Journal of Plant Physiology 185 (2015) 57–64

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Physiology

A balanced JA/ABA status may correlate with adaptation to osmotic stress in *Vitis* cells

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ARTICLE INFO

Article history:
Received 20 April 2015
Received in revised form 13 May 2015
Accepted 11 June 2015
Available online 4 August 2015

Keywords:
Osmotic stress
Grapevine (*V. rupestris*, *V. riparia*)
JAs
ABA
Stilbene compounds
Adaptation

ABSTRACT

Water-related stress is considered a major type of plant stress. Osmotic stress, in particular, represents the common part of all water-related stresses. Therefore, plants have evolved different adaptive mechanisms to cope with osmotic-related disturbances. In the current work, two grapevine cell lines that differ in their osmotic adaptability, *Vitis rupestris* and *Vitis riparia*, were investigated under mannitol-induced osmotic stress. To dissect signals that lead to adaptability from those related to sensitivity, osmotic-triggered responses with respect to jasmonic acid (JA) and its active form JA-Ile, abscisic acid (ABA), and stilbene compounds, as well as the expression of their related genes were observed. In addition, the transcript levels of the cellular homeostasis gene *NHX1* were examined. The data are discussed with a hypothesis suggesting that a balance of JA and ABA status might correlate with cellular responses, either guiding cells to sensitivity or to progress toward adaptation.

1. Introduction

During their life cycles, plants are challenged by different kinds of biotic and abiotic stresses. Water-related stress in particular represents the major type of plant stresses taking place when water quantity (*e.g.*, meteorological drought) or quality (*e.g.*, salinity) is compromised (Bray et al., 2000). Consequently, plants face difficulty in absorbing water as the water potential of soil becomes lower than its counterpart, the plant root. Generally, osmotic stress is considered the common component for all types of water-related stress (Majumder et al., 2010). Depending on its extend and gravity, osmotic stress may result in a wide range of unfavorable cellular and molecular events, such as membrane disorganization, metabolic toxicity, protein dysfunction, and overproduction of reactive oxygen species (ROS). Therefore, photosynthesis inhibition, reduction of growth and fertility, and premature senescence will develop (for review, see Krasensky and Jonak, 2012). On the other hand, osmotic-tolerant plants have evolved an array of strategies to cope with osmoticaly-induced disturbances. For example, at the cellular and molecular levels, many adaptive events may take place, including changes in the cell cycle and cell division, osmotic adjustment, hormonal changes (*e.g.*, ABA, JAs), and modifications of gene expression. In addition, plants inhibit leaf expansion while enhancing root growth and stomatal closure in order to utilize water efficiently (Taiz and Zeiger, 2010).

Abscisic acid (ABA) is a universal plant hormone that has received considerable research interest (for review, see Cutler et al., 2010). ABA synthesis initiates in plastids *via* the terpenoid pathway, while its formation occurs in the cytoplasm (for reviews, see Nambara and Marion-Poll, 2005). The steady-state levels of ABA are kept low and its signaling pathways are switched off by the clade A protein phosphatases 2C (PP2Cs) (for reviews, see Raghavendra et al., 2010). The PP2Cs consist mainly of HAB1, ABI1, and ABI2 those negatively regulating the (sucrose non-fermenting) SNF1-related protein kinases 2 (*e.g.*, OST1/SnRK2.6/SRK2E). However, under stressful conditions such as osmotic stress, the elevated levels of ABA bind to pyrabactin resistance 1 (PYR1), a member of the PYR/PYR1-like (PYL)/regulatory component of ABA receptor (RCAR) family of START proteins that function as PP2Cs inhibitors in an ABA-dependent manner (Ma et al., 2009; Park et al., 2009). Consequently, the ABA-activated SnRK2s phosphorylate downstream transcription factors such as the ABA responsive element-binding protein (AREB) and ABA responsive element-binding factor (ABF), and bZIP-type resulting in activating ABA-responsive genes and ABA-related responses (for review, see Joshi-Saha et al., 2011;

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http://dx.doi.org/10.1016/j.jplph.2015.06.014
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Umezawa et al., 2010). SRK2E/OST1/SnRK2.6 is a key component of ABA signaling mediating ABA-dependent stomatal closure, which is the fast response to reduce the transpirational water loss under water deficit conditions (for review, see Osakabe et al., 2014). Interestingly, nine of the 10 SnRK2s are mannitol- or NaCl-activated, from which five are ABA-activated (Boudsocq et al., 2004). The SRK2E interacts with the K^+ uptake transporter 6 (KUP6) subfamily of transporters that play a key role in osmotic adjustment by balancing potassium homeostasis in cell growth and osmotic stress responses (Osakabe et al., 2013). In addition, another set of stress-responsive genes are ABA-dependent, such as the enzymes required for the biosynthesis of compatible osmolytes (*e.g.*, betaine, dehydrins) and LEA-like proteins (Gao et al., 2004; for reviews, see Mehrotra et al., 2014). However, plants respond to osmotic stress in ABA-dependent and ABA-independent manners, suggesting that there are other central players (for review, see Yoshida et al., 2014).

Jasmonates (Jasmonic acid and related compounds, JAs) are among the plant stress hormones that have been extensively studied over the last few decades (for reviews, see Wasternack and Hause, 2013). In response to environmental or developmental cues where JAs are synthetized, the JA signal cascade starts with its conversion into its bioactive form (+)-7-*iso*-Jasmonoyl-L-isoleucine ((+)-7-*iso*-JA-L-Ile, JA-Ile) *via* JAR1 (Jasmonate-Resistant 1) enzyme (Fonseca et al., 2009). The elevated levels of JA-Ile subsequently bind to the SCF^{COI1}–JAZ complex co-receptor, leading to ubiquitination of JAZ proteins and the subsequent degradation *via* the 26S proteasome (for reviews, see Wager and Browse, 2012). In *Arabidopsis thaliana*, twelve *JAZ/TIFY* genes have been identified, sharing two conserved domains, a ZIM/TIFY (Zinc-finger protein expressed in Inflorescence Meristem), and a Jas (Jasmonate-associated) domain. The Jas domain is required for hormone-dependent interactions of JAZ/TIFY with a bHlH-type transcription factor AtMYC2, F-box protein SCF^{COI1} (Chini et al., 2007; Thines et al., 2007), and for nuclear localization (Grunewald et al., 2009). In contrast, ZIM/TIFY domain mediates homo- and heteromeric hormone-independent interactions among individual JAZ/TIFY proteins (Chini et al., 2009). The degradation of JAZ proteins liberates the JA transcription factors, such as the master regulator MYC2, resulting in the activation of JA-responsive genes (Kazan and Manners, 2013). Interestingly, *JAZ* genes are among the early induced JA-responsive genes, implying a negative feedback loop by their gene products. For example, at least five members of *OsJAZ/OsTIFY* genes were highly induced when rice (*Oryza sative*) underwent different abiotic stress conditions (*e.g.*, salinity, dry, and cold) (Seo et al., 2011; Ye et al., 2009). Repression of JA transcription factors by JAZ factors are mediated by the Groucho/Tup1-type corepressor TOPLESS (TPL) and TPL-related proteins (TPRs) through the Novel Interactor of JAZ/TIFY (NINJA) (Pauwels et al., 2010). This mechanism establishes a negative feedback that ensures the fine-tuning of JA signaling that is a critical player in response to plant stress (for review; see Ismail et al., 2014a; Kazan, 2015).

Grapevine is considered the most important cash crop worldwide. In addition, it is among a limited number of unrelated plant species that produce stilbene phytoalexins (Parage et al., 2012). Those stilbene compounds are a class of low molecular weight plant secondary metabolites that are generated *de novo* in response to biotic (*e.g.*, fungal infection) and abiotic stress factors (*e.g.*, UV-irradiation and salinity), as well as during different developmental stages (Derckel et al., 1999; Duan et al., 2015; Ismail et al., 2014b; Petit et al., 2009). In the current work, we analyzed osmotic stress responses in two grapevine cell lines that differ in their osmotic adaptability (Ismail et al., 2012a,b). The first line was derived from the North America osmotic-tolerant grapevine *Vitis rupestris*, a species that inhabits sunny rocks and slopes. The other was generated from the osmotic-sensitive grapevine *Vitis riparia*, a North American species growing in alluvial forests. We therefore investigated osmotic-triggered responses with respect to plant hormones (JA and ABA) and the expression of their related genes, and stilbene compounds as potential antioxidants.

We found that *V. riparia* accumulates 4 times higher amounts of JA, JA-Ile, and δ-viniferin than those in *V. rupestris* under non-stressful conditions. In addition, mannitol treatments promoted additional 2–3 times induction of JA-Ile and δ-viniferin, as well as the expression level of the *StSy* gene inside *V. riparia*. However, the pattern of ABA was almost reversed and increased drastically in *V. rupestris* to a four-fold higher level after applying mannitol, although it was nearly the same in both cell lines without mannitol stress. Moreover, osmotically-stressed *V. rupestris* cells were able to highly express JA- and ABA-related genes, *JAZ1* and *ABL1*, respectively, as well as the cellular homeostasis gene *NHX1*. Therefore, the balance among stress hormones and stilbenes and their related genes might play a critical role in osmotic adaptation.

2. Materials and methods

2.1. Cell lines and treatment with mannitol

Suspension cell cultures of the two grapevine cell lines, *V. rupestris* and *V. riparia*, which originated from leaves (Seibicke, 2002), were cultivated in Murashige–Skoog (MS) liquid medium (Duchefa, Haarlem, The Netherlands). Cells were subcultured weekly by inoculating 10 ml of stationary cells into 30 ml of fresh medium, and subsequently incubated at 25 °C in the dark on an orbital shaker (KS250 basic, IKA Labortechnik, Staufen, Germany) at 150 rpm. At day 5 of subcultivation, when cells reached their stationary state, osmotic stress was administered by addition of the MS medium containing 120 mM mannitol as final concentration (Sigma–Aldrich, Deisenhofen, Germany). As a negative control, an equivalent volume of mannitol-free MS medium was added to parallel flasks.

2.2. Quantification of plant hormones

Phytohormone contents were quantified for both cell lines with three biological replicates at 1, 3, and 6 h after addition of mannitol-containing MS medium to have 120 mM mannitol as final or mannitol-free MS medium as a control (incubated for 1 h). All samples were collected by removing the supernatant using a Büchner funnel under vacuum. Both, cells and supernatants, were shock-frozen in liquid nitrogen, freeze-dried at −50 °C for 2 days, and weighed. Plant hormones were extracted as described previously (Yoshimoto et al., 2009) with some modifications: lyophilized cultured cells or culture media were homogenized in 4 ml of 80% acetonitrile (MeCN) containing 1% acetic acid, and extracted for 30 min with internal standards ($^{13}C_6$-JA-Il, d_2-JA, d_6-SA, d_6-ABA and d_2-IAA). After centrifugation at 1663 × *g* for 20 min, the supernatant was collected and the sediment extracted again with 4 ml of 80% MeCN containing 1% acetic acid. 1 ml (for cultured cells) or 4 ml (for culture medium) of the supernatant were processed further for hormone analysis. After removing MeCN in the supernatant, the acidic aqueous extract was loaded onto an Oasis HLB column cartridge (30 mg, 1 ml Waters, Milford, MA, USA), and washed with 1 ml of water containing 1% acetic acid to remove highly polar impurities. Plant hormones were eluted with 2 ml of 80% MeCN containing 1% acetic acid. Ten percent of the eluate was used for the analysis of salicylic acid (SA). After removing MeCN in the remaining eluate, the acidic water extract was loaded onto an Oasis WAX column cartridge (30 mg, 1 ml). After washing with 1 ml of water containing 1% acetic acid, neutral compounds were removed with 2 ml of 80% MeCN, and acidic compounds were eluted with 2 ml of 80% MeCN containing 1% acetic acid. Hormones were quantified

by liquid chromatography–electrospray ionization-tandem mass spectrometry as described in detail in Yoshimoto et al. (2009). For mannitol-induced hormones in supernatant samples, see Supplementary Fig. 1.

2.3. Quantification of the levels of gene expression

To analyze osmotically expressed genes, 1.5 ml of cells were collected at day 5 after subcultivation and treated with 120 mM mannitol as the final concentration for 1, 3, or 6 h, respectively. As a control, cells were pre-treated with mannitol-free MS medium for 1 h. At each time point, cells were harvested by low-speed centrifugation (3000 rpm, 1 min), and immediately frozen in liquid nitrogen after removing the liquid media. Cell samples were subsequently lysised with steel beads (Tissue Lyser, Qiagen/Retsch, Germany). The total RNA was extracted using the innuPREP Plant RNA Kit (analytikjena, Jena, Germany) according to the manufacturer protocol. To exclude any potential contamination by genomic DNA, extracted RNA was treated by RNase-free DNase (Qiagen, Hildesheim, Germany). The cDNA was synthesized by reverse transcription using M-MuLV RTase cDNA Synthesis Kit (New England BioLabs; Frankfurt am Main, Germany) according to the instructions of the manufacturer. Primers' efficiency was tested as they do not result in nonspecific amplicons. Afterward, Transcripts were amplified by PCR primers (Table 1**)** with 27–29 cycles of 1 min denaturation at 94 °C, 30 s annealing at 60 °C, and 1 min polymerase reaction at 72 °C. The DNA amplicons were then separated by conventional agarose gel electrophoresis after visualization with SybrSafe (Invitrogen, Karlsruhe, Germany). Images of the gels were recorded on a MITSUBISHI P91D screen (Invitrogen) using a digital image acquisition system (SafeImage, Intas, Germany). The levels of transcript were quantified using the Image J software (http://rsbweb.nih.gov/ij/) and normalized relative to the levels of transcripts for *elongation factor 1α* as internal standard. Relative transcript levels were plotted as fold increase of transcript abundance as compared with the levels in the untreated cells. All quantifications of gene expression represent the mean from at least three independent experiments.

2.4. Extraction and quantification of stilbenes

The production of stilbenes as potential ROS-scavengers was tested for both cell lines after challenge with 120 mM mannitol as final concentration at different time points (0, 2, 4, 6, 8, 10, 24 or 48 h). The treated cells were harvested by centrifugation (5000 rpm, 5 min) to remove the liquid media, weighed, directly frozen in liquid nitrogen, and then stored at −80 °C until analysis. Stilbenes were extracted according to Tassoni et al. (2005) with minor modifications. For each sample (3–5 g fresh weight), 20 ml of 80% (v/v) methanol were added and homogenized by an ultrasonic processor (UP100H, Hielscher, Germany) for 3 min. The homogenate was shaken for 2 h at room temperature/dark and filtered through filter paper under vacuum with 500 Pa. The filtrate was concentrated to a residual volume of 5 ml in a glass tube at 40 °C (Heating Bath B490, BÜCHI, Germany) at 280 rpm (Rotavapor R-205, BÜCHI, Germany), under a vacuum of 80 Pa (Vacuubrand CVC2, Brand, Germany). Water-soluble stilbenes were extracted by adding 2 ml of 5% $NaHCO_3$ (w/v), and three aliquots of 5 ml ethyl acetate. The pooled ethyl-acetate phase was completely dried to yield a stilbenic residue on the bottom of the glass tube. The residue was re-suspended in 2 ml of methanol for analysis by high performance liquid chromatography (HPLC).

Stilbenes were analyzed by HPLC (Agilent, 1200 series, Waldbronn, Germany) using a Phenomenex Synergi hydro RP column (150 × 4.6 mm, particle size 4 μm, Phenomenex; Aschaffenburg, Germany), a DAD detector, and a quaternary valve. The flow

Table 1
Designations, sequences, and literature references for the oligonucleotide primers used to amplify the marker sequences used in this study.

Name	GenBank accession no.	PCR cycles	Primer sequence 5′#786#	Reference
EF1-α (150 bp)	EC959059	29	Sense: 5′-GAACTGGGTGCTTGATAGGC-3′ Antisense: 5′-AACCAAATATCCGGAGTAAAAGA-3′	Reid et al. (2006)
VrJAZ1 (300 bp)	JF900329	29	Sense: 5′-TGCAGTCTGTTGAGCCAATACATA-3′ Antisense: 5′-CACGTTTCCGGACTTCTTTACAC-3′	Ismail et al. (2012)
VrJAZ2 (360 bp)	JF900330	27	Sense: 5′-CATCAACCATGTCATTTGACCTGT-3′ Antisense: 5′-CGGTTGAGCTGCCTGTATTGATT-3′	Ismail et al. (2012)
VrJAZ3 (333 bp)	JF900331	27	Sense: 5′-AACAAGATCTCTGCACTTCCTCAT-3′ Antisense: 5′-AGCACCAACCATGTTCTCACCAG-3′	Ismail et al. (2012)
MYC2 (299 bp)	ABR23669	29	Sense: 5′-CTGCTTCAAGGTCTACCATGTTG-3′ Antisense: 5′-TTCTGGAGCTCCGTGATGTAAGT-3′	Ismail et al. (2012)
COI1 (482 bp)	TA2637_29760	27	Sense: 5′-TGGTTTGACTGATGTGGGTCT-3′ Antisense: 5′-CAGGGTCCAAGGGAGTAACA-3′	Moroldo et al. (2008)
NHX1 (469 bp)	AAV36562	29	Sense: 5′-GCGACATTGTCATTTGTTGCTCAA-3′ Antisense: 5′-GACTGGATGGTTCAGAACAAATCA-3′	Ismail et al. (2012)
VvABL1 (387 bp)	LOC100241147	29	Sense: 5′-AAAGATTCACCCACCTAACCACTC-3′ Antisense: 5′-CCTTGCCACGACATAGAACTGCT-3′	Li et al. (2011)
VvPYL1 (226 bp)	LOC100267793	29	Sense: 5′-CGACGAGAGGCACGTGACCG-3′ Antisense: 5′-CACCGACGCCAGCTTCTGCA-3′	Li et al. (2011)
StSy (216bp)	X76892	29	Sense: 5′-GGAAATTAGAAACGCTCAACGTGC-3′ Antisense: 5′-TGGTACAACATCACTCTTCTGACG-3′	Ismail et al. (2012)
PR10 (249 bp)	AJ291705	27	Sense: 5′-CCTGGAACCATCAAGAAGATTCAC-3′ Antisense: 5′-GTGCTCTTCACAGATCTCTACATC-3′	This paper

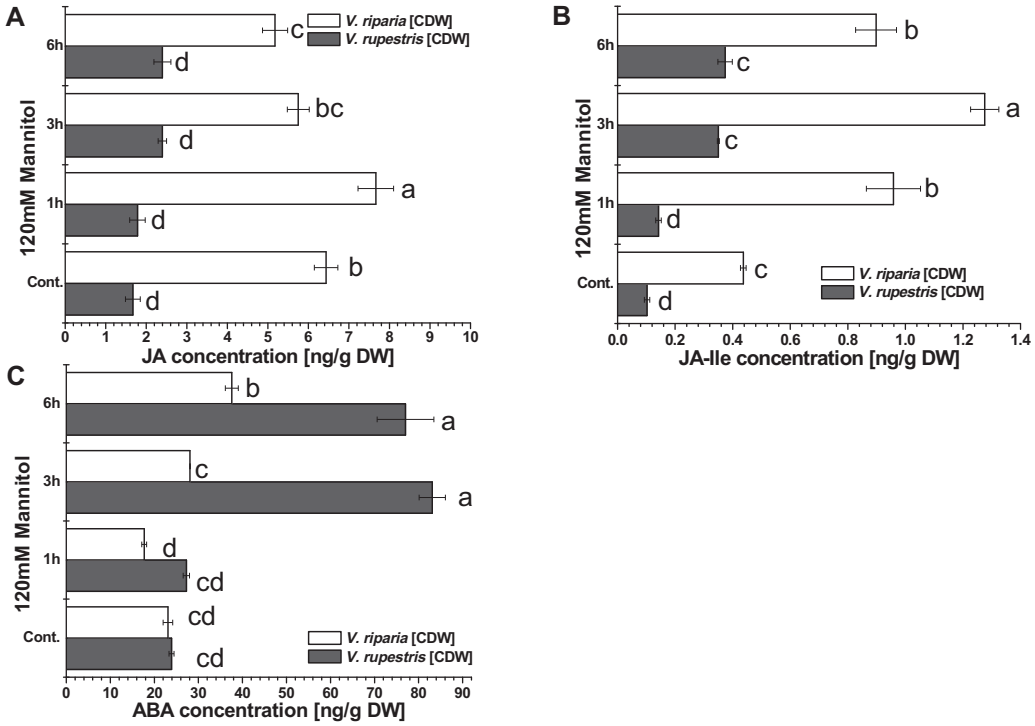

Fig. 1. Time course for the accumulation of endogenous JA (A), JA-Ile (B), and ABA (C) at different time points in *Vitis* cells treated by MS media containing 120 mM mannitol as final concentration at 1, 3 and 6 h for the two cell lines. Control samples were treated by the same volume of mannitol-free MS media for 1 h. Error bars represent SE from three independent biological replicates, and different letters indicate significant differences among treatments (LSD ($P < 0.05$)). Cell dry weight (CDW).

rate was adjusted to 0.8 ml min^{-1}, and the injection volume was 20 μl. The UV–vis spectra were recorded from 200 to 400 nm. The mobile phases included acetonitrile (ACN), methanol and water in the following isocratic gradient: 2 min ACN/water (10/90 v/v); 15 min ACN/water (40/60 v/v); 30 min ACN/methanol (50/50 v/v); 32 min ACN/methanol (5/95 v/v); 35 min ACN/methanol (5/95 v/v); 39 min ACN/water (10/90 v/v); 42 min ACN/water (10/90 v/v). *Trans*-resveratrol, *trans*-piceid, and δ-viniferin were quantified and identified using an external standard on the basis of retention time and UV–vis spectra. The standards for *trans*-resveratrol (Sigma–Aldrich, Deisenhofen, Germany), *trans*-piceid (Phytolab, Vestenbergsgreuth, Germany) and δ-viniferin (kind gift of Dr. Kassemeyer, State Institute of Viticulture, Freiburg, Germany) were dissolved in methanol to a concentration of 100 mg l^{-1}. Calibration curves determined using these standards were linear ($r^2 > 0.99$) and used for quantification of the samples (Chang et al., 2011). At least four biological replicates were analyzed for each time point.

2.5. Statistical analysis

The treatments were arranged as a factorial experiment in a completely randomized design (CRD). Three biological replicates were analyzed for each treatment. Comparisons among means were made *via* the Least Significant Differences LSD ($P < 0.05$) multiple ranges by using the SAS (SAS/STAT® 9.2, 2008) software. Mean values and standard error of the mean were calculated using Microsoft Excel.

3. Results

3.1. Osmotic induction of JA/JA-Ile and ABA depends on genotype

To gain insight into the role of the plant hormones under osmotic stress, the levels of JA, JA-Ile, and ABA, as well as, IAA and SA, were analyzed at different time points after mannitol treatment (Fig. 1 and Supplementary Fig. 2). In control cells of *V. riparia*, the levels of JA were roughly 4 times more abundant than those in *V. rupestris*, slightly increased at 1 h after mannitol application, but decreased later (Fig. 1A). In contrast, *V. rupestris* maintained a much lower JA level, despite a nonsignificant increase from 3 h after adding mannitol. This difference between the two cell lines was even more evident, when the levels of JA-Ile were compared (Fig. 1B). Like JA under the control condition, the levels of JA-Ile in *V. riparia* were 4 times higher than those in *V. rupestris*. Moreover, JA-Ile was highly induced by mannitol application in *V. riparia* cells by around 2 to 3 times. In *V. rupestris*, however, the levels of JA-Ile were increased later after 3 h of applying mannitol, reaching only the same levels observed in non-stressed *V. riparia* cells. In the case of ABA, the pattern was almost reversed under osmotic condition (Fig. 1C). The levels of ABA were nearly the same in both cell lines without mannitol stress, but increased drastically in *V. rupestris* to four-fold higher at 3 to 6 h after addition of mannitol. However, in *V. riparia*, this induction initiated much later, becoming significant only after 6 h of application by only less than two-fold compared to the ground level. In contrast to JA and JA-Ile, the levels of IAA and SA were higher in *V. rupestris* as compared to *V. riparia* before the mannitol treatment (Supplementary Fig. 2A and B), and decreased after the mannitol application in *V. rupestris*, into the levels equivalent to those in *V. riparia*.

3.2. Osmotic stress induces JAZ1 and NHX1 expression

To clarify the sequences of cellular responses, the expression levels of JA- and ABA-related genes, as well as biotic defense and ionic homeostasis markers were followed in both cell lines after applying 120 mM mannitol as the final concentration at different time points. For JA-related genes, three *VrJAZ* genes (*VrJAZ1* (*TIFY10a*), *VrJAZ2* (T*IFY10*b), and *VrJAZ3* (T*IFY*6b)), encoding negative regulators of JA signaling (identified and characterized in *V. rupestris* by Ismail et al., 2012), as well as *MYC2* and *COI1*, encoding

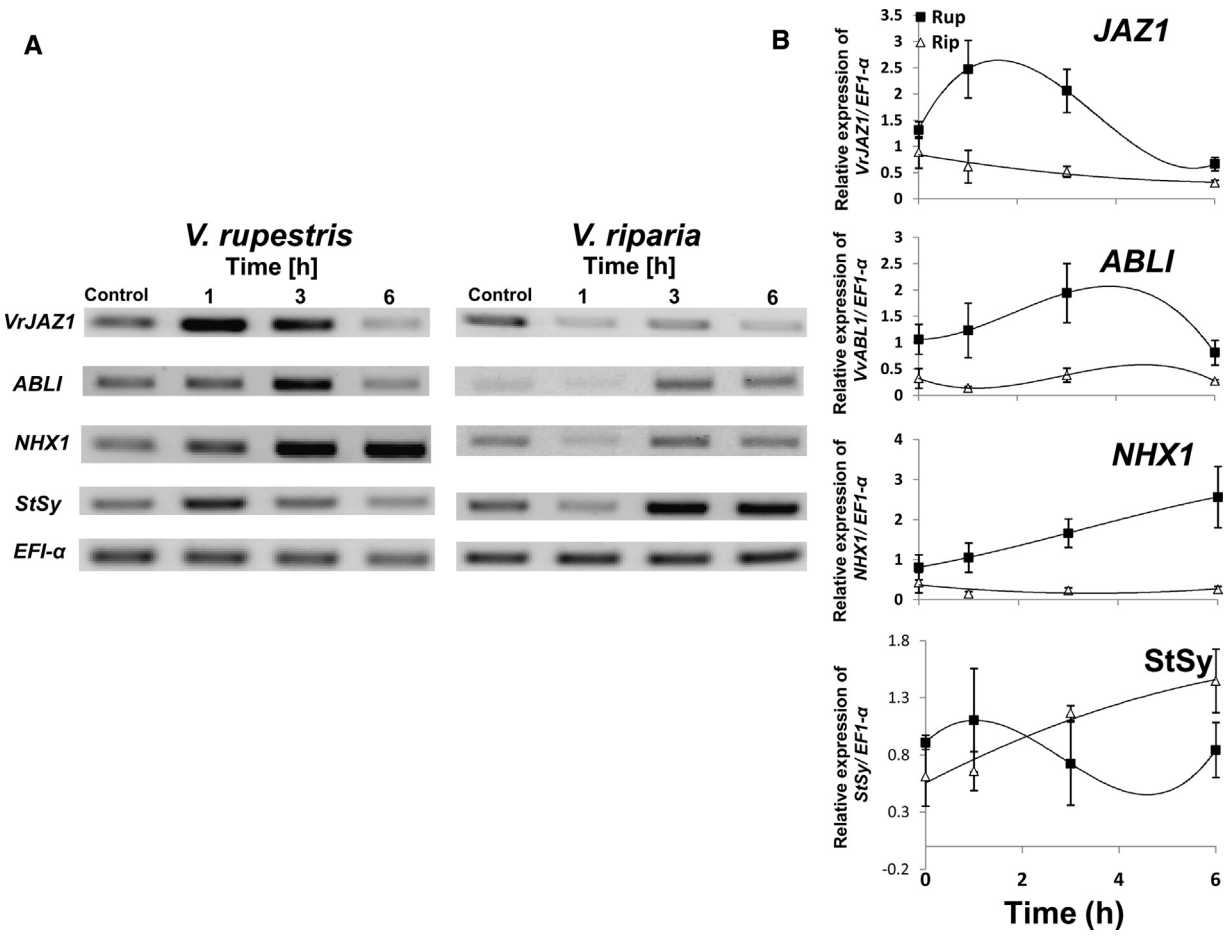

Fig. 2. Time courses of accumulation of transcript for osmotically-responsive genes (*VrJAZ1/TIFY10a*, *VvABL1*, *NHX1*, and *StSy*) at different time points in *V. rupestris* (Rup) and *V. riparia* (Rip). Osmotic stress was introduced to the cells by applying MS media containing 120 mM mannitol as final concentration at 1, 3 and 6 h. Control samples were treated by the same volume of mannitol-free MS media for 1 h. Representative electrophoretograms of the cDNA amplicons are shown at the left side, while relative abundances calibrated for the internal standard (*EF-1α*) are shown at the right side. One representative example of three independent experiments is shown.

positive regulators of JA signaling, were chosen. With respect to ABA-responsive genes, *VvABL1* (*ABI1*-like gene) and *VvPYL1* encoding negative and positive regulator of ABA signaling, respectively (identified and characterized in *Vitis vinifera* by Li et al., 2011) were tested. Finally, we studied markers of biotic defense responses, such as *PR10* (*Pathogen-related gene 10)* and *StSy* (*Stilbene synthase*) (Qiao et al., 2010), as well as an adaptive marker for intracellular ionic homeostasis and adaptive response to salt *NHX1* encoding sodium/hydrogen antiporter localized at the vacuolar membrane (Ismail et al., 2012) (Fig. 2 and Supplementary Fig. S3 and S4). Among *VrJAZ/TIFY* genes, *VrJAZ1* was the most responsive transcript to osmotic stress, induced only in *V. rupestris*, but not in *V. riparia.* Such mannitol-induced expression started earlier (1 h), and decreased later (3 h) (Fig. 2A and B). On the other hand, the expression levels of the other two *JAZ/TIFY* genes were not induced in either cell line (Supplementary Fig. 3A and B). With respect to the JA signaling master components, the expression of *MYC2* was slightly induced in *Vitis rupestris* but seldom in *V. riparia*, while *COI1* expression showed a sluggish response to osmotic stress in *Vitis rupestris*, but not in *V. riparia* (Supplementary Fig. 3A amd B). For ABA-responsive genes, the expression of *VvABL1* was induced by osmotic stress in *Vitis rupestris*, but less in *V. riparia* (Fig. 2A and B). Unlike *VrJAZ1*, *VvABL1* was osmotically induced later (3 h). The *VvPYL1* expression, on the other hand, was sluggish in both cell lines (Supplementary Fig. 4A and B). In contrast, the osmotic adaptation marker (*NHX1*) was clearly induced in *V. rupestris*, but not *V. riparia*, in a continuous manner from 1 h to 6 h after onset of mannitol stress (Fig. 2A nad B). For biotic defense markers, the expression of *StSy* was slightly induced in *Vitis rupestris*, after 1 h of osmotic stress. However, mannitol-stressed *V. riparia* showed a clear increase in the expression levels of the *StSy* gene (Fig. 2A and B). For *PR10*, the expression level was not osmotically responsive in either cell line (Supplementary Fig. 4A and B). Thus, mannitol-induced expression of *VrJAZ1/TIFY10a* precedes the expression of the *VvABL1* gene and *NHX1* in *V. rupestris*. In contrast, in *V. riparia*, expression of *VrJAZ1/TIFY10a*, ABA-responsive genes and *NHX1* was at relatively low levels, but instead *StSy* was highly up-regulated upon mannitol stress.

3.3. Stilbene accumulation in response to osmotic stress

In grapevine, stilbenes such as resveratrol and δ-viniferin not only accumulate in response to biotic stress, but also abiotic stress (Duan et al., 2015). In *V. rupestris*, accumulation of these cytotoxic non-glycosylated stilbenes precedes programmed cell death, and the resveratrol is suggested to act as a secondary signal of elicitor-triggered hypersensitive response in *Vitis* cell (Chang et al., 2011). However, in the context of salinity stress, osmotic-tolerant *V. rupestris* accumulated only a small amount of these stilbenes, whereas salt-sensitive *V. riparia* accumulated significant levels of the glycosylated piceid, and the cytotoxic δ-viniferin (Ismail et al., 2014b; for review, see Ismail et al., 2014a). Therefore the levels of δ-viniferin can be an indicator for cellular damages (Chang et al., 2011). Since salinity is composed of osmotic and ionic stress

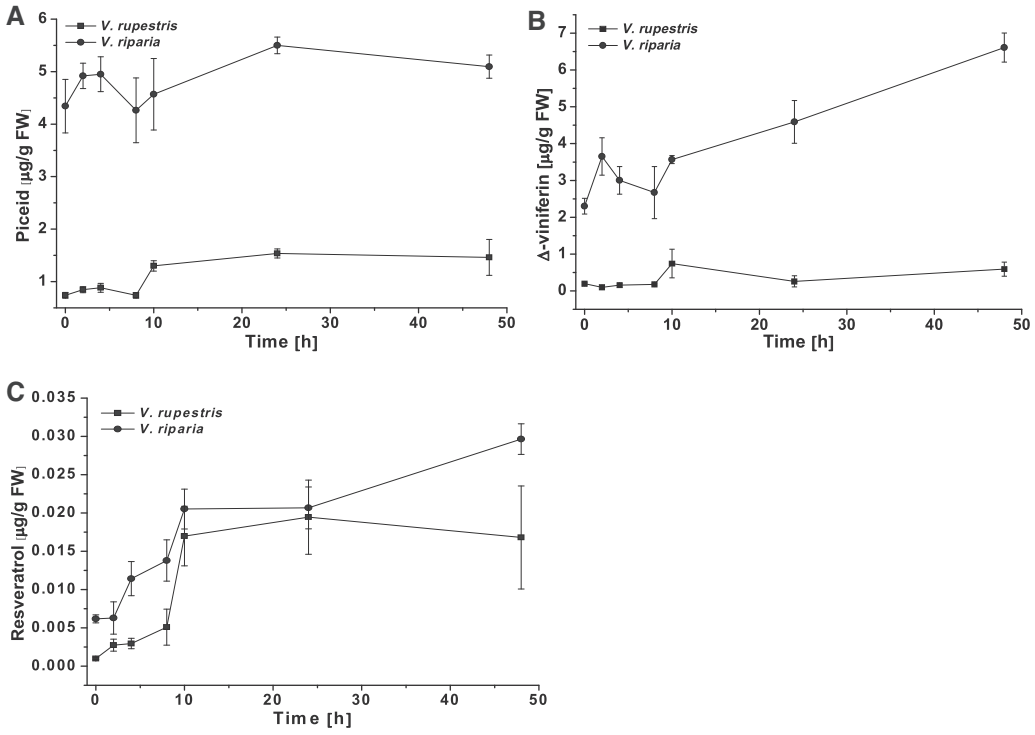

Fig. 3. Time course for the accumulation of the stilbenes piceid (A), δ-viniferin (B) and resveratrol (C) (µg/g FW) in response to 120 mM mannitol as a final concentration. Data represent mean values and standard errors for three independent biological replicates.

(Ismail et al., 2014b), we examined whether mannitol can also trigger the accumulation of these compounds. As shown in Fig. 3, osmotic stress induced the accumulation of α-piceid (Fig. 3A), and δ-viniferin (Fig. 3B), in *V. riparia* cells but seldom in *V. rupestris*. In contrast, both cell lines accumulated only low levels (around 0.020–0.030 µg/g) of *trans*-resveratrol under the osmotic stress condition (Fig. 3C).

4. Discussion

Osmotic stress is imposed by many kinds of water-related stress. In the current study, we followed osmotic-related responses in two grapevine genotypes that differ in their osmotic adaptability (for review, see Ismail et al., 2014a). Particularly, the plant hormones JA and ABA were our focus, as they are pivotal players in the context of abiotic stress responses. In addition, stilbene phytoalexins are important secondary metabolites of grapevine, representing potential antioxidants element of basal immunity (Duan et al., 2015).

The importance of ABA for osmotic adaptation has been well established. For instance, the accumulation of ABA upon osmotic stress results in up-regulation of several osmotic stress-responsive genes such as the ABRE, ABF, bZIP-type, ion channels (SLAC1, KAT1), and a NADPH oxidase (AtrbohF) (for review, see Joshi-Saha et al., 2011). In addition, the phosphoproteome analyses demonstrated that four AREB/ABFs are the main substrate transcription factors downstream of SRK2D/E/I in ABA signaling in response to osmotic stress during vegetative growth (Yoshida et al., 2015). Consequently, several adaptive events will take place such as stomatal closure and the induction of compatible osmolytes or the protective LEA proteins. Consistent with this role of ABA for osmotic adaptation, we observed that ABA increased around fourfold in *V. rupestris* within 3 h of stress, while in *V. riparia*, ABA induction was slower and less pronounced (Fig. 1C). In addition, the expression of ABA-responsive gene *ABL1* (Fig. 2A and B), but not *PYL1* (Supplementary Fig. 4A and B), was osmotically-induced in *Vitis rupestris*. Therefore, elevated accumulation of ABA in *V. rupestris* is correlated with improved osmotic adaptation.

However, ABA is not the only exclusive adaptive signal, as osmotically-stressed plants can respond in ABA-independent manner (for review, see Ismail et al., 2014a,b; Yoshida et al., 2014). For example, *Arabidopsis* DREB1A and rice OsDREB1A are ABA-independent, playing a crucial role in enhancing tolerance to different types of water-related stresses such as drought, high salt, and freezing (Dubouzet et al., 2003). Interestingly, OsDREB1A was found to function in a JA-dependent fashion downstream of OsbHLH148 (Seo et al., 2011). These findings call for a crucial role of JAs in stress adaptation. In our previous study, we examined salinity stress responses using the same systems (Ismail et al., 2014b). The two grapevine cell lines accumulated comparable levels of ABA upon salt stress. However, the salt-sensitive *V. riparia* accumulated four times more JA and six times more JA-Ile in comparison to *V. rupestris* (Ismail et al., 2014b). The findings of the current study demonstrated a similar pattern of JA and JA-Ile in osmotic-sensitive *V. riparia* comparing to osmotic-tolerant *V. rupestris* upon mannitol treatments (Fig. 1A and B). The key to understand the role JA-dependent signals inside the plant is relied on JAZ repressor proteins that fine-tune JA signals (for review, see Ismail et al., 2014a). In rice, when the *OsJAZ9* gene was over-expressed in rice, the resultant transgenic line showed a significant promotion of salinity and drought tolerance, which was accompanied by strong activation of the *NHX1* gene (Ye et al., 2009). In the current study, the osmotic-tolerant *V. rupestris* also exhibited high transcript levels of both *JAZ1* and *NHX1* osmotic stress (Fig. 2A and B), as well as under salinity (Ismail et al., 2014b). While the product of *JAZ1* (the repressor) is expected to re-set JA signals *via* its dimerization with MYC2 by recruiting the TPL and TPRs through the NINJA interactor (Chini et al., 2007; Thines et al., 2007; Pauwels et al., 2010), the product of *NHX1* (the transporter) will re-sequester Na^+ into the vacuole again after utilizing Na^+ as a "cheap osmolite" (Fig. 4 and Ismail et al., 2014b).

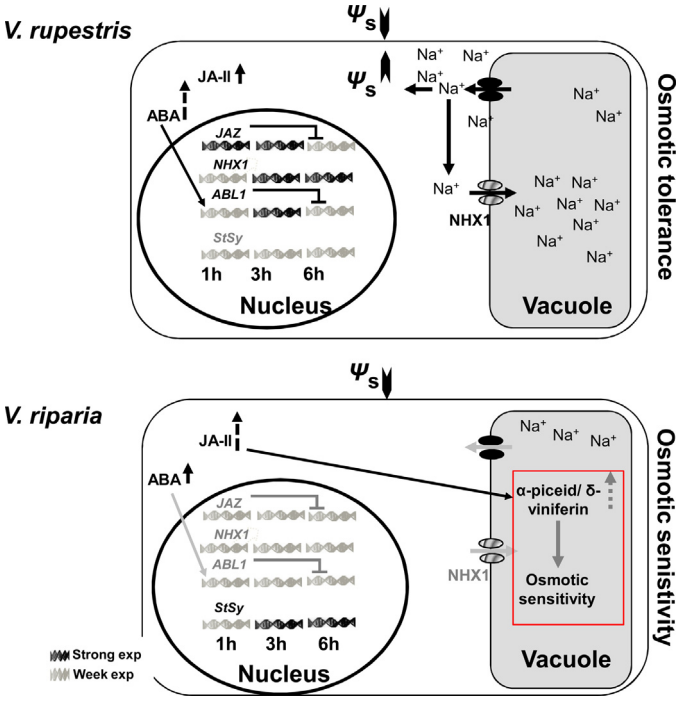

Fig. 4. A simplified model for the osmotically-induced signaling leading to adaptation (in *V. rupestris*) or sensitivity (in *V. riparia*). Ψs osmotic potential between apoplast and cytoplasm triggering osmotic signaling, JA jasmonate acid, ABA abscisic acid. For details refer to the discussion. Dashed arrows (⇑, ⇓) refer to non-significant (later significant after 6 h) and highly significant induction. Black arrows represent activation, gray arrows inactivation.

Moreover, osmoticaly-stressed grapevine *V. riparia* cells, but not *V. rupestris*, accumulated higher amounts of *trans*-piceid and δ-viniferin following the accumulation of JA and JA-Il (Fig. 3A and B). Recently, a screen of a collection of European wild grape (for simplicity termed *V. sylvestris*) with respect to susceptibility to downy mildew of grapevine (*Plasmopara viticola*) revealed that those produce high levels of the bioactive viniferins are less susceptible (Duan et al., 2015). Interestingly, when *V. rupestris* cells elicited by the bacterial effector Harpin, they accumulated both resveratrol and its toxic oxidative dimer δ-viniferin before proceeding to programmed cell death (Chang and Nick, 2012). However, under mannitol-induced osmotic stress, the same line produced very little of these stilbenes as cell death is not the right strategy to cope with abiotic stress (Figs. 3 and 4).

5. Conclusion

The current work was designed to understand cellular events in response to osmotic stress in the context of JAs, ABA, and stilbene phytoalexins, as well as their related genes. The activation of ABA is likely connected to osmotic adaptation, as it is highly accumulated in osmotic-tolerant *V. rupestris*. In addition, the rapid induction of *JAZ1* transcripts might be responsible for fine tuning of JA signals. Such activation of *JAZ1* is accompanied by the observation that the *NHX1*, a central component for salt adaptation, can be activated also by osmotic stress. Furthermore, the late induction of the expression of *ABL1* in comparison to *JAZ1* suggests that the induction of intracellular JA seems to be very critical compared to ABA levels, as the greater accumulation of JA and JA-Il was followed by *trans*-piceid and δ-viniferin in osmotic-sensitive *V. riparia* cells (Fig. 4).

Acknowledgements

The authors gratefully acknowledge Dr. Yusuke Jikumaru for his support of hormone analysis. Ahmed Ismail gratefully acknowledges Prof. Dr. Shin Takeda, Bioscience and Biotechnology Center, Nagoya University, Japan, for revising the manuscript. Ahmed Ismail was partially funded by Science & Technology Development Fund (STDF) Short Term Fellowship (STDF-STF) Cycle 4, Egypt, Research Grant No. 6394(http://www.stdf.org.eg/).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2015.06.014

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