HT microplate reader. Relative anthocyanin content was calculated using the following formula: $(OD_{530} - 0.25 \times OD_{657}) \times$ extraction volume (ml) $\times$ 1/fresh weight of the seedlings (g).

Microscopy

Photographs of approximately 1-month-old plants were taken using an EOS 1100D Canon digital camera (Canon; www.canon.com). Anthocyanin accumulation in seedlings was examined and photographed using a Motic K microscope (Motic, www.motic.com) equipped with a Canon digital camera.

Chromatin immunoprecipitation (ChIP) assay

About 10-day-old *35SE-GL2p:GL2* transgenic plant seedlings were used for the ChIP assay following the procedure described by Saleh *et al.* (2008), with the exception that anti-HA polyclone antibodies and rabbit pre-immune sera, rather than antibodies against histone modifications were used in the assay. Primers used for qPCR amplification of L1-box region in the promoter of *CESA5* have been described previously (Khosla *et al.*, 2014); other primer pairs used for PCR were: *PAP2p:* 5′-GTATTCATGTCAAAGAAATATGTGC-3′ and 5′-GTTACAACTTTCCACATAGTACTAGG-3′, *MYB113p:* 5′-CTAGCGATCTTATCGATGAAGCAATTA-3′ and 5′-TATATACGACCTCATCACTCTGT-3′ *TT8p:* 5′-GGTGAACGACTAAACTCAGAAGA-3′ and 5′-GGGTAATGTATGTACACCTGGTT-3′, and *DFRp:* 5′-CACACGTCTCACCAAACAAATC-3′ and 5′-CGTTGGT TGACGAAGGACTAA-3′.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Trichome and root hair formation and seed mucilage formation in Col wild type, *gl2-3* and *gl2-1D* mutants.

Figure S2. GL2 itself is not a transcriptional activator.

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