ORIGINAL ARTICLE

The grape ubiquitin ligase VpRH2 is a negative regulator in response to ABA treatment

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Abstract

Main conclusion Ubiquitin ligase VpRH2 is a negative regulator in the grape ABA pathway by inhibiting *ABL1*, *PYR1* and *GRP2A* expressions, and its promoter is inhibited by ABA treatment.

Abstract In higher plants, ubiquitin ligases play key roles in various cellular processes. As in our previous study (Wang et al. in J Exp Bot 68:1669–1687, 2017), grape RING-H2-type ubiquitin ligase gene *VpRH2* and its promoter was induced by powdery mildew and showed resistance to the disease. Diverse small-molecule hormones, like salicylic acid (SA), methyl jasmonate (MeJA) or abscisic acid (ABA), play pivotal roles in plant resistance. Here we found that *VpRH2* expression could be induced by SA and MeJA treatment, but inhibited by ABA treatment. The promoter of *VpRH2* revealed a similar variation trend under exogenous hormone treatments as the gene expression by GUS activity assay. By a series of deletion fragments, the promoter fragment of *VpRH2-P656* to *VpRH2-P513* was necessary in response to MeJA treatment, and the inhibition of ABA treatment to the *VpRH2* promoter was independent of the ABRE motif. Over-expression of *VpRH2* in *Arabidopsis thaliana* plants displayed ABA-insensitive phenotypes at the germination stage compared to wild type plants. In *VpRH2* over-expressing *Vitis vinifera* cv. Thompson Seedless plants after ABA treatments, the expression of the ABA pathway related genes *ABL1* and *PYR1* showed a suppresive trend. Moreover, *VpGRP2A* (an VpRH2-interacting protein) also showed a suppresive trend in response to ABA treatment in VpRH2-overexpressing plants. Our results demonstrate that VpRH2 is a negative regulator in the grape ABA signal pathway by inhibiting *ABL1*, *PYR1* and *GRP2A* expressions, and its promoter was also inhibited by ABA treatment.

Abbreviations

ABRE	ABA responsive element
GUS	*β*-Glucuronidase
hpt	Hours post treatment
MeJA	Methyl jasmonate
OERH2	Transgenic Thompson Seedless over-expressing *VpRH2*
SA	Salicylic acid
VpGRP2A	*Vitis pseudoreticulata* Glycine-rich RNA-binding protein GRP2A
VpRH2	*Vitis pseudoreticulata* RING-H2-type ubiquitin ligase

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Introduction

Plant development and physiology are regulated by the formation of new polypeptides and the precise degradation of pre-existing proteins, and the ubiquitination-26S proteasome system (UPS) plays important roles in protein degradation in the eukaryote (Smalle and Vierstra 2004). The UPS is highly conserved in higher plants and is precisely controlled by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) (Smalle and Vierstra 2004), a core regulatory mechanism for a variety of cellular processes (Vierstra 2009). The grape genome owns 3 E1s, 45 E2s and more than 600 E3s (Du et al. 2009). E3, which is the key enzyme for protein ubiquitination, is the most complex factor in the ubiquitin cascade because of its largest number in UPS (Smalle and Vierstra 2004). E3 had been divided into four main classes according to the conserved domain: homology to E6-AP C-terminus (HECT), really interesting new gene (RING)/U-box, Skp–Cullin–F-box (SCF) and anaphase-promoting complex (APC) (Craig et al. 2009; Vierstra 2009; Chen and Hellmann 2013; Duplan and Rivas 2014; Morreale and Walden 2016). In grape, RING-type ubiquitin ligase has the largest number, which possesses 275 numbers (Gao et al. 2013), suggesting a wide function in life cycle in plants. The conserved domain of RING-type ubiquitin ligase contains 40–60 amino acids and a typical zinc finger structure containing cysteine (C) and histidine (H) residues to aid in the binding of two zinc ions (Chen and Hellmann 2013). The RING-HC and RING-H2 are the two major types of RING finger domains (Guzman 2014).

Many hormones play key roles in the regulation of plant defense. SA-mediated defense responses appear to play important roles in biotrophic phytopathogens, and JA plays both antagonistic and synergistic interaction with SA (Clarke et al. 2009; De Torres-Zabala et al. 2009; Pieterse et al. 2009). Some researchers have reported that ABA negatively regulates the SA-mediated resistance, but the role of ABA in biotic stress responses is still unclear (Pieterse et al. 2009; Jiang et al. 2010; Cao et al. 2011). Several RING-type E3s have been reported to take part in ABA signaling pathways. RING-type ubiquitin ligase KEG (KEEP ON GOING) involved in the ABA signaling pathway, and the *keg* mutant *Arabidopsis thaliana* are more sensitive to ABA treatment (Stone et al. 2006). *Arabidopsis thaliana* RING-type ubiquitin ligase SDIR1 is involved in ABA-related stress signaling, its over-expression plants show more hypersensitivity to ABA and have a positive function in ABA signaling (Zhang et al. 2007). Three RING-type ubiquitin ligase Arabidopsis T-DNA insertion mutant plants (AtAIRP1, 2, 3) were insensitive to exogenous ABA treatment (Ryu et al. 2010; Cho

et al. 2011; Kim and Kim 2013). Ubiquitin ligase ECERIFERUM9 (CER9) was involved in ABA signaling in seeds and young seedlings of *Arabidopsis thaliana* (Zhao et al. 2014). RING-type E3 ubiquitin ligase RSL1 interacted with the ABA receptors PYL4 and PYR1, and its overexpression plants reduced the sensitivity to ABA treatment (Bueso et al. 2014). Ubiquitin ligase MIEL1 is capable to mediate the degradation of an ABA signaling protein MYB96 (Lee and Seo 2016). Pepper ubiquitin ligase CaAIRF1 interacted and ubiquitinated CaADIP1, a PP2C protein (2C protein phosphatase), and over-expression of *CaAIRF1* in Arabidopsis resulted in an enhanced tolerance to ABA (Lim et al. 2017). But there are few reports about ubiquitin ligase in grape ABA pathway.

Grapevine is an important fruit crop worldwide and has been used for wine, table grapes, juice and dry grapes (Bouquet et al. 2008). From the FAOstat (https://faostat3.fao.org) in 2017, the production of grapes was 74,276,583 tons and ranked fourth in fruit trees. *Vitis vinifera* is the most important species of grape cultivation in the world. It has the widest acreage, the best commodity value and the long history of cultivation. However, *V. vinifera* has a low resistance to abiotic and biotic stresses (Wang et al. 1995; Cao et al. 2010; Cadle-Davidson et al. 2011). China is one of grape origin center in the world. Chinese wild grapevine *V. pseudoreticulata* Baihe-35-1 was studied for its ability in resistance to abiotic and biotic stresses (Li et al. 2010; Xu et al. 2010, 2011; Yu et al. 2011, 2013a, b; Zhu et al. 2012a, b, 2013; Yao et al. 2017). One of which is VpEIRP1, a RING-type ubiquitin ligase, that was found that interact with VpWRKY11 and play a positive role in grape resistance to infected with powdery mildew (Yu et al. 2013b). A U-box type ubiquitin ligase VpPUB24 was induced by cold stress and maintained the protein balance of VpICE1 (Yao et al. 2017).

The expression of stress-related genes was regulated by transcription factors which bind to the *cis*-elements in the promoter (Noda et al. 2013). In our research group, some promoters had been found as being activated by abiotic and biotic stresses. For example, *VpSTS* promoter was isolated from Chinese wild grape Baihe-35-1 and exhibited an induced trend after powdery mildew. Under SA and low temperature treatments (Xu et al. 2010), the expression of the *GUS* gene under the *VpSTS* promoter was significantly higher than that under *V. vinifera* homologous gene promoters (Xu et al. 2011). The -148 bp region of the *VpRFP1* promoter plays a key role in response to pathogens (Yu et al. 2013a).

In our previous studies, a RING-type ubiquitin ligase VpRH2 and its promoter from *V. pseudoreticulata* Baihe-35-1 were induced by powdery mildew and showed resistance to the disease with an interaction protein VpGRP2A (Wang et al. 2017). The purpose of the present research was

to explore the gene regulatory role of *VpRH2* and its promoter using exogenous hormone treatments including with SA, MeJA or ABA.

Materials and methods

Plant materials

Chinese wild *Vitis pseudoreticulata* accession Baihe-35-1 and *V. vinifera* L. cv. Thompson Seedless were picked from the Grape Repository of Northwest A & F University, Yangling, Shaanxi, China (34°20′ N, 108°24′ E), propagated and transplanted as previously described (Yu et al. 2013a). The over-expression of *VpRH2* transgenic Thompson Seedless grape OERH2 was performed as previously described (Wang et al. 2017). All grapes were grown in a growth chamber at 25 °C under a 16/8 h day/night cycle.

A 35S-VpRH2-3Flag recombinant vector was introduced into *Arabidopsis thaliana* (ecotype Columbia, Col-0) using *Agrobacterium tumefaciens* strain GV3101 (Clough and Bent 1998). Three independent lines of T3 transgenic *A. thaliana* (OERH2-1, OERH2-4, OERH2-5) and wild type Arabidopsis plants were grown in a controlled growth room at 22 °C and a 16 h day/8 h night cycle.

Hormone treatments

The hormone treatments were carried out as previously described (Zhu et al. 2012a). The leaves were sprayed with 100 μM ABA, 1 mM SA or 100 μM MeJA containing the wetting agent Tween-20 at 0.05% (v/v), respectively, while the leaves of control plants were sprayed only with sterile water containing 0.05% (v/v) Tween-20, followed by sampling at the designated times. All the leaves were used for RNA extraction.

The germination assay was performed in triplicate with 100 seeds as previously described (Ryu et al. 2010; Cho et al. 2011; Kim and Kim 2013). Surface-sterilized seeds of three OERH2 lines and the wild type Arabidopsis were grown on MS medium with 0, 0.2, 0.5 or 1 μM ABA. After growth at 22 °C with a 16-h-light/8-h-dark photoperiod, the percentages of radicle emergency and green cotyledons were evaluated and analyzed after 4 days, respectively.

RNA extraction and quantitative RT-PCR analysis

The grape leaves RNA was extracted using the Omega Plant RNA Kit (Omega Bio-tek, Norcross, Georgia), and first-stand cDNA was synthesized using the FastQuant RT Kit (with gDNase) (Tiangen Biotech, Beijing, China). qRT-PCR was carried out using SYBR Premix Ex Taq II on an iCycler iQ5 thermal cycler (Bio-Rad, Hercules, CA, USA) as described before (Yu et al. 2013a). Each sample was analyzed in three replicates. Data were analyzed by $2^{-\Delta\Delta c(t)}$ method, and the grapevine *GAPDH* (glyceraldehyde phosphate dehydrogenase, GenBank accession no: XM_002278316.4) gene was used as an internal control for qRT-PCR normalization (Supplemental Table S1).

Agrobacterium-mediated transient assay

The *Agrobacterium*-mediated transient transformation of grape leaves was performed as previously described (Santos-Rosa et al. 2008; Xu et al. 2010). The detected *Agrobacterium* strains GV3101 harboring *VpRH2* promoter transient transformation vector P_{VpRH2} were grown on the LB plates containing kanamycin and rifampin. When the culture reached an OD600 of approximately 1.0, *Agrobacterium* cells were centrifuged for precipitation and resuspended with the infiltration solution (2.15 g MES, pH 5.6; 2.034 g $MgCl_2$, 20 g sucrose in 1 L and 150 μM acetosyringone added before using). The liquid was then revived for 3 h at 28 °C and mixed as indicated to the same concentration (OD600 ≈ 0.8). The grape leaves were vacuumized for 30 min under 0.085 MPa in a vacuum and cultivated for 2 days before exogenous hormone treatments.

GUS activity assay

GUS histochemical staining of transient transformed grape leaves containing *VpRH2* promoter-GUS constructs was carried out as described by Jefferson et al. (1987) under ABA, SA and MeJA. GUS protein was extracted using extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% SDS, 10 mM *β*-mercaptoethanol, and 0.1% Triton X-100) and GUS activity was measured with 4-methylumbelliferon (4-MU, Sigma-Aldrich) generated per min per mg of soluble protein with an Infinite 200 PRO Microplate Reader (Tecan, Männedorf, Switzerland).

Statistical analysis

The means and standard deviation (SD) were obtained at least three repetitions. Statistical analysis was used by SPSS22.0 software. Statistical analysis was carried out using one-sided paired *t* test ($*P < 0.05$; $**P < 0.01$) between treated and mock samples.

Results

VpRH2 and its promoter respond to exogenous hormone treatments

In previous studies, we had detected that the expression of *VpRH2* and its promoter activity were induced by *U. necator* inoculation (Wang et al. 2017). In order to study whether the expression of *VpRH2* was inducible by defense signaling molecules, ABA, SA and MeJA treatments were carried out on Chinese wild grape Baihe-35-1 leaves (Fig. 1). The samples were collected at different time points after treatments while ddH$_2$O with 0.05% (v/v) Tween-20 treatment was used as the Mock analysis, and the effect of signal molecules on the expression of *VpRH2* was investigated by qRT-PCR. After ABA treatment, expression of *VpRH2* was down-regulated in 2–6 h post treatment (hpt), and the expression was resumed to the original state at 8 hpt (Fig. 1a). After the treatment with SA for 2–8 hpt, the expression showed an upward trend, and the expression peak appeared at 4 hpt (Fig. 1b). After MeJA treatment, *VpRH2* showed an upward trend within 24 hpt, reached the first peak at 2 hpt, slightly decreased at 6–10 hpt, then got another peak at the 12 hpt (Fig. 1c). The experimental results show that the expression of *VpRH2* was increased by SA and MeJA, while reduced by ABA.

To determine the effect of defense hormone molecules on *VpRH2* promoter, quantitation of GUS activity was conducted by transient transformation in Thompson Seedless grape leaves. GUS activity under *VpRH2* promoter was extremely significant down-regulated after ABA treatment (Fig. 2a). On the contrary, GUS activity under *VpRH2* promoter was substantially increased compared with the Mock treatment after SA treatment (Fig. 2b), and a more significant increased trend was observed under the treatment of MeJA (Fig. 2c). These results showed that *VpRH2* promoter activity increased by SA and MeJA treatment, but was inhibited by ABA treatment, a similar variation trend as the gene expression.

In order to find the core functional region of *VpRH2* promoter in response to ABA and MeJA, a series of deletions (*P1,296*, *P1,096*, *P656*, *P513*) was generated by PCR, lost of the W-box and the ABA responsive element (ABRE), TC-rich repeats, two EREs, TC-rich repeats and TGACG-motif, correspondingly (Fig. 2d). After ABA treatment, the *P1,296* region also showed an inhibited GUS activity (Fig. 2e). For MeJA treatment, the *P1,296*, *P1,096*, *P656* regions resulted in a higher GUS activity than the mock-treated plants, but the *P513* regions did almost not change (Fig. 2f).

VpRH2 inhibited ABL1 and PYR1 expression in ABA pathway

In order to further study the biological function of VpRH2 in ABA pathway, the over-expressing *VpRH2* Thompson Seedless grapes were treated with ABA. The transcript levels of ABA-related genes were detected by qRT-PCR (Merlot et al. 2001; Nishimura et al. 2009; Santiago et al. 2009; Bueso et al. 2014). The results showed that *ABI1*, *ABI2*, *ABI3*, *ABL2* and *PYL4* were not obviously differently expressed after ABA treatment in OERH2 and wild type Thompson Seedless. *ABL1* in OERH2 grape plants was suppressive compared with wild type plants. A special gene was *PYR1*, which expression in OERH2 was hard to detect (Fig. 3).
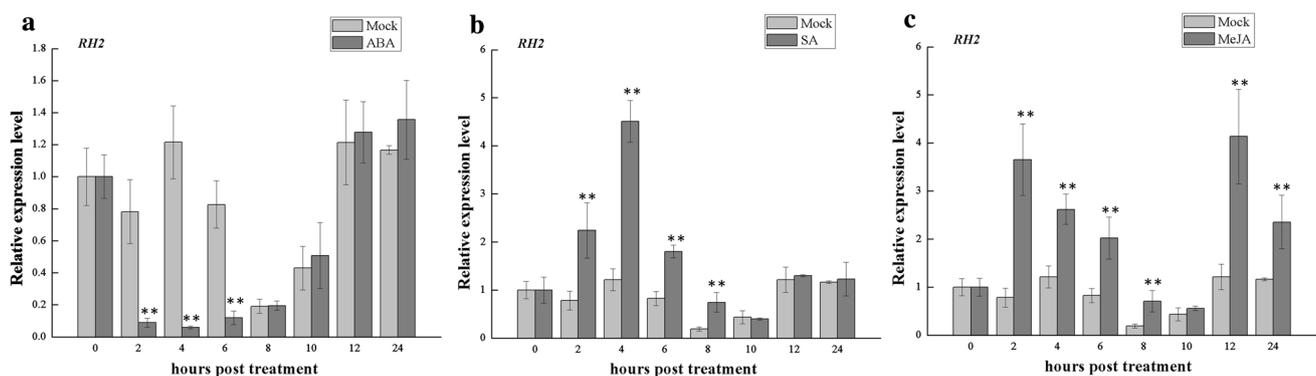

Fig. 1 Expression of *VpRH2* under hormone treatments. **a–c** The expression of *VpRH2* was detected by qRT-PCR. Leaves of *V. pseudoreticulata* accession Baihe-35-1 were sprayed with 100 μM ABA (**a**), 1 mM SA (**b**) or 100 μM MeJA (**c**) and collected at eight time points, respectively; mock, control sprayed with ddH$_2$O with Tween-20 at 0.05% (v/v). The significant difference analysis was assessed by the one-sided paired *t* test (*$P < 0.05$; **$P < 0.01$). Expression values ($\pm$ SD) of three replicates were normalized using *VvGAPDH* as the internal control
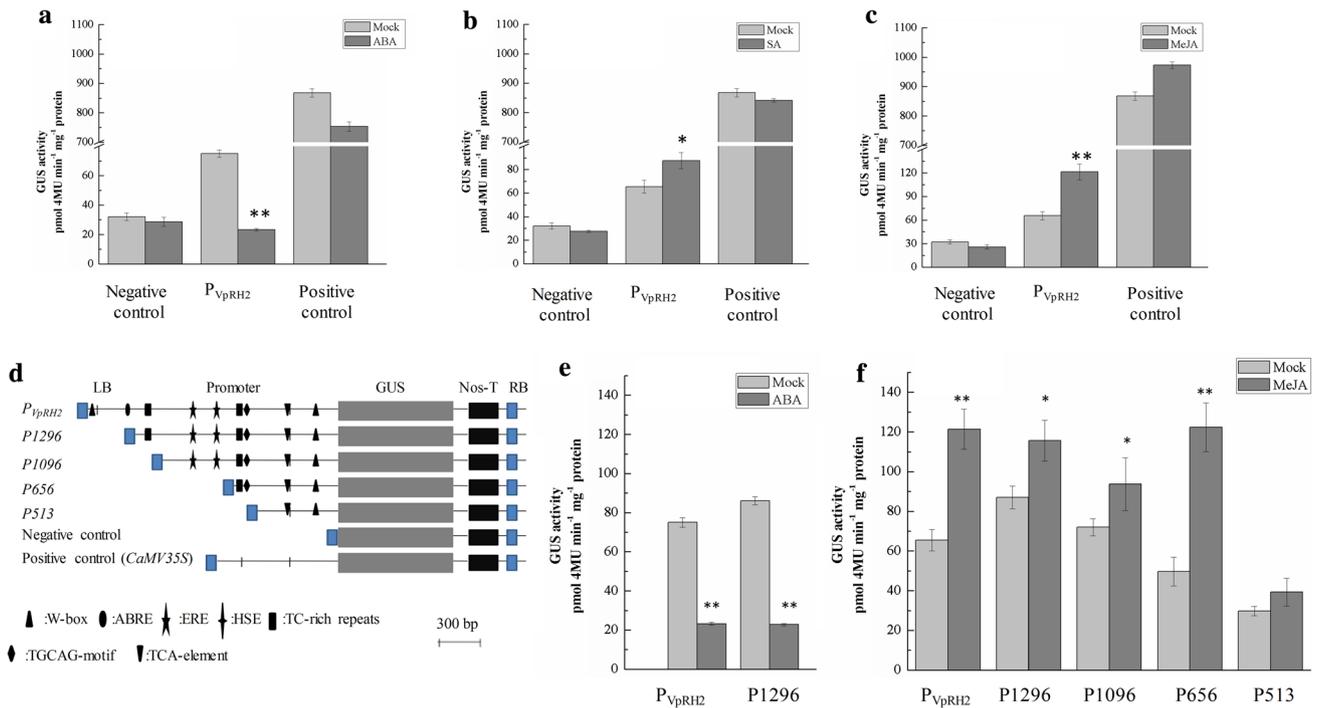
Fig. 2 The activity of *VpRH2* promoter under hormone treatments. **a–c** Measurement of GUS activity driven by the *VpRH2* under 100 μM ABA (**a**), 1 mM SA (**b**) or 100 μM MeJA (**c**) in the transiently transformed grapevine leaves. Leaves were cultivated for 2 days in incubators (22 °C; photoperiod 16/8 h) before hormone treatments for 24 h. The significant difference analysis was assessed by the one-sided paired t test (*$P < 0.05$; **$P < 0.01$). Expression values ($\pm$ SD) were averaged from three experiments ($n = 3$). **d** The constructs of promoters and the deletions used in the study and the location of main defense-regulated elements on the promoters. *LB* left border, *NOS-T* Nos terminator, *RB* right border, *GUS* β-glucuronidase. The main defense-regulated elements were shown as different characters. **e, f** The leaves were cultivated 2 days in the incubators at 22 °C under a 16-h-light/8-h-dark photoperiod before ABA (**e**) and MeJA (**f**) treatments for 1 day. All tests were repeated three times, and its significant difference test was assessed by one-sided paired t test (**$P < 0.01$, or at *$P < 0.05$), expression values and $\pm$ SD were averaged from three experiments ($n = 3$)
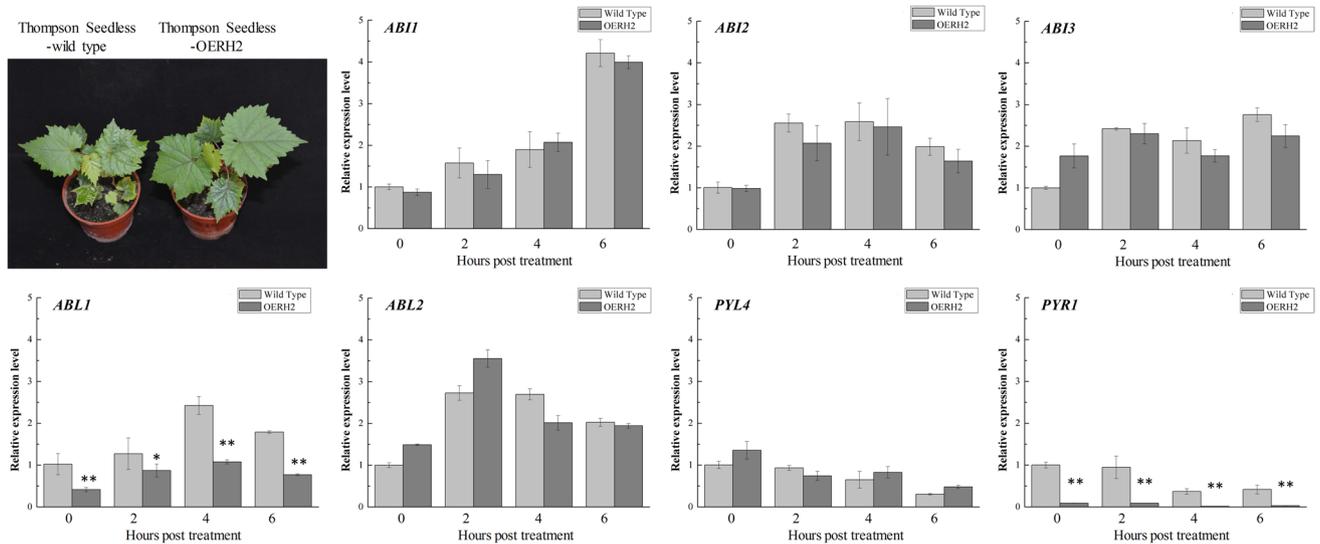

Fig. 3 qRT-PCR analysis of ABA-related genes in wild-type and transgenic plants after ABA treatment. Expression of *ABI1*, *ABI2*, *ABI3*, *ABL1*, *ABL2*, *PYL4* and *PYR1* was detected by qRT-PCR. Leaves of wild-type and transgenic OERH2 *V. vinifera* cv. Thompson Seedless were sprayed with 100 μM ABA and collected at four time points. Expression values ($\pm$ SD) of three experiments were normalized using *VvGAPDH* as the internal control

Over-expression of *VpRH2* in *A. thaliana* resulted in increased insensitivity to ABA

To further study the function of *VpRH2* in ABA tolerance, *VpRH2* over-expressing transgenic *A. thaliana* plants were generated by the *Agrobacterium tumefaciens* mediated method. We compared seed germination rates of three T3 lines of the OERH2 transgenic *A. thaliana* (OERH2-1, OERH2-4, OERH2-5) and the wild type plants in the presence and absence of ABA. To measure germination rates, 100 sterilized seeds of the three OERH2 lines and wild type plants were plated on full-strength solid MS medium with 0, 0.2, 0.5 or 1.0 μM ABA. After 4 days of stratification, a percentage of radicle emergence was calculated as germination

rates (Fig. 4a). Wild type plants and three OERH2 lines almost fully germinated on the solid MS medium with 0 and 0.2 μM ABA. On the medium with 0.5 and 1 μM ABA, germination rates of wild type plants were obviously reduced with the increasing ABA concentrations. More than 60% of wild type seeds did not germinate in the presence of 1 μM ABA (Fig. 4b). As expected, three OERH2 seeds displayed a hyposensitive phenotype toward ABA as compared with wild-type seeds (Fig. 4b). More than 80% seeds germinated normally with 1 μM ABA. Thus, *VpRH2* efficiently resulted in the ABA-insensitive phenotype of *A. thaliana* plants.

In addition, approximately 6.0% of wild type plants and 92.4% of OERH2 plants developed true green cotyledons in the presence of 0.5 μM ABA, 2.0% of the wild type plants,
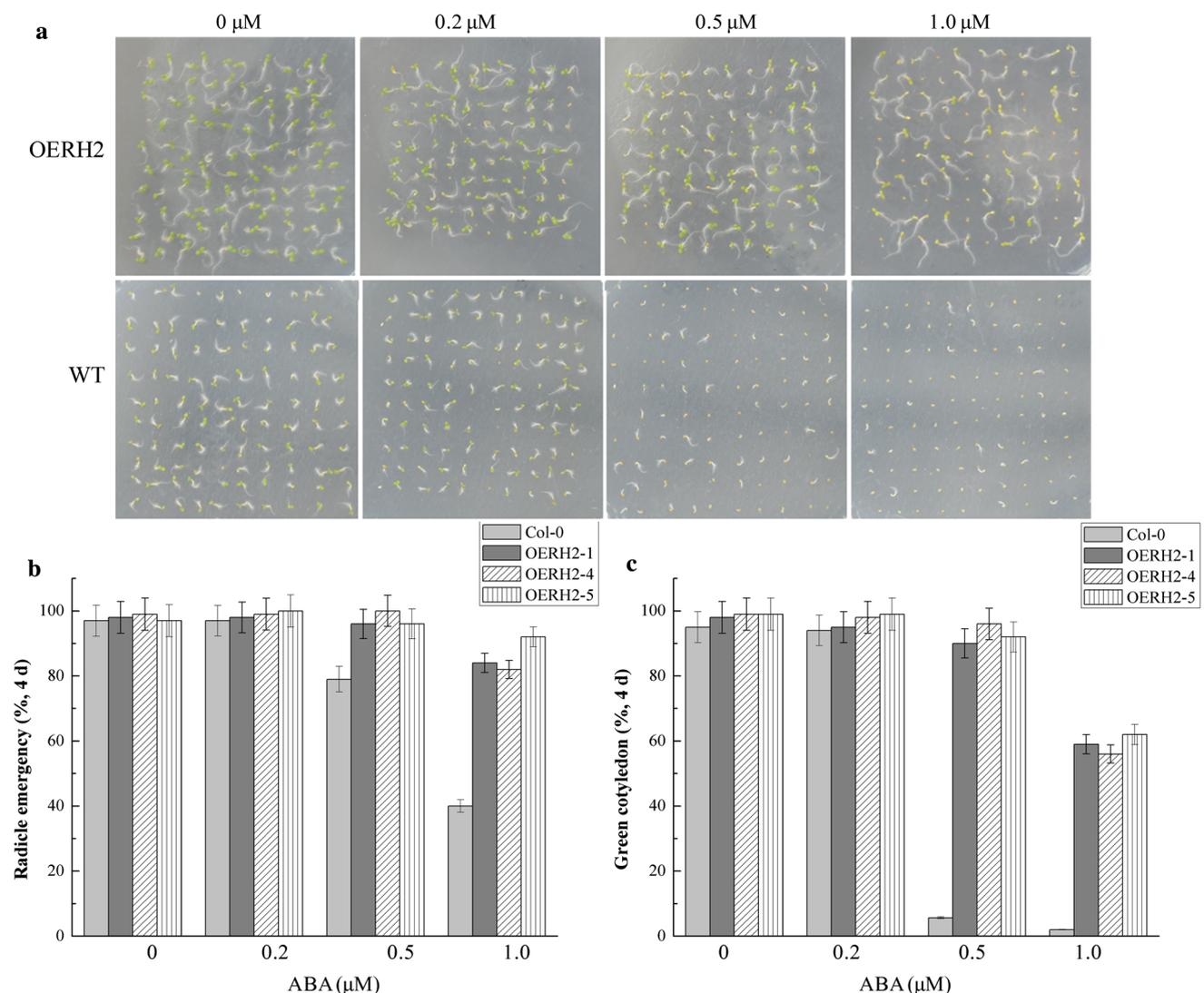

Fig. 4 Phenotypes of wild type Col-0 and OERH2 Col-0 under ABA stress during the seed germination. **a** The phenotypes of wild type (WT) and OERH2 type seeds in the MS medium with different concentration of ABA. **b** Quantitation of germination rates under different concentration of ABA. **c** Quantitation of cotyledon greening rates under different concentration of ABA. Average and SD values were determined from three biological replicates ($n = 100$), and all the experiments were identified after 4 days

while 59.0% in OERH2 plants on the medium with 1 µM ABA (Fig. 4c). Thus, OERH2 plants showed a reduced hypersensitivity to ABA in terms of both, radicle emergence and cotyledon development.

VpGRP2A was reduced by ABA treatment and inhibited in OERH2 plants

VpGRP2A, a glycine-rich RNA-binding protein, was selected as the interaction protein of VpRH2 (Wang et al. 2017). Previous studies with *Malus prunifolia* (Willd.) Borkh. revealed that a glycine-rich RNA-binding protein *MpGR-RBP1* was up-regulated by ABA treatment (Tan et al. 2014). So we also tested the expression of *VpGRP2A* under hormone treatments. After ABA treatment, expression of *VpGRP2A* was up-regulated after 2–8 hpt, and the expression was resumed to the original state at 12 hpt (Fig. 5a). After SA treatment for 4–8 hpt, the expression showed an downward trend, and the expression valley appeared at 8 h after treatment (Fig. 5b). After MeJA treatment, *VpGRP2A*
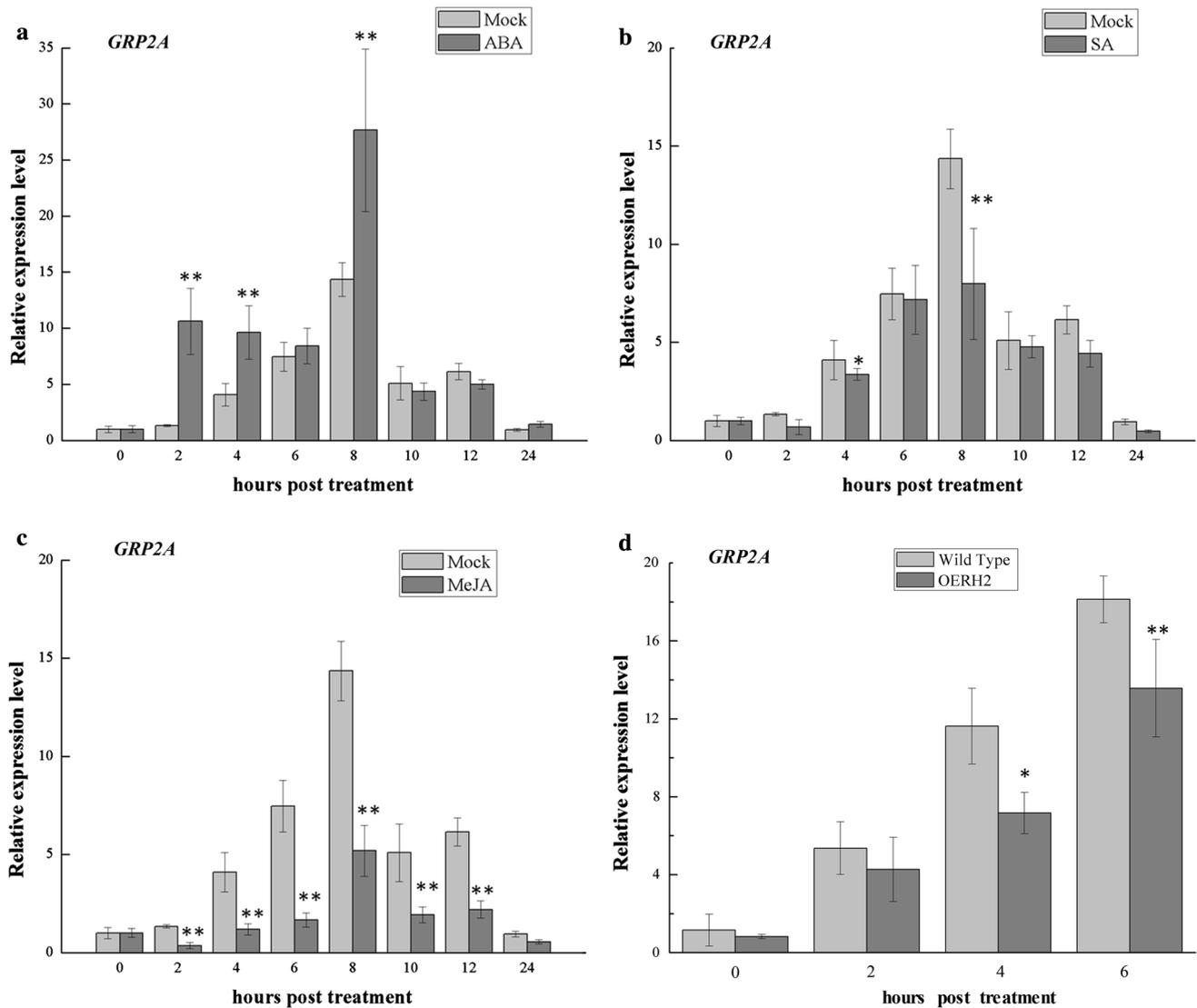

Fig. 5 Expression of *VpGRP2A* under hormone treatments. **a–c** The expression of *VpGRP2A* was detected by qRT-PCR. Leaves of *V. pseudoreticulata* accession Baihe-35-1 were sprayed with 100 µM ABA (**a**), 1 mM SA (**b**) or 100 µM MeJA (**c**) and collected at eight time points, respectively; mock, control sprayed with ddH₂O with Tween-20 at 0.05% (v/v). The significant difference analysis was assessed by the one-sided paired t test (*$P < 0.05$; **$P < 0.01$). Expression values ($\pm$ SD) of three replicates were normalized using *VvGAPDH* as the internal control. **d** The expression of *GRP2A* in wild type and OERH2 Thompson Seedless after ABA treatment. Leaves of wild-type and transgenic OERH2 *V. vinifera* cv. Thompson Seedless were sprayed with 100 µM ABA and collected at four time points. Expression values ($\pm$ SD) of three experiments were normalized using *VvGAPDH* as the internal control. The significant difference analysis was assessed by the one-sided paired t test (*$P < 0.05$; **$P < 0.01$)

showed an downward trend within 24 hpt (Fig. 5c). After ABA treatment, the expressions of *GRP2A* were increased both in the wild-type and OERH2 grapes. However, in the wild-type grapes, the expression of *GRP2A* was higher than that in the OERH2 grapes (Fig. 5d).

Discussion

Plant immune system is a highly sophisticated defense system, and many plant hormones, such as SA, JA and ABA, were originally recognized as regulators of the plant immune system (Verhage et al. 2010). SA and JAs are recognized as major defense hormones; antagonistic interaction and synergistic interaction between SA- and JA-dependent signaling had been described in plant immune system models, while ABA signaling was a negative regulator in plant immunity by suppressing SA-dependent defenses (De Torres-Zabala et al. 2009; Pieterse et al. 2009; Jiang et al. 2010; Cao et al. 2011). As we had found VpRH2 improved the resistance of grapevine to powdery mildew (Wang et al. 2017), we now tested whether its expression was induced by exogenously applied hormones. In the present study, the expression of VpRH2 was induced by SA and MeJA treatments, but inhibited by ABA treatment as we predicted. So we speculated VpRH2 was a positive regulator in plant immune system.

According to PlantCARE databases, the elements of hormones including the TCA-element (SA-responsive element), TGACG-motif (MeJA-responsive element) and ABRE are the pivotal *cis*-regulatory elements present in the *VpRH2* promoter sequence (Fig. 2d) (Merlot et al. 2001; Liao et al. 2008; Miura et al. 2009; Xu et al. 2011; Wei et al. 2012). The *cis*-regulatory elements suggested VpRH2 may also have the functional relationships with hormone-related signaling. By GUS protein activity assay, the activity of the *VpRH2* promoter and its deletions all maintained a relatively low level, particularly in *P513*, thus we predicted this region may have almost no activity (Fig. 2f). In this study, we found that the activity of the *VpRH2* promoter was inhibited by ABA treatment, also the *P1,296* region without ABRE (Fig. 2e). We predicted that the inhibition of ABA by *VpRH2* and its promoter perhaps had nothing to do with the ABRE motif. After MeJA treatment, *P1,296*, *P1,096* and *P656* showed an induced trend, and *P513* showed no significant difference compared to the control (Fig. 2f). So the − 656 to − 513 bp region of the *VpRH2* promoter was the core functional promoter region in response to MeJA because of the TGACG-motif.

For SA treatment, the expression and promoter activity of *VpRH2* showed also induced trend (Figs. 1b, 2b). In previous studies, SA has been found a signal molecule in the plant immunity (Yan and Dong 2014); *NPR1*, *PR1*, *PR2*, *NPR3* and *NPR4* were documented as the central genes in the SA pathway (Zheng et al. 2006; Fu et al. 2012). In our previous studies, the expression of *PR1* and *NPR1* was more sensitive in transgenic OERH2 grapes (Wang et al. 2017). These results suggested that the RING-type ubiquitin ligase VpRH2 may influence the SA-mediated plant immunity. In these studies, we also found that *VpRH2* promoter activity was extremely significantly induced by MeJA, but in our previous studies, a key gene in JA-mediated signaling pathway, *PR3*, did almost not change in transgenic plants and wild-type plants (Wang et al. 2017). Thus the influence of VpRH2 in JA-mediated signaling pathway may need further research. The expression of *VpRH2* was induced by SA and MeJA, but reduced by ABA. Our studies are in agreement with previous reports, indicating that ABA antagonistically interacts with other plant hormones that play pivotal roles in defense responses, including SA and MeJA (Okamoto et al. 2012).

In this report, we identified OERH2 Arabidopsis plants that were less sensitive to ABA treatment than wild-type plants (Fig. 5a). In the past studies, the AtAIRP1-3 responded to ABA (Ryu et al. 2010; Cho et al. 2011; Kim and Kim 2013), but all reacted hypersensitive to ABA treatment after over-expression. Although they all belong to the RING Finger family, VpRH2 and AtAIRP1-3 produce completely different responses in insensitivity to ABA treatment, possibly because of the functional diversity of RING-type ubiquitin ligases. ABA was found to regulate many developmental processes and abiotic stresses and many genes were identified in ABA signaling components (Cutler et al. 2010). ABI1 and its closest homologue ABI2 were reported as negative feedback regulatory loop of the ABA signaling pathway (Merlot et al. 2001). In our studies, we found that the expression of *ABI1*, *ABI2*, *ABI3* and *ABL2* was not obviously different in wild type and OERH2 Thompson Seedless. But the expression of *ABL1* in OERH2 was inhibited compared with wild type grapes (Fig. 3). PYL4 and PYR1 were the interaction proteins of RING-type E3 ubiquitin ligase RSL1 in Arabidopsis. Over-expression of *RSL1* reduced ABA sensitivity and *rsl1* RNAi lines showed an enhanced sensitivity to ABA. RSL1 promoted ubiquitination and degradation of PYL4 and PYR1 in vivo (Bueso et al. 2014). So we also tested the expression of *PYL4* and *PYR1* in OERH2 grape plants. As shown in Fig. 3, we found that the expression of *PYL4* was almost not changed in OERH2 and wild type grapes, but *PYR1* was almost not expressed in OERH2. Since single mutants of PYR/PYL/RCAR receptors did almost not change in the sensitivity in germination responses to ABA, but triple and higher-order mutants were less sensitive (Santiago et al. 2009; Gonzalez-Guzman et al. 2012), VpRH2 may also inhibited a greater number of ABA receptors. In short, we suggest VpRH2 may inhibit the expression of *PYR1*

to reduce sensitivity to ABA. Yet the interaction between VpRH2 and PYR1 needs further research.

In our previous studies, we predicted VpGRP2A functioned as a component of a circadian–regulator (Wang et al. 2017), similarly functioning as AtGRP7. In this study, we found that the expression of *VpRH2* showed an obvious lower trend at 8 h and 10 h in the Mock treatment (Fig. 1a–c), and the expression of *VpGRP2A* also showed a different trend within 24 h (Fig. 5a–c). Thus we confirm the function of VpRH2 and VpGRP2A in circadian regulation as reflected in the expression variations in the Mock samples. We found that *GRP2A* was high-regulated by ABA treatment. In other species, two apple GRPs had been reported that induce expression after ABA treatment (Wang et al. 2010; Tan et al. 2014). We predicted that VpGRP2A might also play role in the ABA pathway in grape. In OERH2 plants, the expression of *GRP2A* was lower than in the WT after ABA treatment, the same as our previous result that *VpRH2* suppressed the expression of *VpGRP2A*. We had reported VpRH2 did not promote the degradation of VpGRP2A, but possibly the interaction between VpRH2 and VpGRP2A might be due to the RRM domain. Therefore we suggested that VpRH2 might take part in the ABA pathway through GRP2A regulated posttranscriptional modification.

In a nutshell, we found that the promoter of *VpRH2* was induced under MeJA by the TGACG-motif, and inhibited under ABA treatment probably not by the ABRE motif. Regarding over-expression of *VpRH2*, grape showed an inhibited expression of the ABA-related genes *ABL1*, *PYR1* and *GRP2A*. Arabidopsis was insensitive to exogenous ABA after over-expression of *VpRH2*. In summary, our results indicate that VpRH2 plays a role in ABA pathway.

Author contribution statement WYJ designed the research. WL and YWK carried out the experiments. WL and YWK analysed the results. WL wrote the manuscript. WYJ revised the manuscript. All authors read and approved the manuscript.

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