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**Authors' contributions**

JH performed ABA measurement, RNA extraction, qRT-PCR analysis, and plant materials collection; QM focused on qRT-PCR analysis, transient expression in grapevine, statistical analysis and the writing of this paper; LH worked on GUS staining analysis and primers design; FZ focused on promoter cloning and plant materials collection; XL worked on experiments design and stomatal bioassays; JH and QM contributed equally to this work

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**Competing interests**

No competing interests have been declared.

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## ORIGINAL RESEARCH PAPER

# VvWRKY13 enhances ABA biosynthesis in *Vitis vinifera*

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**Abstract**

Abscisic acid (ABA) plays critical roles in plant growth and development as well as in plants' responses to abiotic stresses. We previously isolated VvWRKY13, a novel transcription factor, from *Vitis vinifera* (grapevine), and here we present evidence that VvWRKY13 may regulate ABA biosynthesis in plants. When VvWRKY13 was ectopically expressed in *Arabidopsis*, the transgenic lines showed delayed seed germination, smaller stomatal aperture size, and several other phenotypic changes, indicating elevated ABA levels in these plants. Sequence analysis of several genes that are involved in grapevine ABA synthetic pathway identified WRKY-specific binding elements (W-box or W-like box) in the promoter regions. Indeed, transient overexpression of VvWRKY13 in grapevine leaves significantly increased the transcript levels of ABA synthetic pathway genes. Taken together, we conclude that VvWRKY13 may promote ABA production by activating genes in the ABA synthetic pathway.

**Keywords**

VvWRKY13; transcription factor; senescence; ABA; *Vitis vinifera*

**Introduction**

Phytohormone abscisic acid (ABA) regulates a number of plant growth and developmental processes including seed maturation and germination, stomatal movements in response to stress conditions, root growth, and leaf senescence [1,2].

In higher plants, ABA is synthesized via the terpenoid pathway that begins with isopentenyl diphosphate (IPP) converting into violaxanthin [1]. This initial step is catalyzed by zeaxanthin epoxidase (ZEP) encoded by the *ABA1* locus of *Arabidopsis* [3,4]. Violaxanthin is converted to 9-*cis*-neoxanthin and is then cleaved into xanthoxal by 9-*cis*-epoxycarotenoid dioxygenase (NCED) [5-7]. Xanthoxal is converted to ABA aldehyde, which is catalyzed by a short chain dehydrogenase/reductase-like (SDR) enzyme, encoded by the *ABA2* locus in *Arabidopsis* [8,9]. In the end, ABA aldehyde is oxidized to ABA by abscisic aldehyde oxidases (AAOs) [10]. Although ABA biosynthesis takes multiple steps catalyzed by several enzymes, NCED is considered to be the key regulatory enzyme in ABA biosynthesis [5,7]. On the other hand, the produced ABA is inactivated through ABA 8'-hydroxylation pathway, and the ABA 8'-hydroxylase is encoded by a small gene family with four members (*CYP707A1* to *CYP707A4*) in *Arabidopsis* [11,12]. The level of active ABA in a particular tissue thus depends on the activities of ABA synthetic and hydroxylation enzymes. These enzymes are known to be regulated at multiple levels including transcription by transcriptional factors in response to variety of stress conditions.

More than 64 families of transcription factors (TFs) have been identified in plants [13] until now. WRKY proteins represent a large family of TFs that specifically interacts with W-box [(T) (T) TGAC(C/T)] or related sequences to activate genes [14-16]. While much of the work on WRKY family has been done in the model plant *Arabidopsis*

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[17], little is known about the function of these TFs in crop plants. Recent studies have indicated that some WRKY transcription factors also participate in the pathway of ABA signaling and ABA production. The cotton WRKY transcription factor, GhWRKY17, increased sensitivity of transgenic *Nicotiana benthamiana* to drought. It achieved this by reducing the level of ABA and transcript levels of ABA-inducible genes, including *AREB*, *DREB*, *NCED*, *ERD*, and *LEA* [18]. AtWRKY33 acted upstream of *NCED3/NCED5* to negatively regulate ABA biosynthesis in *Arabidopsis* [19]. Recent studies also characterized several WRKY genes in cultivated grapevines and Chinese wild *Vitis pseudoreticulata*. All of them have been shown to regulate biotic or abiotic responses [20–26], but there has been no report about grapevine WRKY transcription factors affecting the ABA signaling and ABA production, until now.

We identified a WRKY protein, VvWRKY13, which belongs to the group IIc of WRKY transcription factors, from the grapevine cultivar ‘Zuoyouhong’ [27]. In this study, we examined its function by overexpression in transgenic *Arabidopsis* plants and transient expression in grapevine leaves. Our data indicates that VvWRKY13 is involved in seed germination, stomatal opening, and several other phenotypes via regulating ABA biosynthesis.

## Material and methods

### Plant material, growth condition, and treatments

The cultivation of the grapevine (*Vitis vinifera* L.) cultivar, ‘Zuoyouhong’ and its tissue culture seedlings, was performed as previously described [27]. The growth condition of *Arabidopsis* was also the same as previously described [27]. The roots were collected from 2-week-old seedlings, and used for qRT-PCR analysis. The leaves were collected from 6-week-old plants, and used for qRT-PCR analysis to detect senescence corresponding to gene expression level.

### DNA constructs, plant transformation, and qRT-PCR analysis

The VvWRKY13 promoter segment was cloned from the grapevine cultivar ‘Zuoyouhong’. The VvWRKY13 promoter sequence was amplified using the primers Promoter<sub>VvWRKY13</sub>-FP and Promoter<sub>VvWRKY13</sub>-RP (Tab. S1), which contain a *Pst*I and *Eco*RI linker sites on the forward and reverse primer, respectively. This fragment was then cloned into the pMD18-T vector (Takara, Japan). The fragment was excised using the restriction enzyme sites in the linkers and ligated downstream of the *CaMV35S* promoter in pCAMBIA1391 linear vector, which contains a GUS gene downstream of the inserted fragment.

The resulting constructs were transferred into *Agrobacterium tumefaciens* strain GV3101. The transformation into Columbia ecotype of *Arabidopsis* along with the selection of transformed homozygous lines was performed as previously described [27].

The 4–5-week-old grapevine tissue culture seedlings were selected for the transient transformation by agroinfiltration. This transformation was performed as previously stated but with minor modifications [28]. The GV3101 strains containing 35S::VvWRKY13 recombinant plasmid were reactivated and amplified at 28°C in YEB liquid culture with kanamycin and rifampicin. Then, collected by centrifugation at 5000 g for 15 min, the pellet was subsequently washed and suspended with induction media (10 mM MES, pH 5.6; 10 mM MgCl<sub>2</sub>; 2% w/v glucose; 150 μM acetosyringone). The new suspension was cultivated again until reaching a density of OD<sub>600</sub> = 0.6. At the same time, P19 strain that functions as gene silencing suppressor, was also activated, amplified, centrifuged, and resuspended into a density of OD<sub>600</sub> = 0.6. Then, the GV3101 and the P19 were mixed at the ratio of 1:1, and infiltrated into the leaves of 4–5-week-old grapevine tissue culture seedlings using a vacuum pump. The leaves were harvested and analyzed after 4 days of infiltration.

Total RNA was extracted using a Plant RNA Extraction Kit (TaKaRa, Japan). The first-strand cDNA was synthesized using M-MLV Reverse Transcription Kit (TaKaRa, Japan). Quantitative RT-PCR was performed using Q-tower 2.0 real-time PCR detection

system (Analytik Jena, Germany) with the presence of SYBR Green I (TaKaRa, Japan) in the amplification mixture according to the manufacturer's protocols. Specific primer pairs were showed in [Tab. S1](#). Amplification of  $\beta$ -actin transcripts served as the internal standard. The data were analyzed using Q-tower software (Analytik Jena).

#### GUS staining analysis

The Promoter<sub>*VvWRKY13*</sub>-GUS transgenic lines were used for GUS activity assays. The GUS staining was performed as previously described but with minor modifications [29]. First, plant tissues were prefixed in 90% (v/v) acetone for 20 minutes, then washed two times with water. Plant material was then placed into the GUS staining buffer [50 mM NaH<sub>2</sub>PO<sub>4</sub>, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM Na<sub>2</sub>EDTA, 0.5 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>], 0.5 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>], 0.1% Triton-100 (v/v), 1 mM X-Gluc] under a vacuum for 10 minutes at room temperature followed by incubation for 12 hours at 37°C. The chlorophyll was then removed using 75% (v/v) ethanol several times. The plant tissues were photographed at this time.

#### Stomatal bioassays

The fully expanded rosette leaves were harvested and immersed in MES buffer, which contained 50 mM KCl, 0.1 mM CaCl<sub>2</sub>, 10 mM MES, pH 6.1. The stomatal bioassay had been performed as previously described [24].

#### Endogenous ABA extraction and determination

Rosette leaves of 4-week-old *Arabidopsis* seedlings were collected, weighed, and immediately frozen in liquid nitrogen. Frozen leaves were grounded to fine powder and ABA was extracted as previously described [9]. Quantitative determination of endogenous ABA was performed by HPLC as previously described [30].

#### Statistical analysis

Statistical analyses were performed using SAS, and the statistical significance evaluated by ANOVA. All tests were repeated at least three times.

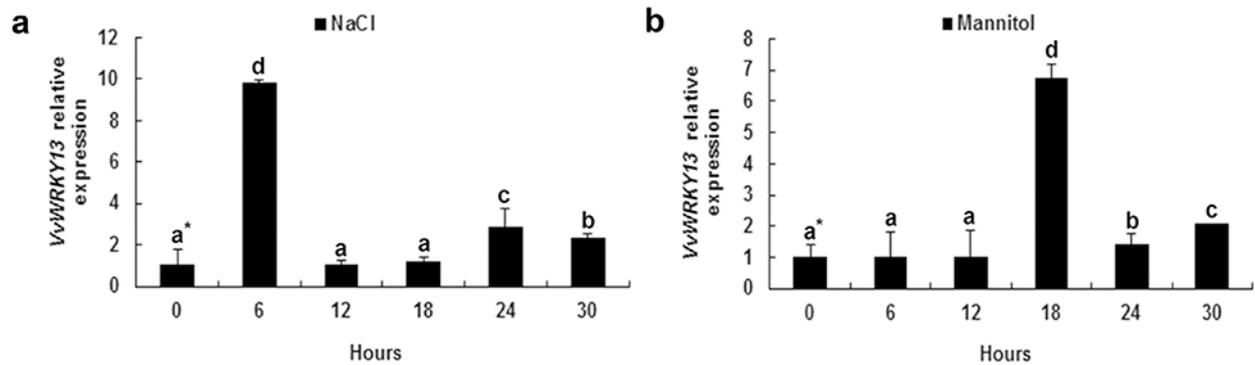
## Results

#### *VvWRKY13* expression induced by stress conditions

Although *VvWRKY13* was broadly detected, expressing in many tissues of grapevine [27], its expression under stress is still unclear. Using grapevine (*Vitis vinifera* L.) cultivar 'Zuoyouhong', 4–5-week-old tissue culture seedlings were treated with 200 mM NaCl and 200 mM mannitol for 0, 6, 12, 18, 24, 30 h. The relative expression level of *VvWRKY13* was detected by qRT-PCR. The results indicated that the expression level of *VvWRKY13* had increased after salt treatment, reaching the peak of transcript accumulation after 6 h of treatment ([Fig. 1a](#)). During treatment with mannitol to mimic drought stress, the expression of *VvWRKY13* showed the peak after 18 h ([Fig. 1b](#)). Similar expression patterns were thus observed both in salt and drought stresses ([Fig. 1](#)).

#### Expression pattern of *VvWRKY13*

In order to investigate the expression patterns of *VvWRKY13*, we constructed a Promoter<sub>*VvWRKY13*</sub>:GUS reporter. We generated transgenic *Arabidopsis* transformed with this construct. Promoter<sub>*VvWRKY13*</sub> contained a 1200-bp genomic DNA sequence upstream



**Fig. 1** Relative expression level of *VvWRKY13* in *Arabidopsis* under NaCl (a) and mannitol (b) treatment. The statistical significance of the difference was confirmed by ANOVA. Means followed by the same letter are not significantly different at  $\alpha = 0.05$  level. \*  $p < 0.05$ .

of ATG starting codon. Various tissues of transgenic plants at different developmental stages were examined via histochemical staining. In early-stage seedlings (12 h and 24 h after germination), *VvWRKY13* promoter directed GUS expression in the cotyledons (Fig. 2a–c). At 48 h after germination, GUS activity was detected in the cotyledons and hypocotyls of seedlings. In older seedlings (3–10 days after germination), GUS was expressed in the leaves but not in the roots (Fig. 2d–h). GUS expression was diminished as plants grew older (4 weeks after germination), but mechanical injury appeared to induce GUS expression (Fig. 2i,j).

In the reproductive organs, GUS expression was detected in petals, pistils, young siliques, but not in mature siliques (Fig. 2k–o). Together, these results suggested that *VvWRKY13* was associated with green tissues in young seedlings and floral organs.

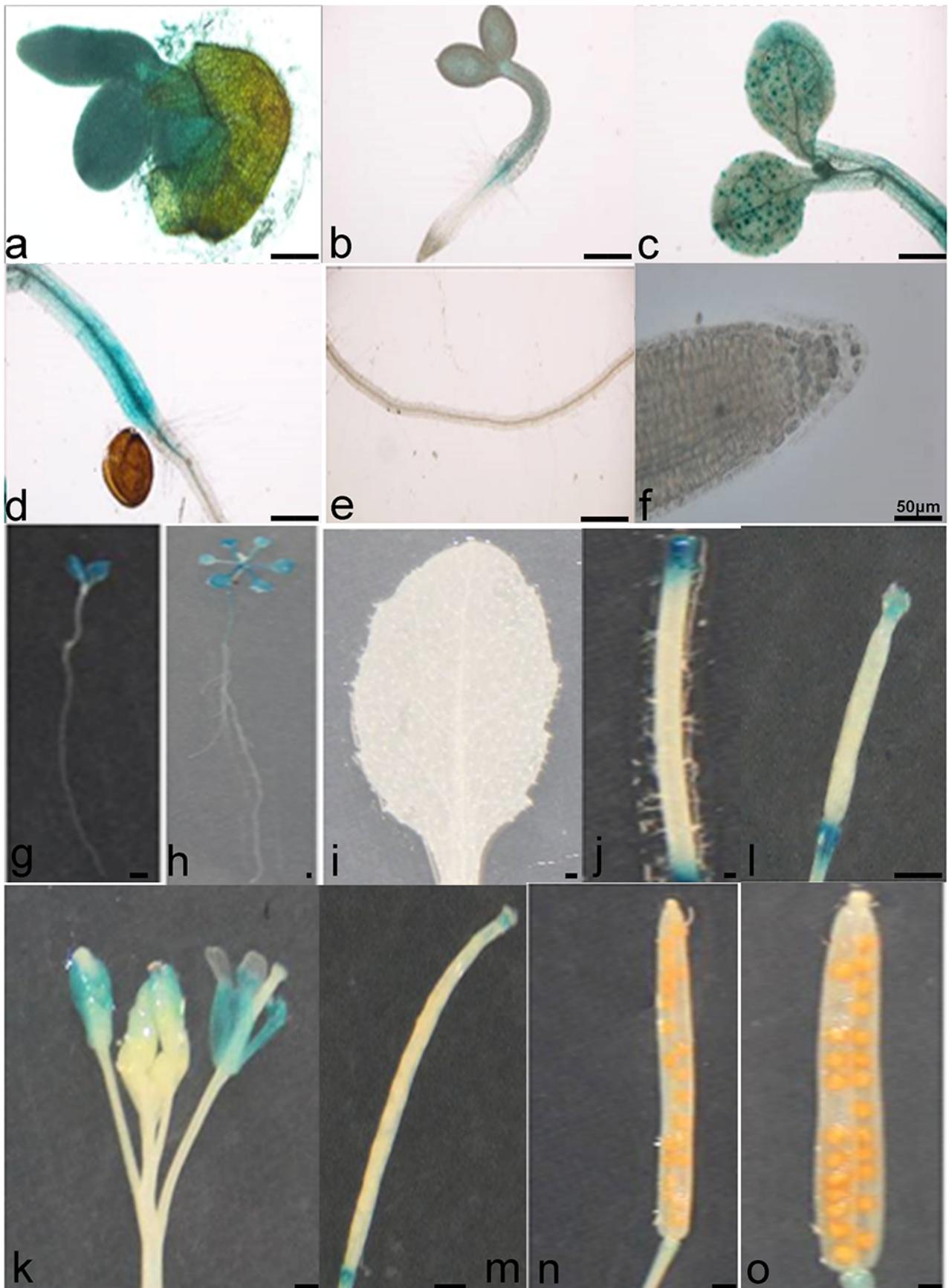
#### The effect of *VvWRKY13* overexpression on seeds germination and stomatal aperture size

To examine the function of *VvWRKY13*, we overexpressed in *Arabidopsis* as a model system. We selected two independent overexpression lines for detailed analysis (Fig. 3a). We found that germination of transgenic seeds was delayed compared to the wild-type control (Fig. 3b). At any time point during germination assay, the germination rates of *VvWRKY13* overexpression lines were lower than the wild type. These results showed that overexpression of *VvWRKY13* inhibited seeds germination, suggesting that the function of *VvWRKY13* might be related to ABA. To test this hypothesis, the stomatal aperture sizes of *VvWRKY13* overexpression lines and the wild-type plants were measured. The results showed that the stomatal aperture size of *VvWRKY13* overexpression lines was significantly smaller compared to the wild type (Fig. 3c).

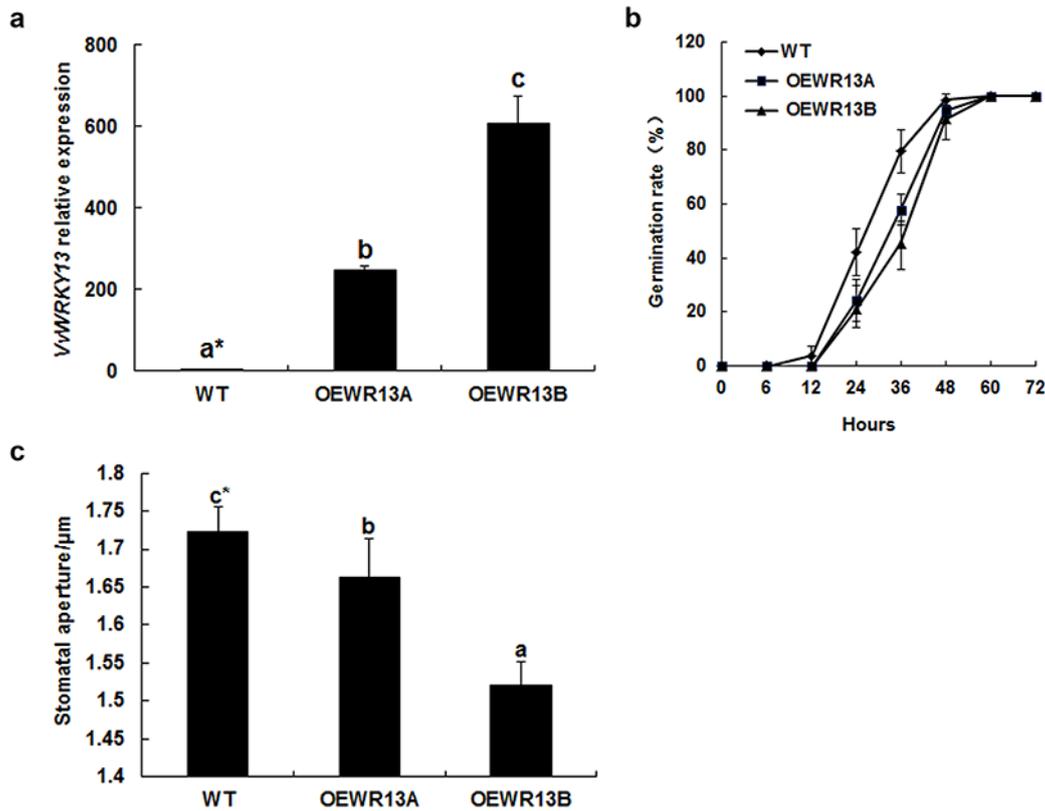
#### The effect of *VvWRKY13* overexpression on root system and plant senescence

It was observed that overexpression of *VvWRKY13* in *Arabidopsis* increased primary root length (Fig. 4a,b) and lateral root number (Fig. 4a,c). The expression levels of other genes involved in root formation such as *AtCDKA1;1* and *AtCYCA2;1* coding cyclins or *AtIAA19* and *AtARF5* controlling auxin biosynthesis [31–34] were higher in the transformed *Arabidopsis* roots than in the wild type (Fig. 4d).

It was also observed that overexpression of *VvWRKY13* in *Arabidopsis* shortened the life period and accelerated plant senescence (Fig. 5a). Checking the expression of senescence related genes *AtSAG12*, *AtSAG13*, and *AtSAG113* [35,36] in the 6-week-old *Arabidopsis* leaves, showed that it was elevated in *VvWRKY13* overexpression lines compared to the wild type (Fig. 5b). In the next step we checked whether overexpression of *VvWRKY13* altered the production of ABA – a plant hormone known to promote senescence [1,2].



**Fig. 2** Histochemical GUS assays in different tissues of Promoter<sub>VvWRKY13</sub>::GUS transgenic *Arabidopsis*. GUS staining analyses of seedlings after 12 h (a), 24 h (b), and 48 h (c) germination; hypocotyl (d), root (e), and root tip (f) of seedlings after 48 h germination; 3-day-old seedlings (g); 10-day-old seedlings (h); 4-week-old leaf (i); 4-week-old stem (j); 3-day-old silique (k); flowers (l); 5-day-old silique (m); and mature silique (n–o). Scale bars: 1 mm (f) 50 μm).



**Fig. 3** Overexpression of *VvWRKY13* (a) inhibited seeds germination (b), and decreased stomatal aperture size (c). The statistical significance of the difference was confirmed by ANOVA. \*  $p < 0.05$ .

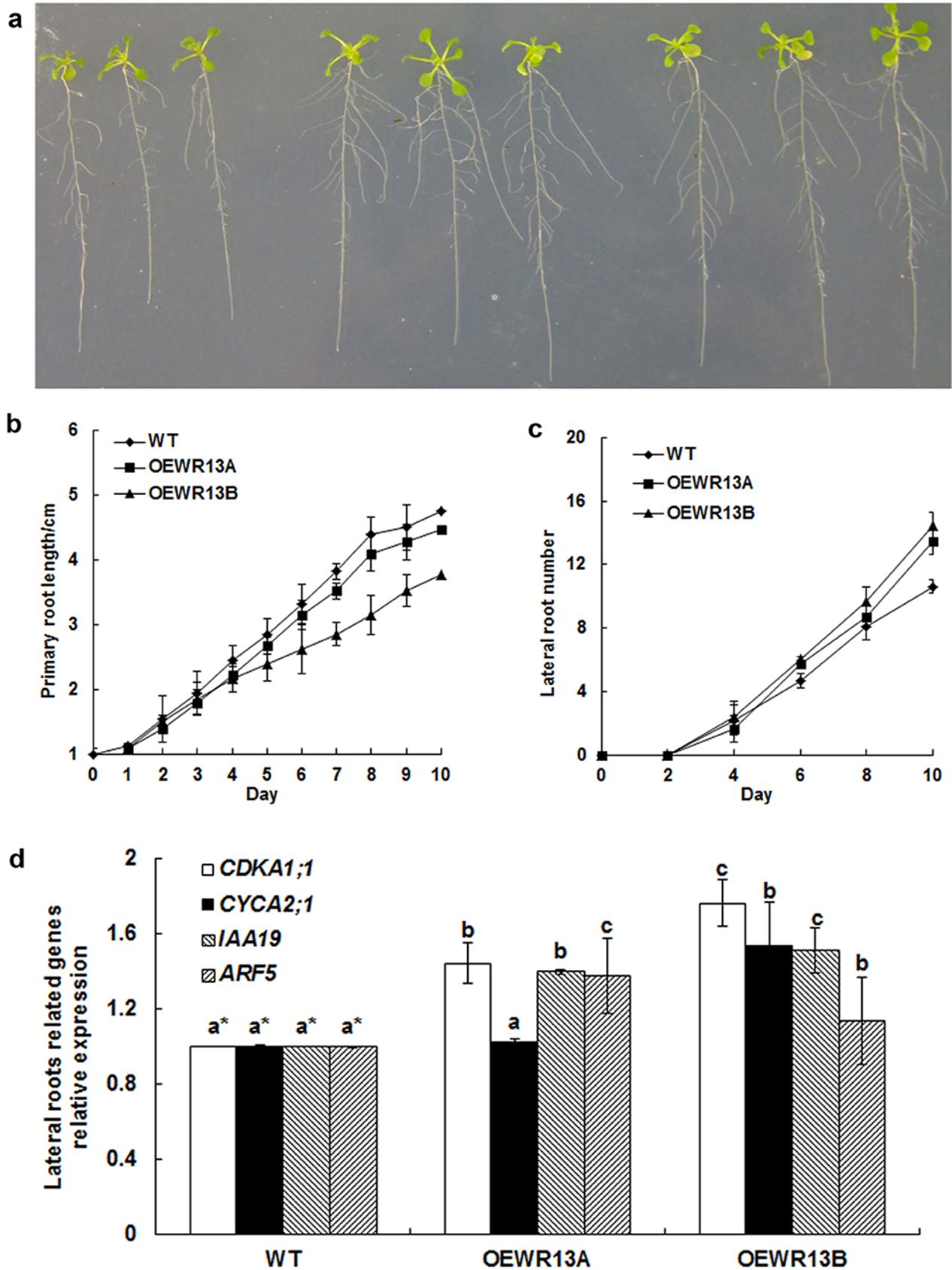
#### The effect of *VvWRKY13* overexpression on ABA production and expression of ABA biosynthesis genes

To find out whether *VvWRKY13* overexpression altered the production of ABA, its level was measured in transgenic and wild-type plants. The results showed that the 4-week-old transgenic *Arabidopsis* seedlings had significantly higher ABA production than wild-type seedlings (Fig. 6a). This result allowed to hypothesize that *VvWRKY13* increased ABA production through regulating its biosynthesis.

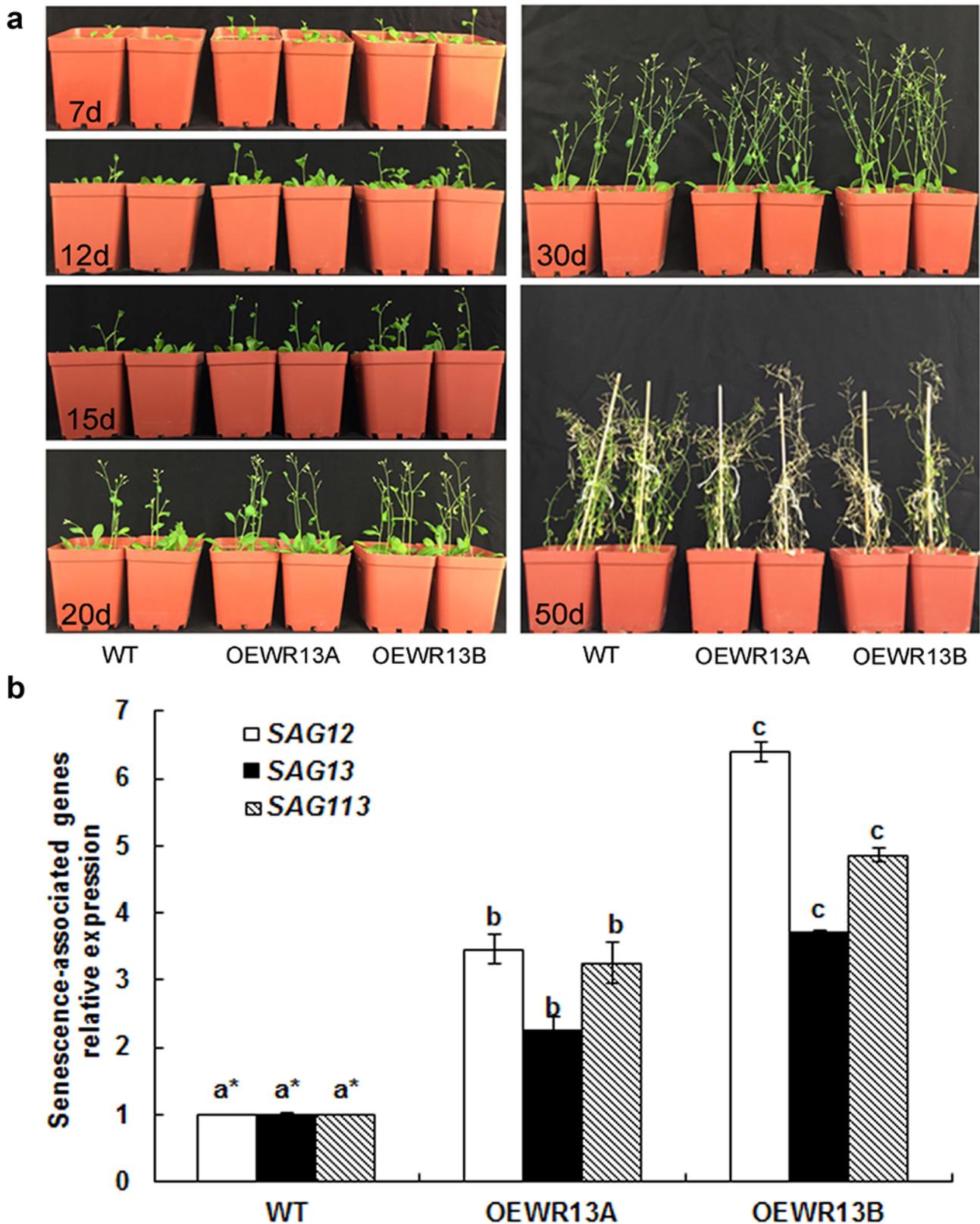
Enzymes functioning in the ABA synthesis pathway include ZEP, NCED, SDR, AAO, etc. The ZEP enzyme is encoded by the *ABA1*, and SDR is encoded by the *ABA2* locus in *Arabidopsis* [1]. Using qRT-PCR, we were able to demonstrate, that the expression of *NCED5*, *ABA1*, and *ABA2* significantly increased in the *VvWRKY13* overexpression lines (Fig. 6b). The expression of *AAO3* increased significantly only in one transgenic line (Fig. 6b). This result confirmed that indeed *VvWRKY13* might increase ABA production by activating the expression of ABA synthesis genes.

#### The expression of ABA synthesis genes in *VvWRKY13* grapevine transient transformed leaves

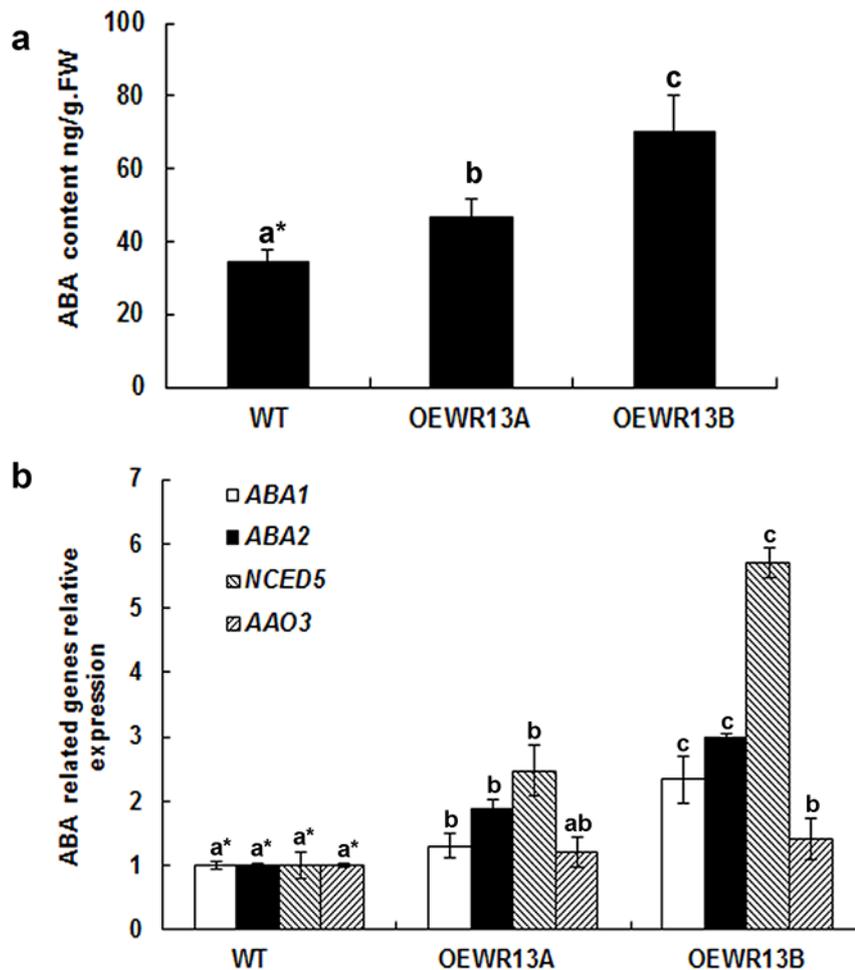
Overexpression of *VvWRKY13* in *Arabidopsis* induced ABA overproduction and increased expression of ABA biosynthesis genes (Fig. 6). How did the corresponding genes' expression level change in the grapevine? Although transformation into grapevine is not as easy as in the case *Arabidopsis*, introduction of transient transforming genes into grapevine leaves caused by agroinfiltration facilitates the research on their function in this plant. Transformation of *VvWRKY13* into the grapevine leaves was confirmed by PCR assay. (Fig. 7a). Overexpression of *VvWRKY13* in grapevine cultivar 'Zuoyouhong' using transient transformed method also showed the increase of *NCED5*, *ABA1*, *ABA2*, and *AAO3*. And these genes all contained W-box or W-like box



**Fig. 4** Overexpression of *VvWRKY13* promoted root formation. *VvWRKY13* increased primary root length (a,b) and lateral root number (a,c). Two-week-old *Arabidopsis* seedlings cultivated in the 1/2 MS medium were used in the picture, and at least 30 plants were measured at each time point in each treatment. **d** The relative expression levels of root formation related genes *CDKA1;1*, *CYCA2;1*, *IAA19*, and *ARF5* by qRT-PCR. The statistical significance of the difference was confirmed by ANOVA. Means followed by the same letter are not significantly different at  $\alpha = 0.05$  level. \*  $p < 0.05$ .



**Fig. 5** Overexpression of *VvWRKY13* accelerated plant senescence. **a** Comparison of plant senescence between the wild type and *VvWRKY13* overexpression lines. **b** The relative expression levels of senescence related genes *AtSAG12*, *AtSAG13*, and *AtSAG113* by qRT-PCR. The statistical significance of the difference was confirmed by ANOVA. Means followed by the same letter are not significantly different at  $\alpha = 0.05$  level. \*  $p < 0.05$

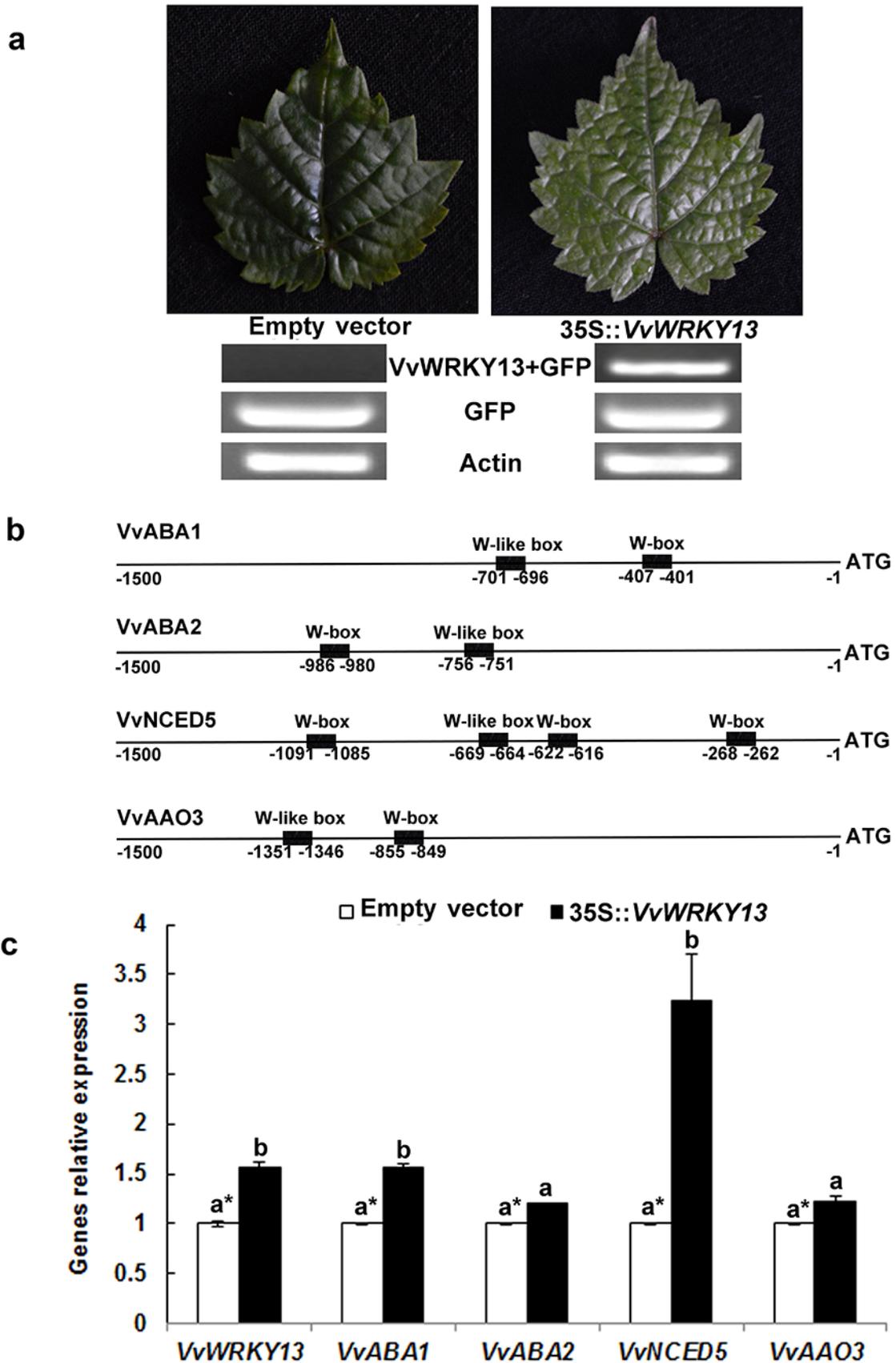


**Fig. 6** VvWRKY13 promoted ABA production by inducing the expression of ABA biosynthesis genes in *Arabidopsis*. **a** ABA production of 4-week-old VvWRKY13 overexpression lines. **b** Relative expression levels of ABA biosynthesis genes *ABA1*, *ABA2*, *NCED5*, and *AAO3* quantified by qRT-PCR. The statistical significance of the difference was confirmed by ANOVA. Means followed by the same letter are not significantly different at  $\alpha = 0.05$  level. \*  $p < 0.05$ .

*cis*-elements in their promoter. (Fig. 7b,c). These results indicated that overexpression of VvWRKY13 in grapevine also promoted ABA biosynthesis. It was concluded that VvWRKY13 might increase ABA production by activating the expression of *NCED5*, *AAO3*, *ABA1*, and *ABA2* genes.

## Discussion

A WRKY transcription factor VvWRKY13 in the grapevine cultivar ‘Zuoyouhong’ was isolated in our previous study [27]. In the later experiments, the functions of VvWRKY13 were continued to be explored. The results of qRT-PCR indicated that VvWRKY13 was induced by salt and drought stress (Fig. 1). VvWRKY13 was related with plant growth and development. According to the results of histochemical staining experiments, the expressed GUS gene was detected in cotyledons and hypocotyls of germinated seedlings, petals, pistils, the top and bottom of tender siliques. The GUS gene was not detected in roots of seedlings, mature leaves and stems of 4-week-old seedlings, and mature siliques (Fig. 2). Together, these results indicate that VvWRKY13 was related with specific stages and organs in the growth and development of the grapevine, such as seed germination, flowering, and siliques development. The mechanical injury increased the expression level of VvWRKY13 in the mature leaves and stems (Fig. 2i,j). Overexpression of VvWRKY13 in *Arabidopsis* also inhibited seed germination, decreased



**Fig. 7** VvWRKY13 induced the expression of senescence and ABA biosynthesis genes in grapevine. **a** States of the grape leaves after being infected by different *Agrobacterium* strains and result analysis with PCR. **b** Sequence analysis of ABA synthase genes *VvABA1*, *VvABA2*, *VvNCED5*, and *VvAAO3* promoters in grape. The WRKY domain interacted W-box and W-like boxes were marked. **c** The relative expression levels of ABA biosynthesis genes quantified by RT-PCR 4 days following infection. The statistical significance of the difference was confirmed by ANOVA. Means followed by the same letter are not significantly different at  $\alpha = 0.05$  level. \*  $p < 0.05$ .

stomatal aperture, and accelerated plant senescence (Fig. 3–Fig. 5). This result showed the function of VvWRKY13 might be related with ABA biosynthesis.

The root system consists of primary, lateral, and adventitious roots in higher plants, and ABA is reported to be functioning in a complex manner [37–39]. In high concentrations, ABA inhibits both primary and lateral root growth [37], nevertheless, it is required in low concentrations for primary root elongation [38,39]. The plants deficient in ABA biosynthesis and signaling also has complex ABA related phenotypes. The primary root of ABA insensitive mutant *rpk1* is less inhibited than the wild type [40]. However, the ABA biosynthesis mutant *aba2* has shorter primary roots and less lateral roots than the wild type [41,42]. In our research, the VvWRKY13 overexpression lines had longer primary roots and more lateral roots because of ABA excessive synthesis, which was coincident with the results of ABA biosynthesis mutant *aba2*. Factors that affect the root growth also include auxin, ethylene, Ca<sup>2+</sup>, H<sub>2</sub>O<sub>2</sub>, reactive oxygen species, etc. [43–45]. The root formation and elongation are probably regulated by a network. The effects of ABA on root growth and development are complex and still need to be further explored.

WRKY transcription factors are reported interacting with W-box and W-like boxes to activate the downstream genes [14–16]. Enzymes functioning in the ABA synthesis pathway include ZEP, NCED, SDR, and AAO, etc. [1]. Bioinformatics and qRT-PCR analysis indicate that the corresponding genes *NCED5*, *AAO3*, *ABA1*, and *ABA2* were activated in the *Arabidopsis* and grapevine transgenic lines (Fig. 6, Fig. 7). This data revealed that VvWRKY13 is likely to promote ABA biosynthesis by the regulation of *NCED5*, *AAO3*, *ABA1*, and *ABA2* expression. Recent studies showed that AtWRKY33 binding the W-boxes of *NCED3/NCED5* promoters negatively regulate ABA biosynthesis in *Arabidopsis* [19]. However, it will be important to prove direct interaction between the VvWRKY13 and the W-box or W-like boxes in the promoters of these genes by yeast one-hybrid electrophoretic mobility shift assay (EMSA), or chromatin immunoprecipitation (ChIP), etc. in the following research.

WRKY transcription factors have different binding intensities to different *cis*-elements and different binding intensities even for same *cis*-elements in various physiological conditions [16]. The diverse of binding intensities may be due to different modification of important amino acid residues or the coexistence of other proteins or cofactors under different physiological conditions [16]. In the end, the different activation of VvWRKY13 to *NCED5*, *AAO3*, *ABA1*, and *ABA2* in ABA biosynthesis pathway will need to be clarified in a future research.

### Acknowledgments

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### Supplementary material

The following supplementary material for this article is available at <http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3546/0>:

**Tab. S1** Oligonucleotide primers used in this study.

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