EOBII Controls Flower Opening by Functioning as a General Transcriptomic Switch[1][C][W]

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R2R3-MYB transcription factors (TFs) are involved in diverse aspects of plant biology. Recently an R2R3-MYB was identified in *Petunia x hybrida* line P720 to have a role in the transcriptional regulation of floral volatile production. We propose a more foundational role for the R2R3-MYB TF *EMISSION OF BENZENOIDS II* (*EOBII*). The homolog of *EOBII* was isolated and characterized from *P. x hybrida* 'Mitchell Diploid' (MD) and *Nicotiana attenuata*. For both MD and *N. attenuata*, *EOBII* transcript accumulates to high levels in floral tissue with maximum accumulation at flower opening. When *EOBII* transcript levels are severely reduced using a stable RNAi (*ir*) approach in MD and *N. attenuata*, *ir-EOBII* flowers fail to enter anthesis and prematurely senesce. Transcript accumulation analysis demonstrated core phenylpropanoid pathway transcripts and cell wall modifier transcript levels are altered in *ir-EOBII* flowers. These flowers can be partially complemented by feeding with a sucrose, *t*-cinnamic acid, and gibberellic acid solution; presumably restoring cellular aspects sufficient for flower opening. Additionally, if ethylene sensitivity is blocked in either MD or *N. attenuata*, *ir-EOBII* flowers enter anthesis. These experiments demonstrate one R2R3-MYB TF can control a highly dynamic process fundamental to sexual reproduction in angiosperms: the opening of flowers.

Regulation of gene transcription is essential for numerous aspects of biology. Transcription factors (TFs) are one way to regulate transcription. To be considered a TF, a protein must bind DNA in a sequence-specific manner (Latchman, 1997). A TF can function to activate or repress the transcription of specific genes (Lee and Young, 2000). Because TFs are essential for the regulation of transcription, TFs are found in all living organisms. The number of TFs per genome follows the power law; the larger the genome the greater the number of TFs (van Nimwegen, 2003).

In plants, an important and relatively large family of TFs is the R2R3-MYB TF family, which in Arabidopsis (*Arabidopsis thaliana*) consists of 126 R2R3-MYB genes (Stracke et al., 2001). R2R3-MYBs have been associated with a large number of divergent plant processes such as primary and secondary metabolism, cell fate and identity, and biotic and abiotic stress response (for review, see Dubos et al., 2010). Recently, a *Petunia x hybrida* line P720 (P720) R2R3-MYB transcript sequence, *EMISSION OF BENZENOID II* (*PhEOBII*), was reported to be involved in floral volatile production during open flower stages of development (Spitzer-Rimon et al., 2010). The authors demonstrate that a PhEOBII:GFP fusion protein localizes to the nucleus in leaf mesophyll cells, transcript accumulation of *PhEOBII* is flower specific and coincides with floral volatile emission from P720, and ectopically expressed PhEOBII can activate phenylpropanoid-related gene promoters. Also, upon a transient reduction of *PhEOBII* transcript levels, reduced levels of emitted and internal volatile benzenoid/phenylpropanoid compounds are detected from the P720 flower. PhEOBII shares high amino acid similarity to the seemingly redundant Arabidopsis MYB21 and MYB24 (AtMYB21/24), which belong to R2R3-MYB subgroup 19 (Stracke et al., 2001).

AtMYB21/24 function is associated with the regulation of jasmonate signaling during stamen and pollen development in an Arabidopsis flower (Mandaokar et al., 2006). Both *AtMYB21/24* transcripts accumu-

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late to high levels in floral tissue of Arabidopsis, and AtMYB21/24 are involved in the activation of phenylpropanoid-related genes (Shin et al., 2002; Yang et al., 2007). Reduction in *AtMYB21/24* transcript (T-DNA insertion lines) results in floral developmental defects like failure of anther dehiscence and flower opening; the latter was an observation and not the focus of experimentation (Mandaokar et al., 2006). Another putative homolog of *EOBII* in ornamental tobacco (*Nicotiana langsdorffii x Nicotiana sanderae*; *NlxNsMYB305*) has been characterized through stable RNAi silencing, and the authors reported on floral developmental defects (Liu et al., 2009). Among which were nectary formation and a failure of flower petals to expand at anthesis, which again, the latter was an observation and not the focus of experimentation. Lastly, EOBII homologs from *Antirrhinum majus* (*AmMYB305* and *AmMYB340*) and *Pisum sativum* (*PsMYB26*) have been reported as flower specific and involved in phenylpropanoid pathway regulation (Sablowski et al., 1994; Uimari and Strommer, 1997).

In a *P. x hybrida* 'Mitchell Diploid' (MD) flower, an R2R3-MYB TF termed *ODORANT1* (*PhODO1*; 27.5% amino acid identity with PhEOBII) is involved with regulating genes in primary and secondary metabolism beginning at anthesis, resulting in the indirect regulation of floral volatile benzenoid/phenylpropanoid (FVBP) biosynthesis throughout open flower stages (Verdonk et al., 2005). Since FVBPs can function as pollinator attractants (Hoballah et al., 2005), it is logical that FVBP production in petunia (*P. x hybrida*) is turned on (Verdonk et al., 2003; Colquhoun et al., 2010b) when the sexual organs are receptive to pollination, which occurs at anthesis (Hoekstra and Weges, 1986; Weiss et al., 1992; Wang and Kumar, 2007).

During flower development in petunia, the biosynthesis of phenylpropanoids is a tightly controlled process. Pigment biosynthesis, anthocyanins and flavonoids, occurs during the first stages of flower de-

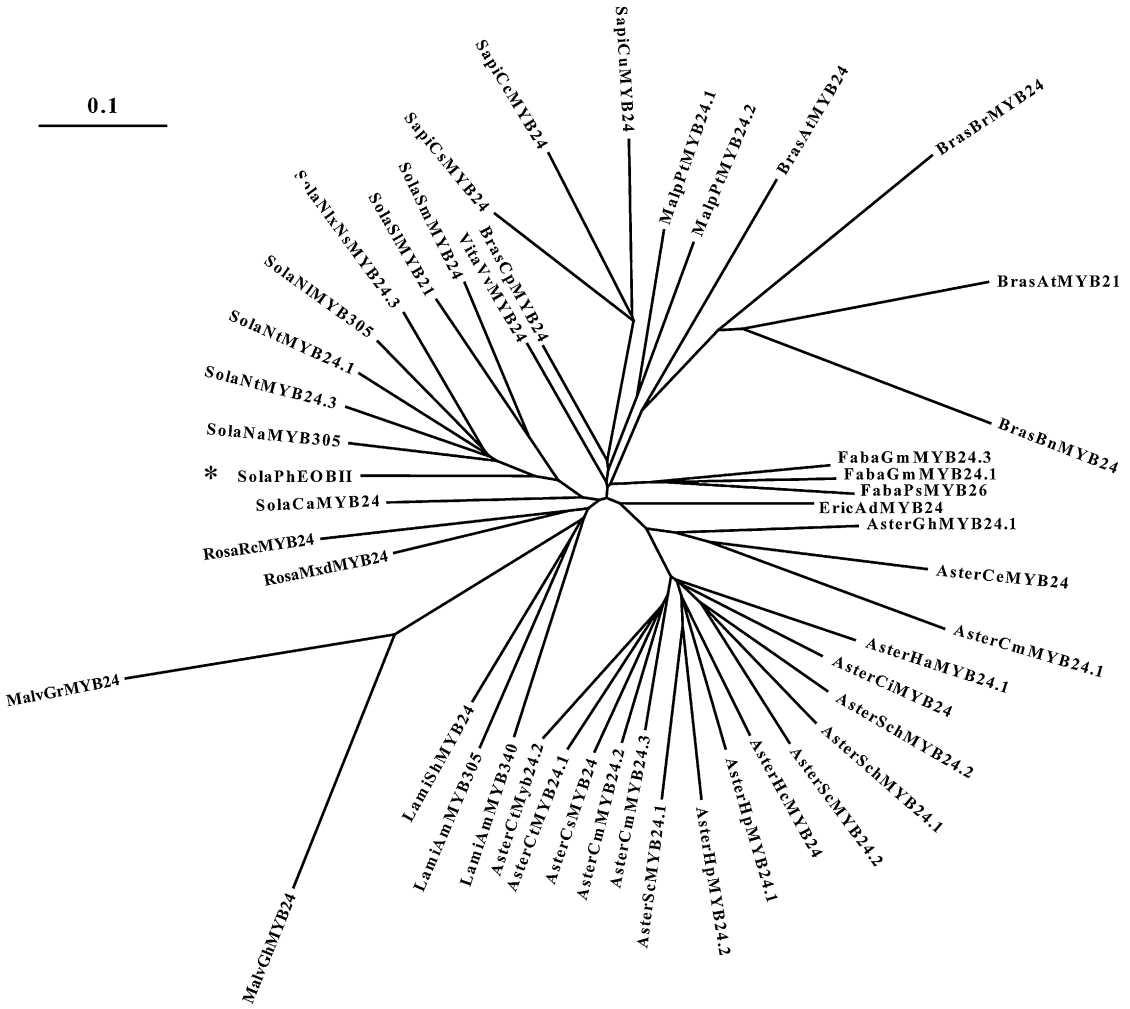

Figure 1. An unrooted neighbor-joining tree of PhEOBII-like amino acid sequences. TREEVIEW (Win32) software version 1.6.6, nearest-joining method, was used to create the resulting phylogenetic tree. Scale bar represents distance as the number of substitutions per site (i.e. 0.1 amino acid substitutions per site). An asterisk denotes the PhEOBII sequence.

velopment (Weiss et al., 1992). Petunia corolla growth starts with a slow growth phase during which cell division occurs followed by a phase of rapid growth that mostly results from cell expansion (Ben-Nissan and Weiss, 1996). The phytohormone, gibberellin (GA) is important during the first stages of petunia flower development. GA is necessary to induce anthocyanin biosynthesis and the initiation of rapid cell elongation, but it is not necessary for their maintenance (Weiss et al., 1992; Weiss, 2000). GA is likely synthesized in the anthers, which is then transported to the corolla (Weiss et al., 1993). At the later stages of petal growth, after the transition to rapid elongation, the corolla is no longer dependent on the anthers or GA for growth and pigmentation. In fact, transcription of the genes associated with anthocyanin production is down-regulated at the onset of anthesis by an unknown mechanism, and the application of GA is insufficient to prevent the decline in transcripts because the floral tissue has become insensitive to GA (Weiss, 2000). These results suggest that, in petunia, GA is not involved in the process of anthesis itself.

The transition phase of the flower, anthesis, has not been well defined from a molecular and genetic prospective. In MD, flower opening occurs after several days of bud elongation when the flower bud is approximately 65 mm from the base of the receptacle to the tip of the corolla (Colquhoun et al., 2010b). In petunia, flower opening is irreversible; the flower stays open until senescence, when the corolla wilts and abscises from the receptacle (Negre et al., 2003; Underwood et al., 2005; Colquhoun et al., 2010b). It is assumed that the opening of the flower occurs due to cell elongation and expansion, and is controlled by phytohormones such as auxin, GAs, cytokines, ethylene, and/or brassinosteroids (for review, see van Doorn and Van Meeteren, 2003).

Here, we have examined *EOBII* function in MD flowers. We propose that *PhEOBII* functionality is dynamic in the MD flower, and ultimately controls flower opening (anthesis). In MD, *PhEOBII* transcript accumulation peaks at anthesis, and a severe reduction of *PhEOBII* transcript using a stable, reverse-genetics approach results in a failure to enter anthesis. Additionally, the transgenic phenotype can be complemented by hormone sensitivity manipulation without complimenting FVBP emission. EOBII amino acid sequence is highly conserved throughout multiple orders of angiosperm species, and the MD *PhEOBII* functional characterizations can be replicated in *Nicotiana* species, which provides support for a conservation of function among angiosperms.

RESULTS

Identification of *PhEOBII*

Our initial aim was to isolate transcriptional regulators associated with the FVBP gene network in *P. x hybrida* MD. Colquhoun et al. (2011) assembled MD R2R3MYB-like transcript sequences, and one of these Contig 4, since named *PhEOBII*, was chosen for further analysis based on the similarity to Arabidopsis *MYB21* and *MYB24*. The predicted protein sequence of PhEOBII is 197 amino acids in length with a predicted nuclear localization (WoLF PSORT). When aligned with putative homologs, orthologs, and paralogs (Supplemental Table S1) a conserved, N-terminal R2R3-MYB domain (Stracke et al., 2001) and a C-terminal transactivation (W/Y-MDDIW) domain (Li et al., 2006) are obvious (Supplemental Fig. S1). The transactivation domain appears highly conserved through 12 orders and 38 species of angiosperms. Three amino acids in particular (Trp-183, Asp-187, and Trp-189) are conserved in every sequence analyzed (one mismatch

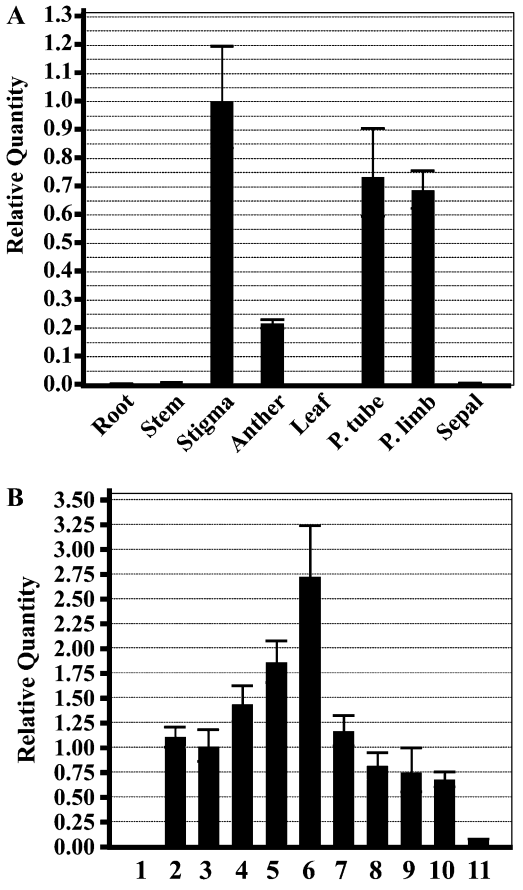

Figure 2. qRT-PCR transcript accumulation analysis of *PhEOBII* from MD plants. A, Spatial analysis used total RNA from root, stem, stigma, anther, leaf, petal (P.) tube, petal (P.) limb, and sepal tissues collected at 4 PM (mean ± SE; $n = 3$). B, Floral developmental analysis used total RNA from staged flower tissues collected at 4 PM of the same day (mean ± SE; $n = 3$). Stage 1 represents a 1-cm floral bud from the base of the receptacle to the tip of the emerging corolla tissue. Each stage is developmentally separated by approximately 1 d, except for stages 10 and 11, where 3 d separates the stages (adapted from Colquhoun et al., 2010b). All histograms are representative of multiple experiments with multiple biological replicates.

for Asp-187). After placing the extensive list of sequences in an unrooted neighbor-joining tree, the family and genus members group together with the compactness of the tree easily identifiable (Fig. 1).

PhEOBII Transcript Accumulation Analysis and Promoter Sequence Features

PhEOBII transcript accumulation has been examined from whole plant, a flower developmental series, and over a time course in flowers of P720 (Spitzer-Rimon et al., 2010). Since *PhODO1* transcript accumulates differentially between petunia lines (Verdonk et al., 2005), we chose to examine *PhEOBII* transcript accumulation in MD utilizing quantitative reverse-transcriptase PCR (qRT-PCR). From a spatial context, high levels of *PhEOBII* transcripts were detected in the stigma, petal tube, and petal limb tissues with a relatively low detection of transcript in anthers (Fig. 2A). To investigate the transcriptional profile of *PhEOBII* in MD further, staged flowers from a 1-cm bud to the end of the floral life cycle (detailed in Colquhoun et al., 2010b) were used to examine *PhEOBII* transcript accumulation over the course of floral development. The results resembled a bell curve with a maximum at stage six, which is anthesis (Fig. 2B). *PhEOBII* transcript was not detected in stage 1 MD flower buds, and accumulation was reduced to comparatively very low levels in senescing flower tissue (Fig. 2B).

The specificity of *PhEOBII* transcript accumulation in MD flowers led us to clone approximately 1.25 kb of DNA sequence upstream of the *PhEOBII* translational start codon in the MD genome, via GenomeWalker. Utilizing the plant cis-acting regulatory DNA elements database (Higo et al., 1999), putative cis-acting regulatory elements were identified including the abundant etiolation induced, light regulated, Dof binding, enhancers, basic helix-loop-helix, and WRKY binding motifs. Less-abundant elements include: ethylene responsive, GA induced, jasmonic acid (JA) responsive, a plastid responsive, and a prolamine box element (Wu et al., 2000).

Functional Characterization of *PhEOBII*

A 3′ segment of the *PhEOBII* transcript (Supplemental Fig. S2) was used to create approximately 50 independent T0 *ir-PhEOBII* (stable RNAi, inverted repeat) plants. These transgenic plants were generated in the MD genetic background by leaf-disc transformation (Jorgensen et al., 1996; Underwood et al., 2005; Dexter et al., 2007; Colquhoun et al., 2010a). A striking phenotype was visually observable when the *ir-PhEOBII* plants were grown to a mature, reproductive stage; flowers from multiple independent transgenic events failed to enter anthesis (Fig. 3) and prematurely senesced as closed buds. *PhEOBII* transcripts are not detected in a stage 1 MD flower (Fig. 2B), so a physiological study was conducted by tagging stage 1 flower buds and measuring the floral growth rate from T0 plants and MD (Fig. 3A). Flowers from MD and *ir-PhEOBII-1* (a transgenic plant with a wild-type [Fig. 3B] flower opening) developed at an equivalent rate, entering anthesis between the sixth and seventh stage with an almost exponential growth curve between stage 4 and 7. In contrast, floral bud growth from the *ir-PhEOBII-5* and *ir-PhEOBII-12* plants (complete nonopening floral phenotype; Fig. 3D) diverged from that of MD between the fourth and fifth stage, and resembled more of a linear function between stage 4 and 7 (Fig. 3A). A representative of an intermediate phenotype, *ir-PhEOBII-3* floral growth rate bisected the difference between MD and the complete nonopening lines (Fig. 3A). The *ir-PhEOBII-3* flowers opened but they failed to completely expand the petal limb tissue (Fig. 3C).

Twelve T0 plants showing the nonopening phenotype and a reduction of *PhEOBII* transcript during an initial semiquantitative (sq)RT-PCR screen (Supplemental Fig. S3) were chosen for further analysis and

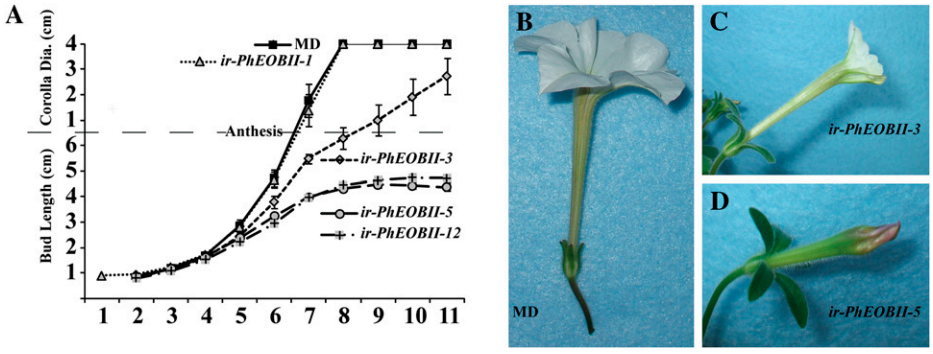

Figure 3. Phenotypic growth comparison of MD and T0 *ir-PhEOBII* flowers. Shown are representatives from transgenic plants showing a wild type (*ir-PhEOBII-1*), an intermediate (*ir-PhEOBII-3*), and two strong floral phenotypes (*ir-PhEOBII-5* and *12*). A, All flower buds were tagged at a 1-cm bud and followed through development (mean ± SE; $n = 12$). B to D, Pictures of the *ir-PhEOBII* phenotype. B, An entire open MD flower. C, A semiopening flower from an intermediate line, *ir-PhEOBII-3*. D, A nonopening flower from a strong phenotype line, *ir-PhEOBII-5*. [See online article for color version of this figure.]

Table I. *Phenotypic characterization of the vegetative growth of T1 ir-PhEOBII RNAi lines*

Measurements of three independent plants were made; averages and SE were then calculated. Significant differences from MD ($P < 0.01$) were not found except for the field marked with an asterisk.

Plant Aspects	MD	*ir-3*	*ir-5*	*ir-12*
No. of branches	12.7 ± 0.82	11.3 ± 0.41	12.0 ± 0.71	13.0 ± 0.71
No. of subbranches	27.0 ± 3.74	11.3 ± 8.44	24.0 ± 8.86	21.7 ± 1.78
No. of nodes per branch	16.2 ± 0.32	16.4 ± 0.84	16.1 ± 0.98	18.6 ± 1.08
No. of flowers or buds	116.3 ± 14.57	61.0 ± 22.26	148.7 ± 6.1	107.3 ± 19.8
Aerial fresh weight (g)	220.3 ± 18	248.3 ± 96.65	325.9 ± 28.53	353.6 ± 15.27*
Total branch length (cm)	381.0 ± 54.55	507.7 ± 71.37	715.7 ± 63.25*	835.7 ± 87.37*
Average branch length (cm)	60.6 ± 2.7	45.1 ± 7.51	59.5 ± 2.19	64.3 ± 5.74

self pollinated by cutting the corolla open of an approximately 3-cm flower bud, allowing the anthers to dehisce, and pollinating an adjacent mechanically opened flower bud. Segregating T1 populations were used for gross physiological and more intricate floral measurements such as: the total number of branches per plant, the total number of flowers per plant, and the average branch length per plant (Table I); or petiole length, sepal length, and stigma length (Table II). The only per plant measurement that demonstrated a significant difference ($P < 0.01$) between MD and both nonopening transgenic lines was total branch length, where *ir-PhEOBII-5* and *ir-PhEOBII-12* plants demonstrated a longer total branch length than MD (Table I). Comparative floral measurements between MD and the two nonopening lines illustrated significant differences in petiole length, stigma length, and anther filament length (Table II). Note, no difference was measured for sepal length between MD and transgenic flowers. All T1 populations segregating in a Mendelian fashion were self pollinated as previously described, and a T2 homozygous *ir-PhEOBII* line (*ir-5*, name originated from T0 number) was produced and used for further analysis (Fig. 4B). When flowers from MD and *ir-5* were tagged, developmentally staged, and visualized photographically, the nonopening phenotype was evident (Fig. 4).

The development of an *ir-5* flower is disconnected from that of a MD flower (Figs. 3 and 4). To normalize a targeted transcript accumulation analysis, we compared *ir-5* flowers to MD flowers staged over time (Fig. 4). One-centimeter flower buds were tagged and allowed to progress through what would be stage 7 in MD flowers. Again, qRT-PCR was utilized, but a two-step reaction was preferred to eliminate any *PhEOBII* RNA expressed from the transgene (Colquhoun et al., 2011). *PhEOBII* transcripts were severely reduced (approximately 82% reduction at floral developmental stage 5) in *ir-5* flowers compared to MD flowers (Fig. 5A). A petunia transcript very similar to a rose (*Rosa hybrida*) *AQUAPORIN* necessary for flower petal expansion in rose, *RhPIP2;1* (Ma et al., 2008), was unaltered between MD and *ir-5* flowers (Fig. 5B). AtMYB21, NlxNsMYB305, and PhEOBII can positively regulate a *PHENYLALANINE AMMONIA-LYASE* (*PAL*) promoter in the respective species (Shin et al., 2002; Liu et al., 2009; Spitzer-Rimon et al., 2010). In MD, two *PhPAL* transcripts accumulate to high levels around anthesis, but no increase in transcript accumulation was observed for the two *PhPALs* in *ir-5* flowers (Fig. 5C). As expected from the nonopening phenotype (Fig. 4) and the lack in up-regulation of the *PhPALs* (Fig. 5C), *ir-5* flowers do not emit FVBPs (Supplemental Fig. S4). Petunia sequences very similar to xyloglucan endotransglycosylases/hydrolases (cell wall modifiers) *PhXTR6* and *PhXTR7* (Yokoyama and Nishitani, 2007) were also altered in *ir-5* flowers compared to MD (Fig. 5D). Whether *PhEOBII* directly or indirectly regulates the *PhPALs* and the *PhXTRs* has yet to be determined, however, we conclude when levels of *PhEOBII* are reduced in MD, these transcripts (associated with divergent biological processes) are altered and may collectively contribute to the nonopening phenotype.

Complementation of *ir-PhEOBII* Phenotype

PhEOBII has a wide-ranging effect on the development of a petunia flower, e.g. slowed corolla growth to

Table II. *Phenotypic characterization of the floral organs of T1 ir-PhEOBII RNAi lines*

Measurements of five independent flowers at stage 8 were made; averages and SE were then calculated. Significant differences from MD ($P < 0.01$) are marked with an asterisk. In reference to anthers, d1 denotes tube segment attachment.

Flower Aspects	MD	*ir-3*	*ir-5*	*ir-12*
Petiole length (cm)	4.47 ± 0.26	3.63 ± 0.20	3.28 ± 0.13*	3.26 ± 0.14*
Sepal length (cm)	1.12 ± 0.03	1.10 ± 0.02	1.13 ± 0.03	1.13 ± 0.02
Ovary length (cm)	0.47 ± 0.02	0.48 ± 0.02	0.48 ± 0.02	0.52 ± 0.02
Stigma length (cm)	4.10 ± 0.04	3.98 ± 0.06	2.73 ± 0.08*	2.80 ± 0.11*
Anther filament length (cm)	4.35 ± 0.06	4.31 ± 0.06	3.02 ± 0.08*	3.22 ± 0.01*
Anther attached [d1] (cm)	1.77 ± 0.07	1.80 ± 0.03	1.47 ± 0.07*	1.50 ± 0.04*

the failure to enter anthesis (Fig. 3, A and D), or phenylpropanoid metabolism to cell wall modification (Fig. 5, C and D). We hypothesized that the supplementation of downstream compounds from the effected molecular steps would rescue the transgenic nonopening phenotype (Fig. 4). After investigating numerous chemicals and combinations, petiole feeding with a Suc, *t*-cinnamic acid, and GA (STG) solution resulted in an increased bud elongation of the *ir-5* flowers compared to flowers in water (Fig. 6). Additionally, the *ir-5* flowers in the STG solution enter anthesis; however, complete limb expansion was not observed (Fig. 6, inset picture). JA feeding resulted in a hypersensitive response (bud growth impairment was exacerbated) from *ir-5* flowers, which was abolished in the presence of the STG solution (Fig. 6).

The nonopening, transgenic flowers senesce prematurely (Fig. 4). Therefore, we hypothesized the phytohormone ethylene was responsible for the premature senescence of the *ir-5* flowers. The ethylene-insensitive *P. x hybrida* MD line 44568 (Wilkinson et al., 1997), which displays an increased floral longevity phenotype (Wilkinson et al., 1997; Colquhoun et al., 2010b), was utilized to cross pollinate with the *ir-5* flowers. The 44568 × *ir-5* flowers did not senesce early compared to MD. The F1 generation 44568 × *ir-5* flowers displayed a partial complementation by entering anthesis on the same day the *ir-5* flowers displayed signs of senescence (Fig. 7). Again, anthesis was not complete, but the petal limb did expand further than the mechanical complementation (Fig. 6). The premature senescence of the closed *ir-5* flower bud is most likely attributed to the ethylene senescence program. Also, if the ethylene signal is not perceived, anthesis is triggered by an unknown mechanism.

Conservation of *PhEOBII* Function

To test the functional conservation suggested by amino acid conservation through many angiosperm species, the *PhEOBII* homolog from the ecological model system *Nicotiana attenuata* (Baldwin, 1998) was isolated (*NaMYB305*) and cloned. *NaMYB305* transcript accumulation in a developing *N. attenuata* flower is very similar to *PhEOBII* in MD (Figs. 2 and 8). Much like in petunia, a stable RNAi approach was used to reduce the *NaMYB305* transcript accumulation in *N. attenuata*, which resulted in 14 independent T0 plants with flowers that failed to enter anthesis, senesced prematurely, and were reduced in *NaMYB305* transcript levels compared to wild-type *N. attenuata* (Fig. 9A). The *ir-NaMYB305* plants displaying the nonopening phenotype were extremely difficult to sexually propagate because the closed flower buds would drop if mechanically manipulated as was done for the MD *ir-PhEOBII* flowers.

Of interest was the phenotypic complementation by ethylene insensitivity in petunia (Fig. 7). When the *ir-NaMYB305* plants were treated with 1-methylcyclopropene (MCP; an ethylene receptor blocker), a partial complementation was observed (Fig. 9B). However, the frequency (flowers/plant) of complementation was low. Regardless, the results obtained from experimentation with *N. attenuata* resemble that of MD, and further support a conservation of function for EOBII between these two genuses.

Figure 4. Relation of MD floral development and a T2 *ir-PhEOBII* homozygous line. A, Developmentally staged MD flowers from a small bud, stage 1; 1 cm, to a senescing flower, stage 11 (adapted from Colquhoun et al., 2010b). B, Developmentally staged flowers from the *ir-PhEOBII-5* (*ir-5*) homozygous line. Approximately 1 d separates each stage except for stages 10 and 11 from MD flowers, where these stages are separated by 3 d. [See online article for color version of this figure.]

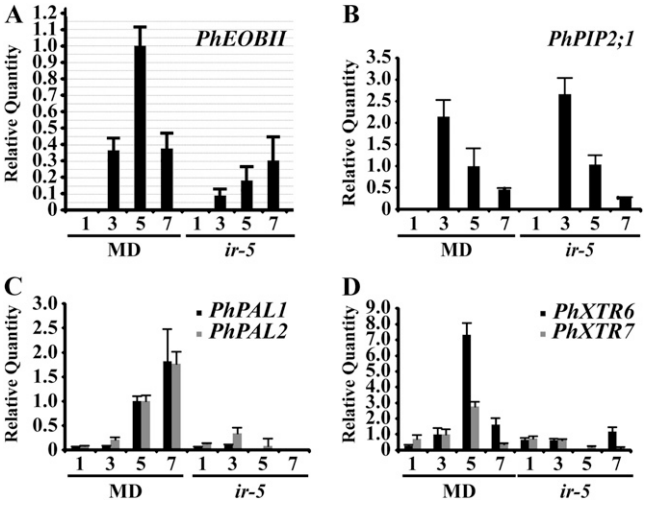

Figure 5. Comparative transcript accumulation analysis using MD and *ir-5* flowers. A to D, Developmentally staged flowers (an abbreviated staging since *ir-5* flowers initially differs from MD between stage 4 and 5) were collected on one day at 4 PM. qPCR was carried out with 10 times diluted cDNA samples and run with the ΔΔCt method using *PhFBP1* as an internal reference. Histograms are representative of two biological replicates (mean ± SE; $n = 3$).

DISCUSSION

Since we were interested in FVBP biosynthesis regulation, we initially isolated the Contig 4 nucleotide (nt) sequence (Colquhoun et al., 2011) from MD be-

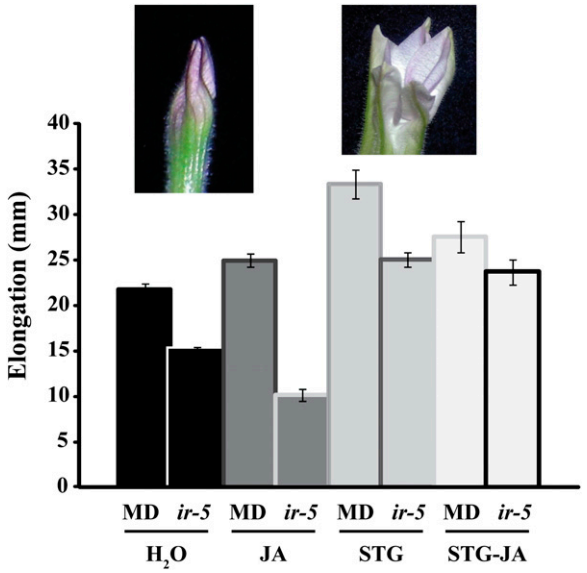

Figure 6. Feeding experiments with excised MD and *ir-5* flowers. All flowers were excised at 3 cm and placed in solutions indicated: water, 50 μM JA, and STG. Growth was measured every 24 h in millimeters (mean ± SE; $n = 15$). Inset pictures are representative of the *ir-5* flowers used in this experiment after four days of feeding. [See online article for color version of this figure.]

cause it was most similar to *AtMYB21* and *AtMYB24*, of which AtMYB21 can positively regulate the *AtPAL1* promoter (Shin et al., 2002). During our time of experimentation with *P. x hybrida* MD, another group published quality results communicating a floral volatile function for *PhEOBII* in an open flower of a commercially available *P. x hybrida* line P720 (Spitzer-Rimon et al., 2010). This group used a virus-induced gene silencing-based system to illustrate that upon reduction of *EOBII* transcript a concomitant reduction of emitted and internal levels of FVBP compounds were measured from open flowers. A nonopening phenotype was not reported (Spitzer-Rimon et al., 2010). A double T-DNA insertion line for Arabidopsis *EOBII* homologs (*AtMYB21* and *AtMYB24*) was generated and a nonopening flower phenotype was observed, but no further analysis was conducted (Mandaokar et al., 2006). Additionally, a stable RNAi-based approach was used to reduce the transcript of another *EOBII* homolog in ornamental tobacco *N. langsdorffii x N. sanderae*, *NlxNsMYB305*, and the authors reported on a "failure of the flower petals to expand at anthesis," with no further analysis conducted (Liu et al., 2009, p. 2682). We have demonstrated through a stable RNAi approach that a drastic reduction of *EOBII* transcript in MD and *N. attenuata* plants results in a nonopening floral phenotype (Figs. 3, 4, and 9), and that *EOBII* function is highly dynamic (Figs. 3, 5–7, and 9).

A floral volatile function for EOBII in an open flower is acknowledged (Spitzer-Rimon et al., 2010). Indirectly our experimentation supports for a similar conclusion. In the MD genetic background, the nonopening phenotype of *ir-5* was partially complimented by ethylene insensitivity (Fig. 7), but the lack in emission of FVBPs was not complemented (Supplemental Fig. S4). This result implies the petal limb tissue can transition into and partially through the physical processes of opening, but induction of the FVBP pathway is not necessary for that complementation. In contrast, EOBII may be necessary for the induction of the FVBP pathway.

The *EOBII* gene is an R2R3-MYB TF that is expressed in flowers at high levels in genus like *Petunia*, *Arabidopsis*, *Antirrhinum*, *Nicotiana*, and *Pisum* (Figs. 2A and 8; Sablowski et al., 1994; Uimari and Strommer, 1997; Shin et al., 2002; Schmid et al., 2005; Liu et al., 2009; Spitzer-Rimon et al., 2010). Interestingly, *EOBII* transcripts accumulate to relatively high levels in the petal tube tissue of MD (Fig. 2A), but in P720 and *Antirrhinum* very little transcript was detected in petal tube tissue (Sablowski et al., 1994; Spitzer-Rimon et al., 2010). Also noteworthy, *PhEOBII* transcript was not detected in sepal tissue of MD (Fig. 2A) and sepal development was not affected in the *ir-PhEOBII* plants by our observation (Table II), which suggests the functionality of this transcript is regulated after organ identity (Coen and Meyerowitz, 1991) has been established.

The primary amino acid sequence of EOBII is well conserved at the R2R3 domain and the transactivation domain through 12 orders of eudicots (Fig. 1; Supplemental Fig. S1). Work carried out in the aforementioned plant species indicates the EOBII protein can activate promoter regions of genes like *PAL1*, *PAL2*, *ISOEUGENOL SYNTHASE1*, *ODO1*, *NECTARIN1*, and *NEC5*. Therefore, EOBII has been established to have multiple target genes, and with evidence like protein sequence and functional conservation, appears conserved in many angiosperms. The *N. attenuata* experimentation was an initial attempt to test the conservation of function in an angiosperm system that

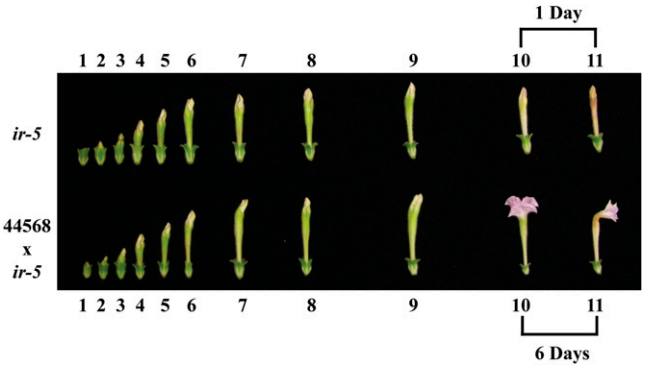

Figure 7. Complementation of the *ir-PhEOBII* phenotype by ethylene insensitivity. Shown are developmentally staged *ir-5*, and 44568 × *ir-5* flowers, from a small bud (stage 1, 1 cm) to a senescing flower (stage 11). Approximately 1 d separates each stage except for stages 10 and 11 in the 44568 × *ir-5* line, where 10 and 11 are separated by approximately 6 d. [See online article for color version of this figure.]

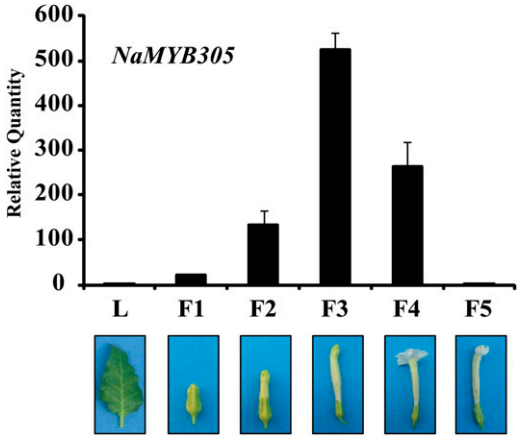

Figure 8. Transcript accumulation of the *PhEOBII* homolog in *N. attenuata*, *NaMYB305*. qPCR analysis of *NaMYB305* transcript accumulation through the development of a *N. attenuata* flower (mean ± SE; $n = 3$). The level of *NaMYB305* transcript in floral tissue is set relative to the level in leaf tissue. [See online article for color version of this figure.]

was accessible (Figs. 8 and 9), which resulted in a conservation of function down to the ethylene insensitivity complementation (Fig. 9).

Our results indicate that a more foundational role for *PhEOBII* exists, which is the control of flower opening. The concept of TFs affecting different numbers of promoters at differing protein concentrations is not novel, e.g. the morphogen gradient model from *Drosophila melanogaster* or haploinsufficiency in *Homo sapiens* (Deutschbauer et al., 2005; Ashe and Briscoe, 2006). However, various other explanations exist such as: necessary coregulators produced at differing developmental stages effecting different target pathways, or cascades of factors with different developmental timing. Intriguing though, is that one gene (*PhEOBII*), through a developmental stimulus of some sort (floral developmental stage 2; Fig. 2B), controls divergent aspects of flower biology (phenylpropanoid metabolism to cell wall modifications) to set forth a different cellular paradigm (FVBP biosynthesis). This model resembles that of a trans-epigenetic state (Bonasio et al., 2010).

CONCLUSION

In petunia, *Nicotiana*, and Arabidopsis EOBII function is required for sexual reproduction. That is not to say in all cases of severe *EOBII* deficiency the sexual organs are defective. The tissue that encases developing sexual organs and later may attract potential pollinators (the corolla in general) is most likely affected in secondary metabolite production, cell wall modifications, turgor pressure manipulation, and phytohormone response. Together, these biological limitations result in a failure to display sexual organs to the environment by undergoing anthesis. Whether EOBII has a direct role in all these systems, or EOBII is a central member of a cascade of molecular events, all culminating in the dynamic process of flower opening, is not clear. However, if the molecular mechanism of anthesis is conserved among most angiosperms, exploitation of *EOBII* expression in genetically modified angiosperm crops may aid in reducing the risk of outcrossing for particular applications.

MATERIALS AND METHODS

Plant Material

Inbred *Petunia x hybrida* MD plants were used as a wild-type control in all experiments (Mitchell et al., 1980). The homozygous ethylene-insensitive *cauliflower mosaic virus 35S:etr1-1* line, 44568, generated in the MD genetic background (Wilkinson et al., 1997), was used as a negative control for ethylene sensitivity (Underwood et al., 2005; Dexter et al., 2007, 2008; Colquhoun et al., 2010a, 2010b, 2011) and in cross pollinations to the MD *ir-PhEOBII* lines. All petunia (*P. x hybrida*) plants were grown in glass greenhouses as previously described (Dexter et al., 2007). *Nicotiana attenuata* (30th inbred generation) seeds, originally collected from a native population from a field site located in Utah, were used for all described experiments. Wild-type and *ir-NaMYB305* RNAi plants were grown in glass greenhouses as previously described (Kaur et al., 2010).

Identification of *EOBII*

Sequences with similarity to Arabidopsis (*Arabidopsis thaliana*) R2R3-MYB TFs (*AtMYBs*) were gathered using the National Center for Biotechnology Infor-

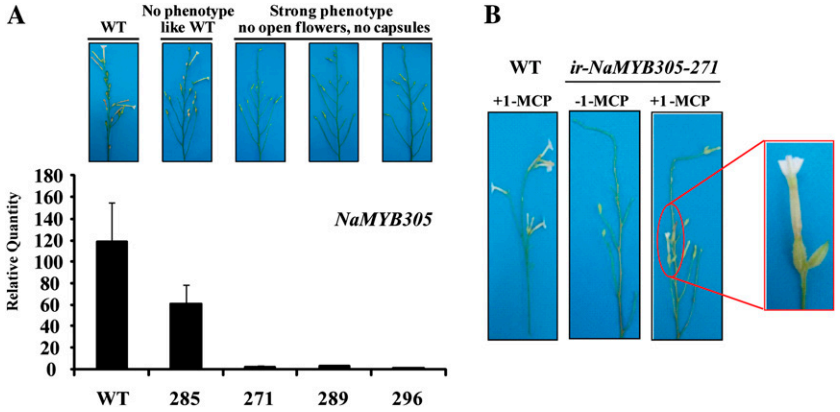

Figure 9. A reduction of *NaMYB305* transcript levels in *N. attenuata* by RNAi. A, Transcript accumulation of *NaMYB305* levels in representative T0 *ir-NaMYB305* lines compared to wild-type *N. attenuata* (mean ± SE; $n = 3$) with phenotypic pictures and descriptions included. B, Complementation study using MCP to block ethylene sensitivity in *N. attenuata* and *ir-NaMYB305* with a magnified inset picture of an *ir-NaMYB305* that has entered anthesis. [See online article for color version of this figure.]

mation, Sol Genomics Network, and the 454 petunia databases (www.ncbi.nlm.nih.gov, http://solgenomics.net, and http://140.164.45.140/454petuniadb). The resulting sequences were used to construct a petunia nt alignment (ContigExpress module, Vector NTI Advance 11, Invitrogen Corp., http://invitrogen.com) representing the MD *PhEOBII*, and sequence-specific primers (*PhEOBII* forward primer 5′-TCCCTATCCCATCTCTTTCTCTCTCCCTCT-3′; *PhEOBII* reverse primer 5′-CACTCATGAGATGGTTCAATCTAGGG-3′, ordered through Integrated DNA Technologies) were designed approximately 80 to 100 nt 5′ and 3′ of the deduced 594-nt coding region (GenBank accession number: *P. x hydrida* MD *NON-OPENING 1* [*PhNON1*], EU374207). Replicates of the expected, approximate 700-nt product were amplified using Advantage 2 polymerase mix (Clontech Laboratories Inc., http://www.clontech.com) from gene transcript pools of MD flower tissue, and purified using QIAquick spin columns (Qiagen, http://qiagen.com). Amplicons were ligated into pGEM-T-easy vector (Promega Corp., http://promega.com), transformed into One Shot Mach1-T1^R chemically competent *Escherichia coli* (Invitrogen Corp., http://invitrogen.com), multiple clones were isolated and sequenced (Big Dye V1-2; University of Florida Sequencing Core Facility, http://www.biotech.ufl.edu) to at least a 4× coverage to check for errors.

nt sequence of *NaMYB305* was assembled from 109 individual reads (size 400–450 bp) obtained from 454 GS FLX titanium series sequencing of *N. attenuata* cDNAs (vertis Biotechnologie AG; http://www.vertis-biotech.com/). Individual 454 reads were assembled by the PAVE program for assembling and viewing ESTs (Soderlund et al., 2009). Gene-specific primers were designed to amplify *NaMYB305* gene fragments that were ligated into pGEM-T-easy vector (Promega Corp., http://promega.com) and sequenced using a standard sequencing method. The sequence of *NaMYB305* was deposited in GenBank under accession number: *N. attenuata MYB305* (*NaMYB305*), Na_454_02248.

Promoter Cloning and Motif Scan

Approximately 1.25 kb of the MD *EOBII* promoter was cloned with a gene-specific primer 5′-ACGTTTGAGACCAGCAGATTTAGCTAAGG-3′ and a nested specific primer 5′-TTCTTCCATAGTCCAAGGTCCTTTCC-3′ using the GenomeWalker kit and protocol according to the manufacturer's instructions (Clontech Laboratories Inc., http://www.clontech.com). Plant cis-acting regulatory DNA elements, Signal Scan Search, an online database of nt sequence motifs found in plant cis-acting regulatory DNA elements was used to screen the *PhEOBII* promoter sequence (www.dna.affrc.go.jp).

Generating Transgenic *PhEOBII* RNAi Plants

To directly test the gene function of MD *PhEOBII*, stable RNAi-based gene silencing was utilized. A 283-nt sequence at the 3′ end of the *PhEOBII* transcript was developed as the RNAi-inducing fragment (*PhEOBII* forward primers: 5′-GCTCTAGAGCACATTAAGCAAGCAGA-3′, 5′-CGGGATCCGCACATTAAGCAAGCAGA-3′; and reverse primers: 5′-GGAATTCAGATGGTTCAATCTCAGG-3′, 5′-GGAATTCATAGGCACCTCCATGCAT-3′). The corresponding nt region in the most similar petunia sequence is 65.6% identical. Also, when the 283-nt *PhEOBII* RNAi trigger is fractionated into 22- to 25-nt fragments, then used as queries for Blast analysis on the petunia EST databases, the only identical sequences that return belong to *EOBII* petunia sequences. Additionally, the corresponding nt region in *AtMYB24* is 60.9% and 46.1% identical to the two closest Arabidopsis sequences (*AtMYB21* and *AtMYB57*, respectively). In planta expression of this fragment is driven by a constitutive promoter, pFMV. Fifty independent *PhEOBII* RNAi (*ir-PhEOBII*, inverted repeat) plants were generated in the MD background by leaf disc transformation (Jorgensen et al., 1996). Further details of the technical cloning have been previously described (Underwood et al., 2005; Dexter et al., 2007).

At least 12 T0 plants showing a transcriptional (sqRT-PCR) and physiological phenotype were self pollinated by cutting the corolla open of an approximate 3-cm flower bud, allowing the anthers to dehisce, and pollinating an adjacent, mechanically opened flower bud. The T1 generation was analyzed for a 3:1 segregation based on the presence of the transgene and the observable phenotype. Segregating T1 lines were self pollinated as before, and T2 generation was examined for nonsegregating lines with the phenotype. In this manner, a T2 homozygous *ir-PhEOBII* line (*ir-5*) was produced.

An RNAi approach was utilized to reduce the *NaMYB305* transcript accumulation, resulting in 30 independently transformed *ir-NaMYB305* plants. An RNAi-inducing 161-bp long fragment of *NaMYB305* coding sequence was introduced into *N. attenuata* plants using *Agrobacterium tumefaciens*-mediated plant transformation method as previously described (Krügel et al., 2002). PCR-amplified inverted repeat of *NaMYB305* gene (forward primers: 5′-CTGCAGCAGAAAACATGAATGGACAA-3′, 5′-GAGCTCGCAGAAAACATGAATGGACAA-3′; and reverse primers: AAGCTTGTTTCAGTGAGAAAAGGTC, CTCGAGTTTCAGTGAGAAAAGGTC) was cloned into *Hin*dIII/*Pst*I and *Sac*I/*Xho*I restriction sites of pSOL8 (*S12*) as inverted repeat to generate transformation vector pSOL8MYB3.

RNA Isolation, Tissue Collection, and Treatments

In all cases, total RNA was extracted as previously described (Verdonk et al., 2003) and subjected to TURBO DNase treatment (Ambion Inc.) followed by total RNA purification with RNeasy mini protocol for RNA cleanup (Qiagen). Total RNA was then quantified on a NanoDrop 1000 spectrophotometer (Thermo Scientific) and 50 ng/μL dilutions were prepared and stored at −20°C. Generation of cDNA samples used 2 μg of total RNA with SuperScript reverse transcriptase II (Invitrogen Corp., http://invitrogen.com) and was conducted more than three times for technical replications.

The physiological study was performed by tagging newly emerging flower buds of *ir-PhEOBII* and MD plants at stage 1: a bud 1 cm from the base of receptacle to corolla tip. Buds were measured in length of the corolla limb for 11 d. *ir-PhEOBII* were self pollinated by slicing open approximate 3-cm flower bud corolla, allowing the anthers to dehisce, and pollinating an adjacent, mechanically opened, emasculated flower bud.

The feeding experiments were performed using the compounds: Glc, Fru, Suc, sorbitol, auxin, JA, GA, salicylic acid, abscisic acid, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid, Phe, *t*-cinnamic acid, and benzoic acid. Excised *ir-PhEOBII* flowers were harvested at 3 cm and exogenously fed designated solutions and water as a control. Elongation was measured every 24 h in millimeters from the original 3-cm flower bud.

All petunia tissue collections were done as previously described (Colquhoun et al., 2010b). The spatial transcript accumulation analysis consisted of total RNA isolated from petunia root, stem, stigma, anther, leaf, petal tube (stage 8), petal limb (stage 8), and sepal tissues from multiple greenhouse-grown MD plants harvested at 4 PM. The developmental transcript accumulation analysis used total RNA isolated from whole (from the base of the receptacle up) petunia flowers (MD, 44568, *ir-5*, and 44568 × *ir-5*) collected at 11 consecutive stages beginning at a small bud to floral senescence from multiple greenhouse-grown plants at 4 PM. For all tissue collections, individual samples consisted of three flowers. All samples were frozen in liquid N_2 and stored at −80°C. Total RNA was then isolated from all samples, with multiple biological replicates included.

RNA extractions, cDNA synthesis, and qRT-PCR analyses of transcript abundances of *NaMYB305* gene were performed essentially as described in Kaur et al. (2010). The developmental transcript accumulation analysis used whole *N. attenuata* flowers collected at five consecutive stages beginning at a small bud to floral senescence from multiple greenhouse-grown plants at 1 PM. Rosette leaf samples were collected and analyzed to compare expression levels of *NaMYB305* in reproductive and vegetative tissues. Silencing efficiency of *NaMYB305* gene relative to wild-type levels was determined in four independently transformed *ir-NaMYB305* lines and wild type using floral buds at F2 stage of development.

To inhibit ethylene perception, we grew isolated branches of *ir-NaMYB305* plants with F1-F2 stage flower buds in 0.1× Murashige and Skoog salt solution and exposed them to ethylene receptor antagonist 1-MCP vapors: 10 mg of SmartFresh (3.3% 1-MCP; AgroFresh; Rohm and Haas) was dissolved in 1 mL of alkaline solution (0.75% KOH + NaOH in a 1:1 ratio) to release the active volatile substance, 1-MCP. Alkaline solution without 1-MCP was used in control experiment. The branches with flower buds were maintained in closed containers during whole treatment (6 d); once a day the containers were fully opened and new vial with freshly activated 1-MCP solution or alkaline solution alone were inserted before closing.

Transcript Accumulation Analysis

All transcript accumulation analyses were conducted multiple times with multiple biological replicates and equivalent results were observed. sqRT-PCR was performed using a Qiagen one-step RT-PCR kit (Qiagen Co.) with 50 ng total RNA. To visualize RNA-loading concentrations, samples were amplified with *Ph*18S primers (forward primer 5′-TTAGCAGGCTGAGGTCTCGT-3′; reverse primer 5′-AGCGGATGTTGCTTTTAGGA-3′) and analyzed on an agarose gel. The following primers were designed and utilized for the

visualization of the mRNA levels corresponding to *PhEOBII* (forward primer 5′-TCCCCCATATATGTGAGTTAAGTG-3′; reverse primer 5′-CCATAGGCACCTCCATGCAT-3′) ΔΔCt. qRT-PCR was performed and analyzed using a StepOnePlus real-time PCR system (Applied Biosystems). Power SYBR green RNA-to-Ct 1 and 2-step kits (Applied Biosystems) were used to amplify and detect the products according to the manufacturer's protocol. The following qRT-PCR primers were constructed in Primer Express software v2.0 (Applied Biosystems): *PhEOBII* forward primer, 5′-CATTTGCCTGGAAGAACAGA-3′; *PhEOBII* reverse primer, 5′-TGCTTGTCCATTCATGGTTT-3′; *PhFBP1*, forward primer, 5′-TGCGCCAACTTGAGATAGCA-3′; *PhFBP1* reverse primer, 5′-TGCTGAAACACTTCGCCAATT-3′; *PhPIP2;1*, forward primer, 5′-TGCTGGTCTCAAACGTACCG-3′; *PhPIP2;1* reverse primer 5′-GGACATCAGGCCGGAGATAA-3′; *SGN-U207589* (*PhXTR6*), forward primer, 5′-GCATCTTCTACTTCTGCCAGAA-3′; *SGN-U207589* (*PhXTR6*), reverse primer 5′-TCTGCACCCATTTCATCCTT-3′; *SGN-U207700* (*PhXTR7*), forward primer, 5′-GCAAATCCCAATTCTCAGATG-3′; *SGN-U207700* (*PhXTR7*), reverse primer 5′-GGAAAACCCTGTGGAAACCT-3′: *PhCM1*, forward primer, 5′-CCCTGATGAGCACCCATTC-3′; *PhCM1*, reverse primer 5′-ACTGCATGGGTGGCAACAC-3′; *PhPAL1*, forward primer, 5′-GCTAGGCGGTGAGACGCTAA-3′; *PhPAL1* reverse primer 5′-CTCGGACAGCTGCACTGTCA-3′; *PhPAL2*, forward primer, 5′-ACTGGCAGGCCTAATTCCAA-3′; *PhPAL2* reverse primer 5′-GCGAAACGCTTCTTCAGCAT-3′. Optimization of primers was conducted and demonstrated gene specificity during melt curve analysis.

A qPCR core kit for SYBR green I (Eurogentec; http://www.eurogentec.com) was used following the manufacturer's instructions on a Stratagene Mx3005P real-time PCR system (http://www.stratagene.com) to determine relative expression of *NaMYB305* gene. Relative expression was calculated using the elongation factor-1 housekeeping gene (GenBank accession number: *Nicotiana tabacum*, D63396) as an endogenous reference. *NaMYB305* gene-specific primers were used: forward primer 5′-ATGCTAAGTGGGGAAACAG-3′ and reverse primer 5′-GCAATTGCATGGACCAGA-3′.

Volatile Emission

For all volatile emission experiments, emitted floral volatiles from excised flowers were collected at 6 PM and quantified as previously described (Underwood et al., 2005; Dexter et al., 2007). All samples consisted of three flowers per sample with at least three biological replicates.

GenBank Accession Numbers

PhODO1, AY705977; *PhEOBII* (P720), EU360893; *PhEOBII* (MD), EU374207; *promPhEOBII* (MD), HQ450382; *NlxNsMYB305*, EU111679; *RhPIP2;1*, EU572717; *AtMYB21*, AT3G27810; *AtMYB24*, AT5G40350; *PhPAL1*, AY705976; *PhPAL2*, CO805160; *AtXTR6*, AT4G25810; *AtXTR7*, AT4G14130; and *NaMYB305*, Na_454_02248.

Supplemental Data

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

Supplemental Figure S1. Predicted amino acid sequence alignment of putative, homologous EOBII proteins.

Supplemental Figure S2. Schematic representation of the *PhEOBII* gene model.

Supplemental Figure S3. sqRT-PCR transcript accumulation analysis in floral tissues of independent T0 *ir-PhEOBII* lines and MD plants.

Supplemental Figure S4. Floral volatile emission analysis of representative plants from MD, *ir-5*, and 44568 × *ir-5*.

Supplemental Table S1. Accession numbers and respective identifiers of sequences used for the phylogenetic comparisons.

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