

**Regular paper**

***VASCULAR PLANT ONE-ZINC FINGER1 and VOZ2 repress the FLOWERING LOCUS C clade members to control flowering time in Arabidopsis***

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## **Abstract**

Floral transition is regulated by environmental and endogenous signals. Previously, we identified *VASCULAR PLANT ONE-ZINC FINGER1 (VOZ1)* and *VOZ2* as phytochrome B-interacting factors. *VOZ1* and *VOZ2* redundantly promote flowering and have pivotal roles in the downregulation of *FLOWERING LOCUS C (FLC)*, a central repressor of flowering in *Arabidopsis*. Here, we showed that the late-flowering phenotypes of the *voz1 voz2* mutant were suppressed by vernalization in the Columbia and *FRIGIDA (FRI)*-containing accessions, which indicates that the late-flowering phenotype of *voz1 voz2* mutants was caused by upregulation of *FLC*. We also showed that the other *FLC* clade members, *MADS AFFECTING FLOWERING (MAF)* genes, were also a downstream target of *VOZ1* and *VOZ2* as their expression levels were also increased in the *voz1 voz2* mutant. Our results suggest that the *FLC* clade genes integrate signals from *VOZ1/VOZ2* and vernalization to regulate flowering.

## **Key words:**

*Arabidopsis thaliana*; *FLOWERING LOCUS C (FLC)*; flowering; phytochrome B (phyB); *VASCULAR PLANT ONE-ZINC FINGER (VOZ)*

## **Introduction**

Plants have developed mechanisms to adapt to diverse environments to survive as sessile organisms. For successful reproduction, plants control the timing of the floral transition by a sophisticated regulatory network. Seasonal change is an important environmental cue for flowering. Plants monitor seasons mainly by sensing day length, light quality, and prolonged cold temperature (vernalization). The day length is a reliable source of environmental information, and plants use it to anticipate the arrival of season for flowering. In temperate climates, many plants also use cold as an environmental cue to flower at the correct time of year. Many

vernalization-requiring plants also accelerate flowering under long day (LD) conditions. Coupled requirement of vernalization and LD conditions further ensures that flowering occurs only after winter.

In *Arabidopsis thaliana* (*Arabidopsis*), a facultative LD plant, flowering under LD conditions is promoted by the FLOWERING LOCUS T (*FT*) protein. It is widely accepted that *FT*, and *FT* homologs, are the major component of the long-distance signal, florigen, that moves from leaves via phloem to the shoot apical meristem. [1-5] In the shoot apical meristem, *FT* protein activates several floral regulators to initiate flower development. [6, 7] *FT* expression is induced by the transcriptional activator CONSTANS (*CO*) protein. Under the control of the circadian clock, *CO* expression peaks in the afternoon of long days to induce *FT* expression. [8, 9] *CO* protein stability is also regulated by light quality through multiple photoreceptors, including the red/far-red photoreceptor phytochrome B (*phyB*). [9, 10]

Among *Arabidopsis* accessions there are natural flowering-time variations. The winter-annual (late flowering without vernalization) and summer-annual (early flowering) habit is determined by allelic variation at the *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) loci. [11-13] Winter-annual accessions have functional *FRI* and *FLC* genes, whereas summer-annual accessions have mutations in either *FRI* or *FLC* or both [13]. *FRI* positively regulates the MADS-box transcription factor *FLC*, [12, 14] which plays a central role in flowering-time regulation through direct repression of flowering-time integrators, including *FT*. [15] *FLC* is epigenetically silenced during vernalization and plants become competent to flower in warm conditions. [16] The commonly used accession Columbia (*Col*) has the *fri* genotype and hence flowers rapidly without vernalization because *FLC* expression is insufficient to repress downstream genes for flowering. Conversely, many natural accessions carrying functional *FRI* are late flowering because of high *FLC* expression levels, even in inductive LD conditions, without vernalization treatment. This *FLC*-induced repression of flowering can be overridden by FR

enrichment, [17, 18] although the molecular mechanism for this regulation is largely unknown.

Previously, we have reported that *VASCULAR PLANT ONE-ZINC FINGER1* (*VOZ1*) and *VOZ2* have pivotal redundant roles in the upregulation of *FT* and downregulation of *FLC* expression to promote flowering. [19] *VOZ1* and *VOZ2* interact with phyB, and act downstream of phyB in flowering regulation. [19] *VOZ1* and *VOZ2* encode the subgroup VIII-2 of NAC proteins, which comprise one of the largest transcription factor families in plants. [20, 21] In addition, *VOZ1* and *VOZ2* are involved in changing the expression of various stress-related genes and controlling both abiotic and biotic stress-responsive pathways, such as those related to freezing responses and pathogens. [22, 23]

Recently, it was reported that the *voz1 voz2* mutant is responsive to vernalization. [24] In the present study, we further investigated *VOZ* function in the regulation of *FLC*, analyzing the responses to vernalization using *voz1 voz2* mutants both in Col and *FRI;FLC* backgrounds. In these backgrounds, the late-flowering phenotype of *voz1 voz2* mutants is completely suppressed by vernalization. We also showed that *VOZ1* and *VOZ2* regulate the expression of *FLC* and its paralogous genes. *VOZ1* and *VOZ2* may control flowering time by coordinating the *FLC* clade gene expression levels in response to environmental cues.

## **Results**

*FLC-mediated flowering delay in the voz1 voz2 mutant is suppressed by vernalization*

*VOZ1* and *VOZ2* repress *FLC* expression in leaves and the *voz1 voz2* mutant shows a late-flowering phenotype. [19] As *FLC* is a major determinant of the vernalization response, we evaluated the role of *VOZ1* and *VOZ2* in this response. The vernalization-treated *voz1-1 voz2-1* mutants flowered much earlier than the non-vernalized mutants, which indicates that the vernalization pathway is intact in

*voz1-1 voz2-1*. Col and *voz1-1 voz2-1* showed a similar flowering time after vernalization treatment (Fig. 1). As vernalization completely suppresses the late-flowering phenotype of *voz1-1 voz2-2*, signals in vernalization- and *VOZ1/VOZ2*-mediated pathways must be integrated at a certain point.

Fig. 1

Next, we compared the *FLC* transcript levels after vernalization treatment. As previously reported, in the wild type the expression level of *FLC* is significantly decreased after vernalization treatment. [12] (Fig. 2) Consistent with the flowering phenotype (Fig. 1), repression of *FLC* by vernalization was also observed in the *voz1-1 voz2-1* mutant (Fig. 2). Moreover, even after plants were returned to a moderate temperature environment, the repression of *FLC* expression was maintained in *voz1 voz2* mutants as in Col (Fig. 2). These results indicate that *VOZ1* and *VOZ2* are not required for the vernalization-mediated *FLC* repression and the stable maintenance of repressed *FLC*.

Fig. 2

*Suppression of the late-flowering phenotype of voz1 voz2 by vernalization is further confirmed in the FRI background*

Columbia, a summer-annual *Arabidopsis* accession, has a loss-of-function mutation in *FRI* and does not require vernalization to promote rapid flowering. To further investigate the role of *VOZ1* and *VOZ2* in the regulation of *FLC*, we analyzed the effects of the *voz1 voz2* double mutation in the Col-*FRI* (Col plants harboring a functional *FRI* allele from ecotype San Feliu-2) background. Without vernalization treatment, the *FRI*-containing *voz1-1 voz2-1* mutant showed an extreme late-flowering phenotype compared with Col-*FRI* (Fig. 3). Interestingly, after vernalization, the *FRI voz1-1 voz2-1* plants significantly accelerated flowering and the flowering time was comparable to that of Col-*FRI* plants (Fig. 3).

Fig. 3

Considered together with the flowering phenotype of the Col background (Fig. 1), these results show that the late-flowering phenotype caused by the *voz1 voz2* double mutation is completely suppressed by vernalization. This indicates that *FLC*

upregulation is the cause of the late-flowering phenotype of the *voz1 voz2* mutants, and that *VOZ1* and *VOZ2* control *FLC* expression in a vernalization-independent pathway.

*VOZ1 and VOZ2 repress FLC/MAF clade gene expression in leaves to promote flowering*

Given the evidence from the physiological analysis that *FLC* is a downstream factor of *VOZ1* and *VOZ2*, we investigated the genetic interaction between *VOZs* and *FLC*. We generated the *flc-3 voz1-1 voz2-1* triple mutant and compared the flowering time with the *voz1-1 voz2-1* mutant and the *flc-3* mutant. As previously reported, the *flc-3* mutant shows a slightly early-flowering phenotype compared with the Col background (Fig. 4). [25] The *flc-3 voz1-1 voz2-1* triple mutant flowered earlier than the *voz1-1 voz2-1* double mutant, but later than the *flc-3* single mutant (Fig. 4), suggesting that *FLC* is not a sole downstream target of *VOZ1* and *VOZ2*.

Fig. 4

In the *Arabidopsis* genome, there are five paralogs of the *FLC* gene, comprising *FLOWERING LOCUS M (FLM)/MADS AFFECTING FLOWERING1 (MAF1)* and *MAF2* to *MAF5*. As for *FLC*, *MAF1–MAF5* also have functions in the control of flowering, [26-31] and the proteins in the *FLC* clade interact with each other and form complexes that coordinate the flowering response. [29] We examined whether the expression levels of *MAF* genes were altered in *voz1 voz2* mutants, using leaves of 10-day-old seedlings grown under LD conditions. Similar to the *FLC* expression level, [19] the mRNA levels of *MAF1–MAF5* were also elevated in *voz1-1 voz2-1* mutants (Fig. 5). These results indicate that these *MAF* gene family members represent additional regulatory targets of *VOZ1* and *VOZ2*.

Fig. 5

## Discussion

We have previously reported that *VOZ1* and *VOZ2* are phyB-interacting factors and redundantly promote flowering by repressing *FLC* expression. [19] However, details

of the relationship between *FLC* and *VOZ* functions in flowering pathways have been elusive. The present study describes the role of *FLC* downregulation by *VOZ1* and *VOZ2* to control flowering time in *Arabidopsis*. We also provide evidence that *VOZ1* and *VOZ2* negatively regulate expression of *FLC* and its paralogous genes, *MAF1–MAF5*, to promote flowering in a vernalization-independent pathway (Fig. 6).

Fig. 6

It should be noted that the *voz1 voz2* double mutation affects flowering time in the *FLC;FRI* background as in the Col background under the non-vernalized condition, and that vernalization treatment completely suppressed the late-flowering phenotype of *voz1-1 voz2-1* mutants in both *fri* and *FRI* backgrounds (Figs. 1 and 3). Our results show that *VOZ1* and *VOZ2* are not required for *FLC* repression by vernalization (Fig. 2), which indicates that these factors are not involved in the vernalization pathway itself. It has also been reported that the *FRI*-containing lines and the autonomous pathway mutants, in which late-flowering phenotypes are tightly associated with the elevated *FLC* expression, show sensitivity to vernalization. The complete suppression of the late-flowering phenotype of the *voz1 voz2* mutant by vernalization confirmed that *VOZ1* and *VOZ2* promote flowering through repression of *FLC*.

Our genetic data suggested that *FLC* is not the sole downstream target of *VOZ1* and *VOZ2* (Fig. 4) and the expression analysis indicated that the other *FLC* clade members, *MAF1–MAF5*, are additional regulatory targets of *VOZ1* and *VOZ2* (Fig. 5). The members of the *FLC* clade are MIKC<sup>c</sup>-type MADS-domain proteins, which can assemble into quaternary complexes to regulate the target genes. [32, 33] Future research using multiple mutants for *VOZ* and *FLC* clade genes will help to further understand the genetic relationship between *VOZ* and *FLC* clade genes and the regulatory mechanism of their downstream targets.

A recent study reported that *FLC* and *MAF1–MAF4* proteins directly interact with each other and form nuclear complexes to directly regulate *FT* expression. [29] The transcript levels of the *FLC* clade members are regulated by environmental cues.

*FLC*, *MAF1*, *MAF2*, and *MAF3* are repressed by vernalization. [27, 28] Changes in ambient temperature affect the alternative spliced form of *MAF1* and control the flowering time. [34-36] The photoperiod modulates diurnal abundance of *MAF1*, *MAF2*, and *MAF3*. [29] *VOZ1* and *VOZ2* are phyB-interacting factors and the VOZ protein stability is regulated by light quality. [19] *VOZ1* and *VOZ2* may coordinate responses to an environmental signal including light quality, and regulate the expression levels of the *FLC* clade members to control flowering time. A recent field experiment supports our suggestion. Wilczek et al. showed that *FLC*-induced flowering repression, even in a winter-annual accession, could be overridden other than by vernalization, possibly as a result of natural temperature fluctuations and light conditions. [37] Future research on the relationship between VOZ-mediated phyB signal and *FLC*-mediated flowering regulation may help to increase our understanding of the crosstalk between the light signal and other environmental signals such as temperature in flowering pathways.

## **Materials and methods**

### *Plant materials and growth conditions*

The wild-type plant used was the Col accession of *Arabidopsis thaliana*. Genotypes and alleles used were as follows: *voz1-1 voz2-1*, [19] *flc-3*, [12] and Col-*FRI* [38] were previously reported. The *flc-3 voz1-1 voz2-1* and *FRI voz1-1 voz2-1* mutants were produced by crossing. Plants were grown in soil or on half-strength Murashige and Skoog (MS) agar medium, chilled for 3–4 days at 4°C, then transferred to growth chambers under LD (16 h light/8 h dark) with an illumination intensity of 90–110  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of white fluorescence light at 22°C. For vernalization treatment, seedlings were grown for 7 days after germination and subjected to cold treatment for 12 weeks.

### *RNA isolation and expression analysis*



Methods for RNA preparation and quantitative RT-PCR were described previously. [19] Expression of *ACT2* was used for normalization. The following thermal profile was used for all PCRs: 95°C for 10 s, 40 cycles of 95°C for 5 s, and 60°C for 30 s. Primer sets used in this study were as follows: *FLC*, 5'-CCGAACTCATGTTGAAGCTTGTTGAG-3' and 5'-CGGAGATTTGTCCAGCAGGTG-3'; *MAF1*, 5'-GGAAAGAATACGTTGCTGGCAACA-3' and 5'-CCGTTGATGATGGTGGCTAATTGA-3'; *MAF2*, 5'-CGAAATACATCATGCTGATGAACTTG-3' and 5'-GCTTTGGACTATTTCTAGTAACTCTTTGA-3'; *MAF3*, 5'-GGAAATAAAGGTAAAACAAAACGAAGCTCTT-3' and 5'-GAACTCTGATATTTGTCTACTAAGGTACA-3'; *MAF4*, 5'-GATGGGGAAGATGAAGAAGTCTGT-3' and 5'-AGTCTCCGGTGGCTTGTTGT-3'; *MAF5*, 5'-GAAACAGGGGATGAAAGAGCAGTA-3' and 5'-TGGGCTGTGGCCAGAGCTAT-3'.

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### **Author contributions**

Y.Y. and T.K. designed the research. Y.Y. performed research. Y.Y. analyzed data.

Y.Y and T.K. wrote the article.

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### Figure caption

Fig. 1. Flowering time of the *voz1 voz2* mutant in response to vernalization. Flowering time was measured as the number of days to bolting after vernalization treatment. After vernalization, plants were grown under LD conditions (16 h white light/8 h dark). Data are the mean  $\pm$  SD ( $n \geq 20$ )

Fig. 2. Relative expression level of *FLC* in the wild-type and *voz1 voz2* mutant in response to vernalization. Relative mRNA expression levels of *FLC* in Col and *voz1 voz2* mutant were measured using real-time quantitative RT-PCR. Non-ver, non-vernalized; Ver, 12 weeks of vernalization; Ver $\rightarrow$ 22°C, 12 weeks of vernalization followed by 5 days at 22°C. Real-time quantitative RT-PCR experiments were performed with three biological replicates. *ACT2* was used as a control. Data are the mean  $\pm$  SE ( $n = 3$ ).

Fig. 3. Flowering time of the *voz1 voz2* mutant in the Col-*FRI* background in response to vernalization. Flowering time was measured as the number of days to bolting after vernalization treatment. After vernalization, plants were grown under LD conditions (16 h white light/8 h dark). Data are the mean  $\pm$  SD ( $n \geq 7$ ). Flowering of non-vernalized *FRI voz1 voz2* plants did not occur within the duration of the experiment (200 days).

Fig. 4. Rosette leaf numbers at bolting of the *voz1 voz2* mutant and *flc voz1 voz2* triple mutant. Rosette leaf numbers at bolting stage were counted for the plants grown under LD conditions. Data are the mean  $\pm$  SD ( $n \geq 13$ )

Fig. 5. Relative expression level of *MAF* family genes in the *voz1 voz2* mutant. Relative expression levels of *MAF1-MAF5* were determined by real-time quantitative RT-PCR in Col and the *voz1 voz2* mutant. Plants were grown for 10 days under LD conditions. RNA was extracted from rosette leaves.

*ACT2* was used as a control. RNA extraction was performed three times independently. Data are the mean  $\pm$  SE ( $n = 3$ ).

Fig. 6. A proposed model of *VOZ* function in the flowering network. Our data indicate that *VOZ1* and *VOZ2* repress the expression of *FLC* and *MAF* genes in a vernalization-independent pathway.



Figure 1

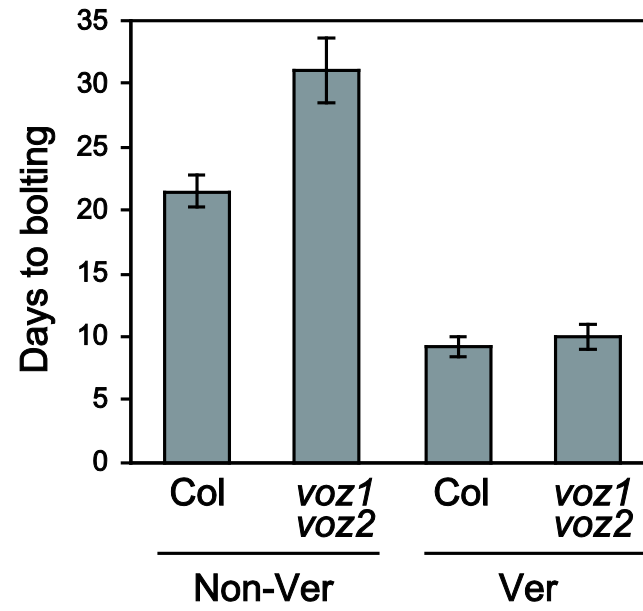
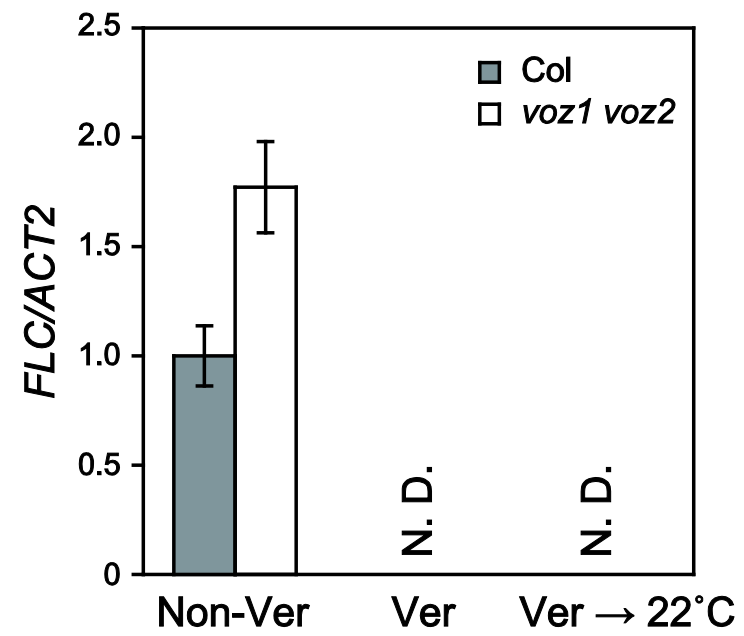


Figure 2



# Figure 3

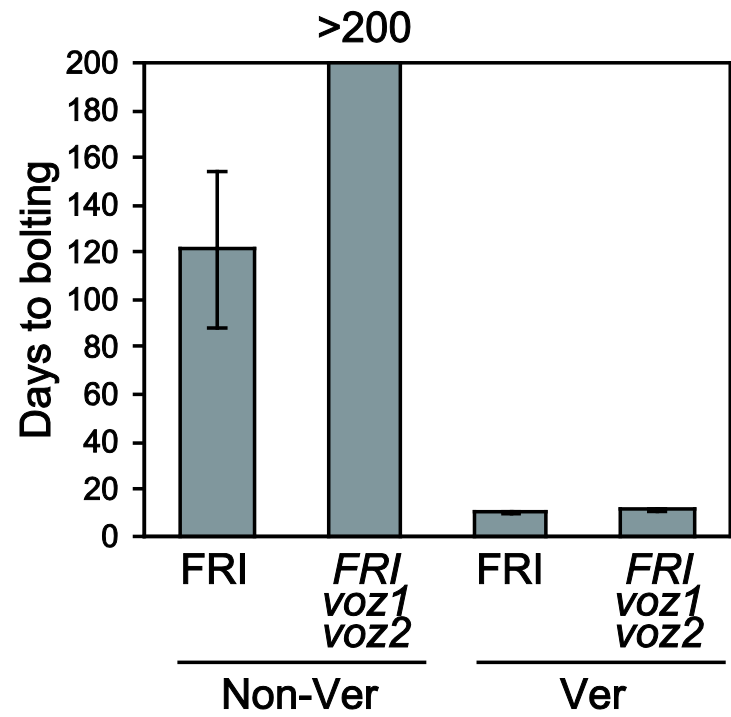


Figure 4

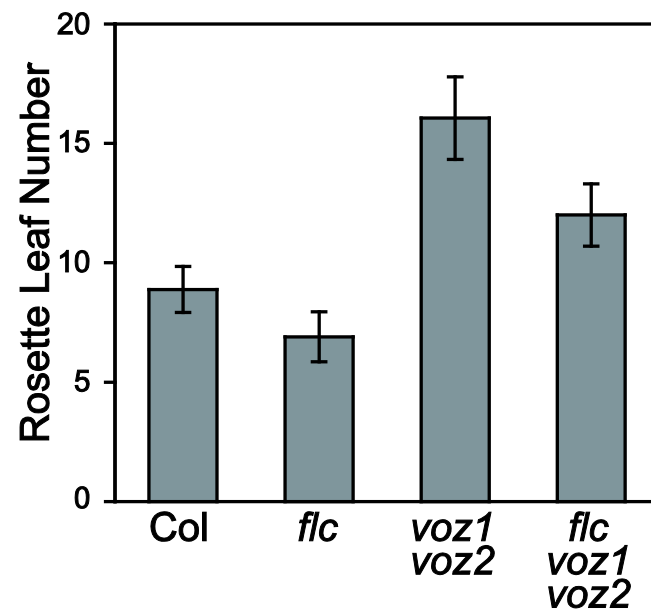


Figure 5

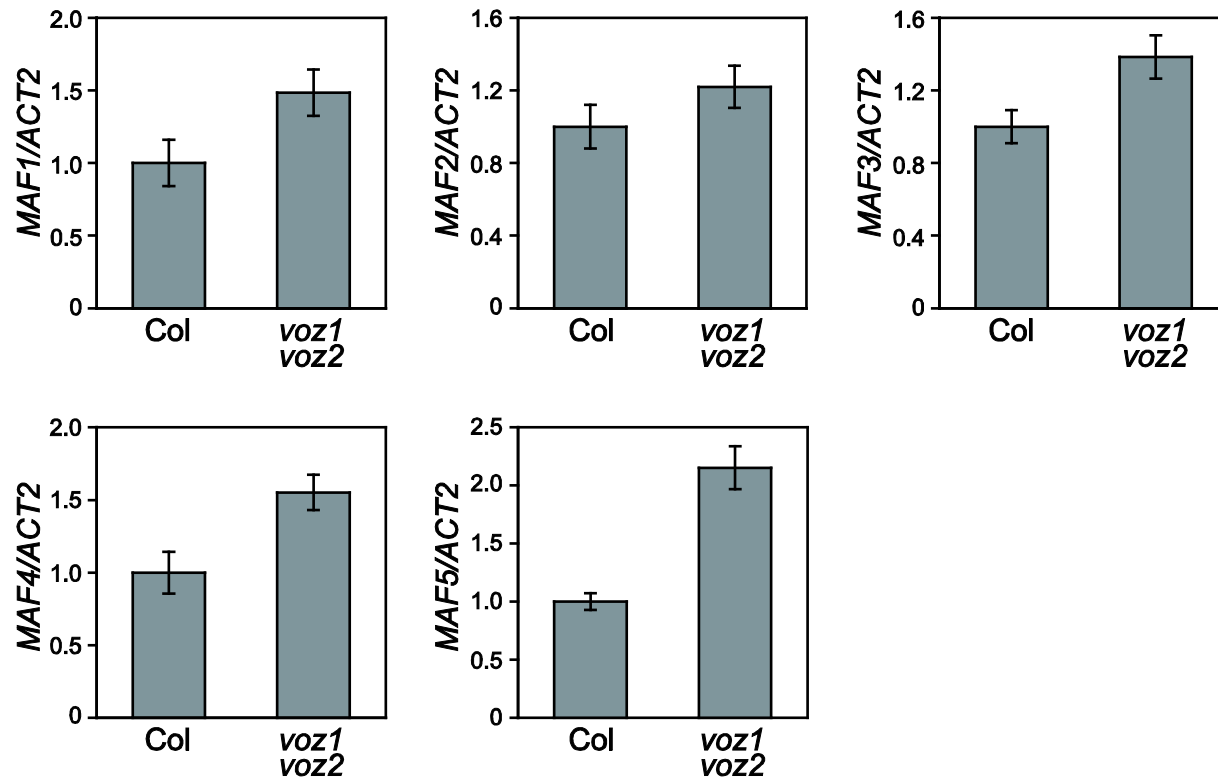


Figure 6

