RESEARCH PAPER

Drought stress tolerance in grapevine involves activation of polyamine oxidation contributing to improved immune response and low susceptibility to *Botrytis cinerea*

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Abstract

Environmental factors including drought stress may modulate plant immune responses and resistance to pathogens. However, the relationship between mechanisms of drought tolerance and resistance to pathogens remained unknown. In this study, the effects of drought stress on polyamine (PA) homeostasis and immune responses were investigated in two grapevine genotypes differing in their drought tolerance; Chardonnay (CHR), as sensitive and Meski (MSK), as tolerant. Under drought conditions, MSK plants showed the lowest leaf water loss and reduction of photosynthetic efficiency, and expressed a lower level of *NCED2*, a gene involved in abscisic acid biosynthesis, compared with CHR plants. The improved drought tolerance in MSK was also coincident with the highest change in free PAs and up-regulation of the genes encoding arginine decarboxylase (ADC), copper amine-oxidase (CuAO), and PA-oxidases (PAO) and their corresponding enzyme activities. MSK plants also accumulated the highest level of amino acids, including Arg, Glu, Gln, Pro, and GABA, emphasizing the participation of PA-related amino acid homeostasis in drought tolerance. Importantly, drought-tolerant plants also exhibited enhanced phytoalexin accumulation and up-regulation of *PR* genes, especially *PR-2* and *Chit4c*, compared with the sensitive plants. This is consistent with a lower susceptibility of MSK than CHR to *Botrytis cinerea*. Data suggest a possible connection between water stress tolerance and immune response in grapevine. Pharmacological experiments revealed that under drought conditions CuAO and PAO pathways were involved in the regulation of photosynthetic efficiency, and also of immune response and resistance of grapevine to a subsequent pathogen attack. These results open new views to improve our understanding of crosstalk between drought tolerance mechanisms and immune response.

Key words: Drought stress, immune response, photosynthesis, phytoalexins, polyamines.

Introduction

Plants are constantly challenged with numerous environmental stresses, both biotic and abiotic. Sometimes plants can be challenged with different stresses simultaneously or consecutively. To survive under such conditions, plants have evolved a variety of mechanisms to perceive distinct stimuli, to integrate the stress signal and to modulate the expression of the optimal response to each type of stress accordingly. Often, partially overlapping sets of responses are activated in different stress conditions and several points of crosstalk exist between abiotic and biotic stress responses (Fujita et al.,...
Takahashi and Kakehi, 2010). Water deficit represents one of the most harmful abiotic stresses affecting plants performance and yield. The level of drought tolerance depends on the genetic make-up of the plant and how fast reprogramming of gene expression and metabolism occurs. Drought stress can negatively affect the response to pathogens, suggesting that plants have less effective defence towards pathogens when growing under drought conditions (Fujiita et al., 2006). In contrast, abiotic stress may also interact positively with pathogen stress. For example in tomato, drought stress can enhance resistance to the necrotrophic fungus Botrytis cinerea (Achuo et al., 2006).

Emerging evidence indicates that metabolic pathways associated with amino acid and polyamine (PA) metabolism play integral roles in plant response to biotic and abiotic stresses (Marina et al., 2008; Moschou et al., 2008a; Liu et al., 2010; Zeier, 2013). Assorted functions have been attributed to these molecules ranging from their involvement in carbon/nitrogen homeostasis (Aziz, 2003; Liu et al., 2010; Moschou et al., 2012) to functioning as a signalling molecule during plant–microbe interactions (Walters, 2003; Urano et al., 2004). Under water-limiting conditions, PAs as well as some related amino acids serve in the adaptation to osmotic stress by acting as signalling molecules or as compatible solutes (Aziz et al., 1999; Bouchereau et al., 1999; Hussain et al., 2011). As PAs are involved in multiple metabolic pathways, their homeostasis needs to be regulated. The diamine putrescine (Put), can be synthesized directly from l-ornithine (l-Orn) by ornithine decarboxylase (ODC, absent from Arabidopsis genome) or from l-arginine (l-Arg) through several biosynthetic steps that involve arginine decarboxylase (ADC, two genes in Arabidopsis) (Takahashi and Kakehi, 2010). Decarboxylated S-adenosyl methionine serves as an aminopropyl donor for the production of the triamine spermidine (Spd) and the tetraamine spermine (Spm) from Put and Spd, respectively. The relative importance of ADC and ODC pathways for Put synthesis varies according to the plant species and the tissue. PA homeostasis is also dependent on PA catabolism through copper-containing amine oxidases (CuAOs) and flavin-containing PA oxidases (PAOs). CuAOs oxidize Put and cadaverine at the primary amino groups, and PAOs catalyse the oxidation of Spd, Spm, and/or their acetylated derivatives at the secondary amino groups (Cona et al., 2006). The action of CuAO on Put yields pyrroline, hydrogen peroxide, and ammonia, whereas PAO action on Spd and Spm yields pyrroline-1-(3-aminopropyl)pyrrolinium, respectively, as well as 1,3-diaminopropane (Rea et al., 2004; Cona et al., 2006).

Protective role of PAs against osmotic or drought stress has been reported in different plant species (Urano et al., 2004; Kusano et al., 2007; Takahashi and Kakehi, 2010). A loss-of-function mutation in Arabidopsis of one of the two ADC-encoding genes, the stress inducible ADC2, resulted in plants that were more sensitive to osmotic stress (Urano et al., 2004). In grapevine, only one ADC-encoding gene was characterized (Primikirios and Roubelakis-Angelakis, 2001), and genotypes with high or increased ADC or ODC activities were more tolerant to water stress (Toumi et al., 2010). On the other hand, rice plants overexpressing ADC showed increased endogenous Put levels as well as drought tolerance, but the higher Put levels were not enough to trigger the conversion of Put into Spd and Spm (Capell et al., 2004). Also, the enhanced Spd and Spm but not Put levels, was associated with enhanced recovery from water stress, but not increased tolerance during stress, correlating Put with direct tolerance, and Spd and Spm with recovery efficiency (Peremarti et al., 2009). This is consistent with other results providing evidence for the direct involvement of PA oxidation in plant adaptation to abiotic stress (Moschou et al., 2008a; Yoda et al., 2006). The PA metabolic pathways are also interconnected with other metabolic routes such as ethylene (ET), GABA, nitric oxide (NO), the Krebs cycle, and abscisic acid (ABA), as the most prominent pathways responsible for drought tolerance and disease resistance to fungal pathogens (Cona et al., 2006; Alcázar et al., 2010).

More recently, several studies have associated PAs with plant defence against microbial pathogens (Takahashi and Kakehi, 2010; Hussain et al., 2011). In grapevine, it has been proposed that PA oxidation may affect the host-signalling network that leads to enhanced immune response (Hatmi et al., 2014). Spm was also found to accumulate in intercellular spaces of tobacco leaves infected with tobacco mosaic virus (TMV), and its exogenous application further increased PR gene expression in an SA-independent manner (Yamakawa et al., 1998). Spm also triggered expression of other HR marker genes as well as the activation of SA- and wound-induced protein kinases in tobacco. These responses were blocked in the presence of PA oxidase inhibitors, suggesting that H2O2 derived from PA catabolism might be involved in Spm-induced defence signalling (Takahashi et al., 2004). Moreover, exogenous Spd and Spm induce H2O2 accumulation and cell death in tobacco, Arabidopsis, and rice (Yoda et al., 2006 2009). Furthermore, the elicitor cryptogein elevated apoplastic PAO activity in tobacco, and cryptogein-induced H2O2 production and cell death was attenuated by silencing of PAO (Yoda et al., 2006). Infection of tobacco leaves by the necrotrophic fungus Sclerotinia sclerotiorum resulted in increased ADC activity, as well as in Put and Spm accumulation (Marina et al., 2008). An enhancement of leaf PA levels, either by overexpressing ADC in transgenic tobacco or by exogenous application of Put, Spd, or Spm, potentiated S. sclerotiorum-induced necrosis formation and disease severity. Moreover, the pharmacological inhibition of PA oxidation reduced the severity of S. sclerotiorum-induced leaf rot, suggesting a disease-promoting role for PA and PA oxidation upon necrotrophic pathogen infection. Conversely, enhanced PA levels induced resistance to infection of tobacco leaves by the biotrophic bacterium Pseudomonas viridiflava, and this effect was reduced when PA oxidation was inhibited (Marina et al., 2008). These results suggest that PA and PA oxidation exert positive and negative influence on plant resistance to (hemi)biotrophic and necrotrophic pathogens, respectively.

Although there has been much progress in the grapevine–Botrytis cinerea interaction in the context of induced resistance (Aziz et al., 2003 2004; Magnin-Robert et al., 2007; Varnier et al., 2009; Bordiec et al., 2011; Verhagen et al., 2011), to our knowledge, there is no published research...
regarding the relationships between mechanisms involved in tolerance to abiotic stress, such as drought, and the grapevine resistance to this necrotrophic pathogen. The induced resistance usually involves the activation and/or priming state of immune response (Verhagen et al., 2010). However, in vineyard conditions, this resistance seemed to be irregular and this has been attributed, at least in part, to abiotic stressing factors (Magnin-Robert et al., 2007). Otherwise, the role of PAs in grapevine development and ripening has already been described (Aziz et al., 2001, 2003; Moschou et al., 2012), but the contribution of PA homeostasis to the grapevine resistance to pathogenic fungi following water starvation remains unknown. In this study, the effects of drought stress on PA homeostasis and immune responses were investigated in cuttings from two grapevine genotypes differing in their drought tolerance; Meski (MSK) from Tunisia as tolerant, and Chardonnay (CHR) as sensitive. The contribution of PA oxidation through CuAO and PAO to the control of PSII efficiency and resistance of grapevine leaves to B. cinerea was also evaluated using a pharmacological approach.

Materials and methods

Plant material and stress application

Two genotypes of grapevine (Vitis vinifera L.) with different degrees of drought stress tolerance were used: Chardonnay (CHR) as susceptible and Meski (MSK), which originated from south semi-arid regions of Tunisia, as tolerant to drought. This latter genotype requires hot and dry soils to achieve successfully mature berries and wood, and to express all its aromatic potential. It is fairly vigorous and characterized by rapid ageing. Plants of both genotypes were grown from cuttings in pots containing sand/perlite mixture (2/1, v/v) in growth chamber at 24 °C day/20 °C night, with relative humidity and characterized by rapid ageing. Plants of both genotypes were

Materials and methods

Polyamine extraction and analysis

Grapevine leaves were sampled at different times of treatments and ground in liquid nitrogen. Free PAs were extracted with cold 1M HCl (0.25:1, w/v) on ice, as described by Aziz et al. (2001). The aliquots of 200 μl from the supernatants were dianyslated using 600 μl of 10 mg ml⁻¹ dansyl chloride with 100 mg sodium carbonate. -Proline (300 μl of 100 mg ml⁻¹) was added to remove the excess dansyl chloride. The dianyslated PAs were further extracted with ethyl acetate. The organic phase was dried under nitrogen stream and the residue solubilized with 1 ml of methanol, filtered through 0.22 μm PTFE filters and stored at −20 °C until analysis. Dianysyl PAs were analysed using an Acquity UPLC system (Waters). They were eluted on a BEH C18 1.7-μm 2.1 × 100-mm column heated at 30 °C, with acetonitrile:water solvent gradient, changing from 50–95% over 5 min at a flow rate of 0.5 ml min⁻¹; elution was completed in 0.5 min and the column was washed with 100% acetonitrile for 1 min. Dansyl PAs were detected by an Acquity fluorimeter (Waters) with an excitation wavelength of 365 nm and an emission wavelength of 510 nm. PAs were quantified after calibration with external standards (Sigma).

Amino acid extraction and analysis

Free amino acids were extracted from 50 mg of freeze-dried tissue powder with 400 μl of methanol, 200 μl of chloroform and 400 μl of water at room temperature. Aliquots of 200 μl of the upper phase were dried under vacuum and residues were resuspended in 50 μl of ultra-pure water. Amino acids were derivatized using the AccQTM Ultra derivitization kit (Waters Corporation, Milford, USA) according to the manufacturer’s instructions. Derivatized amino acids were analysed using an AcquityTM UPLC system (Waters Corporation, Milford, USA) as described by Jubault et al. (2008). For each sample, amino acids were reliably identified by comparison of sample chromatograms with standards, and then quantified after normalization against internal standard and plant material fresh weight.

Phytoalexin extraction and analysis

Stilbenic phytoalexins were extracted from 250 mg of freeze-dried powder with 2 ml of methanol:water 85 % v/v. Tubes were placed in shaker for 1 h at room temperature and then centrifuged for 10 min at 8000 g. The supernatant was dried under nitrogen stream and residues were solubilized with 1 ml of methanol, filtered through 0.22 μm PTFE filters. Resveratrol and ε-viniferin were analysed using an Acquity™ UPLC system (Waters Corporation, Milford, USA) with a gradient from 10–90% acetonitrile over 7 min at a flow rate of 0.5 ml min⁻¹. The column was an Acquity BeH C18 1.7 μm 2.1×100 mm, heated at 30 °C. Phytoalexins were detected with an Acquity fluorimeter (Waters) at an excitation wavelength of 365 nm with an emission wavelength of 375 nm. Resveratrol and ε-viniferin were identified and quantified with reference of retention time and calibration with external standards.

CuAO and PAO activity

Amine oxidase assays were performed as described in Hatmi et al. (2014). Briefly, 250 mg of powdered leaves were homogenized in 1 ml of cold buffer (100 mM potassium phosphate, pH 6.5 for PAO or pH 7.0 for CuAO) on ice and the extract was centrifuged at 12 000 g for 10 min at 4 °C. Extractable CuAO and PAO activity was measured
spectrophotometrically by following the formation of a pink adduct ($\varepsilon_{515} = 2.6 \times 10^6 M^{-1} cm^{-1}$) as a result of the oxidation and condensation of 0.1 mM 4-aminoantipyrine and 1.0 mM 3,5-dichloro-2-hydroxybenzene sulfonic acid (Sigma-Aldrich) catalysed by 0.06 mg ml$^{-1}$ horseradish peroxidase (Corna et al., 2006). The assays were performed with 2mM putrescine or 2mM spermidine as the substrates of CuAO and PAO, at 25°C. Enzyme activities were expressed in µmol substrate oxidized per min per g dry weight.

RNA extraction and real-time quantitative RT-PCR
Total RNA was isolated from leaves using Plant RNA purification reagent (Invitrogen) and 150 ng was used for reverse-transcription using the Verso cDNA Synthesis kit (ThermoElectron) according to the manufacturer’s instructions. The transcript levels were determined by real-time PCR using the CFX96 system (BIO-RAD) and absolute blue qPCR SYBR Green dye as recommended by the manufacturer (ThermoElectron). PCR reactions were performed using a 30-fold cDNA dilution in duplicates as template in 96-well plates in a buffer containing 1×SYBR Green I mix (including Taq polymerase, dNTPs, SYBR Green dye) and 280 nM forward and reverse primers. The EF1 and 60SRP genes were used as reference genes and experiments were repeated twice. The specific primers of analysed genes are listed in Supplementary Table S1. Relative gene expression was determined with the formula fold induction: \(2^{-\Delta\Delta Ct}\), using CFX Manager 3.0 software (BIO-RAD) where \(\Delta\Delta Ct = (Ct \text{ GI [unknown sample]} – Ct \text{ GI [reference sample]}) – (Ct \text{ GR [unknown sample]} – Ct \text{ GR [reference sample]})\). GI is the gene of interest and GR is the reference gene. The reference sample is the control sample at 0 day from CHR cultivar chosen to represent 1× expression of the gene of interest. Results correspond to means±standard deviation of the two experiments (n=3).

Fungal disease assay
The B. cinerea strain 630 was used for disease assays. Fungal culture and preparation of conidial spore suspension were as described previously (Aziz et al., 2003). Leaf disks from the youngest fully expanded leaves of control or stressed plants were placed on wet absorbing paper in glass Petri dishes. One needle prick wound was applied to the abaxial side of each leaf and covered with 5 µl drops of a conidial suspension of B. cinerea (5 × 10$^5$ conidia ml$^{-1}$). Quantification of disease symptoms was measured on 35–45 leaf disks from three independent experiments at 3 or 7 days post-inoculation. Quantification of disease development in grapevine leaf disks was measured as average diameter of lesions formed during infection. Disease rating was expressed as the fraction of leaf disks falling in the following classes: I, spreading lesion covering less than 25% of the leaf area; II, spreading lesion covering 26–50% of the leaf area; III, spreading lesion covering 51–75% of the leaf area; IV, spreading lesion covering more than 75% of the leaf area.

Results
Water status, photosynthetic efficiency, and ABA synthesis were differentially affected in drought-sensitive and tolerant cultivars
Drought stress was induced by withholding water on 8-week-old cutting plants for up to 8 days. Under well-watered conditions, the foliar RWC was similar between genotypes (Fig. 1A, B). However, a significant decrease in the RWC was observed from day 3 of water deprivation for both genotypes. The decrease of RWC was much faster in CHR and reached its maximum on day 3, while it appeared much more slowly in MSK plants, indicating the potential of the latter to better tolerate drought stress.

The photosynthetic machinery within the chloroplast was associated with the plant adaptation to drought stress. Photosynthetic quantum efficiency of photosystem II (ΦPSII) analysed in the dark-adapted state revealed clear differences between the drought sensitive and tolerant genotypes. Under well-watered conditions, ΦPSII was not affected in both genotypes relative to controls. With water deficit, ΦPSII in CHR was significantly inhibited by day 3 relative to its control, whereas in MSK leaves ΦPSII decreased progressively (Fig. 1C, D). The stress-induced inhibition of ΦPSII was significantly stronger in CHR than in MSK plants, it remained high in MSK with a minimum of 0.78 on day 6, whereas
corresponding values for healthy plants were 0.81 (Fig. 1D). In addition, the expression pattern of the oxygen-evolving complex in PSII (PsbP1), which links the light-sensing network and the plant adaptation to abiotic stress (Abbink et al., 2002), was monitored in both MSK and CHR plants in response to water stress. Quantitative real-time PCR (qRT-PCR) analysis established that PsbP1 transcript abundance is reduced by water deficit in both plants (Fig. 1E). Although the initial levels of PsbP1 transcript were slightly higher in MSK plants, the down-regulation of this gene expression was much stronger in the MSK than in CHR leaves. These results demonstrate that PsbP1 may be involved in the abiotic stress response.

Knowing that ABA plays an important role in regulating plant responses to different stresses, we further examined whether NCED2 (involved in ABA biosynthesis) is responsive to drought stress in both CHR and MSK. The basal level of NCED2 expression was significantly lower in the MSK than in the CHR leaves. However, NCED2 transcript was highly up-regulated in both genotypes in response to drought stress (Fig. 1F). The increase in NCED2 transcription was stronger in the sensitive CHR than in the tolerant MSK plants.

Polyamine homeostasis in drought-stressed sensitive and tolerant plants

To further ascertain whether PA homeostasis plays a role in the drought tolerance mechanism, concentrations of free PAs were determined during withholding of water in both grapevine genotypes (Fig. 2). Control plants of MSK (at day zero) contained a higher amount of free PAs (≥1.5-fold) than the sensitive cultivar; Spd (Fig. 2B, F) being the most abundant, whereas Put (Fig. 2A, E) and Spm (Fig. 2C, G) were present almost in comparable amounts. Under water-deficit conditions, Put amount significantly increased in the leaves of both genotypes. It reached a maximum from the third day of water privation and remained elevated thereafter in MSK plants, whereas it declined later in the sensitive genotype (Fig. 2A, E). Spd (Fig. 2B, F) and Spm (Fig. 2C, G) levels, however, decreased along the time course in both genotypes when they were exposed to water deficit. This decrease was progressive for Spd and very low for Spm in CHR leaves, but it was more marked in MSK plants following water stress. Indeed, whereas Spd and Spm levels continued to decrease at day 8 in the tolerant plants, they slowed in the susceptible genotype. Significant increases in Dap (1,3-diaminopropane; Fig. 2D, H), a product of Spd and/or Spm oxidation, were observed in the two genotypes at day 3 and day 6 of water stress. The level of this amine was significantly higher in MSK than in CHR plants. These results support the contention that both PA biosynthesis and catabolism are tightly coordinated in grapevine plants and both of these processes could be required for an optimal level of PAs, and possibly for enhanced stress tolerance. The tolerant plants, when subjected to drought stress, exhibited significantly higher Put accumulation and decrease in Spd and Spm levels, accompanied with enhanced level of Dap, than the sensitive plants. Thus, the tolerant genotype exhibited higher potential for both PA synthesis and PA degradation under drought stress than the sensitive one.

The expression of genes encoding the key ADC and ODC enzymes controlling Put biosynthesis was also monitored by qRT-PCR. Using specific primers we recently identified an ADC and two ODC genes in grapevine (Gruau et al., unpublished). Under well-watered conditions, the three genes showed similar transcript levels in susceptible and tolerant plants. However, ADC mRNA levels increased significantly at 6 days of stress in both genotypes, and its expression was significantly higher in MSK than in CHR plants (Fig. 2I). In contrast, expression levels of ODC1 and ODC2 did not change in MSK, but slightly induced in CHR under stress conditions. The cellular PA level is also regulated by the action of amine oxidases including CuAO and PAO. Relative expressions of CuAO, PAO1, and PAO2 and their respective enzyme activities were monitored in the two genotypes as well. Under well-watered conditions, the CuAO and PAO2 transcript levels were comparable in both genotypes and did not change after 6 days in controls (Fig. 2I). However, the basal transcript levels of PAO1 (at day zero) in the MSK plants were almost 2-fold higher than in CHR, and increased significantly to similar extents in both genotypes under control conditions. After 6 days of drought, the expression levels of CuAO and PAO2 in the MSK and CHR plants were significantly induced, whereas PAO1 was reduced in both genotypes compared with their respective controls. In control plants both CuAO and PAO activities were significantly higher in MSK than in CHR leaves (Fig. 2J). Their levels increased in both cultivars after 6 days of drought stress. The drought-induced up-regulation of CuAO and PAO2 transcripts and enzyme activities was significantly higher in the MSK than in the CHR plants. These results suggest that the elevated Put and decreased Spd and Spm levels may involve not only PA synthesis, but also an activation of PA oxidation, and both pathways may contribute to drought tolerance in MSK plants.

Amino acid homeostasis in drought-stressed sensitive and tolerant plants

Next, we profiled free amino acids in grapevine leaves to investigate how their levels are modulated upon drought stress, and potentially contribute to plant defence. Free amino acids were analysed at day 6 of stress, specifically looking at PA-related amino acids, i.e. Arg, Orn, Glu, Gln, Pro, and GABA contents (Fig. 3; supplementary Table S2). Among the 22 amino acids detected in our UPLC method, the most abundant in well-watered plants were Glu, Asp, and Cys, which together accounted for 60% of the total amount of amino acids present in the two genotypes. Arg, Orn, Gln, Pro, and GABA contents were significantly lower in both well-watered genotypes. However, after 6 days of water deficit, the levels of the amino acids Orn, Gln, Pro, and GABA showed a sharp rise in both drought-stressed CHR and MSK plants. Arg and Glu levels increased only in MSK plants. The drought tolerant MSK plants showed a tendency to accumulate higher levels of the PA-related amino acids than the CHR plants (Fig. 3; Supplementary Table S2), reflecting a general
deficiency of free amino acids in CHR. However, the amount of Orn was somewhat higher in CHR than MSK in response to water stress. In the same condition, other striking differences between genotypes were observed for Asp, Apn, Leu, Ile, and Val, which were more abundant in the tolerant than in the sensitive genotype (Supplementary Table S2).

Drought stress induces differential defence responses in grapevine varieties

The enhanced tolerance of MSK plants to drought, along with the enhanced PA oxidation and accumulation of some PA-related amino acids, prompted us to evaluate whether the expression of immune responses was affected in both genotypes. One of the most studied defence response that pathogens encounter in plants is the production of antimicrobial compounds, such as stilbenic phytoalexins in grapevine. To investigate the effect of drought stress on the defence response of the plant, we analysed the main stilbenes, trans-resveratrol and trans-ε-viniferin, in grapevine leaves in a time-course experiment. Data show that drought stress induced significant accumulation of stilbenes in the leaves of both genotypes (Fig. 4). The amounts of trans-resveratrol and trans-ε-viniferin produced were significantly more important in MSK leaves (Fig. 4A, B) than in CHR ones (Fig. 4C, D) where both stilbenes accumulated slowly. The highest accumulation of trans-resveratrol and trans-ε-viniferin in MSK occurred at day 6 and day 8, respectively.

We further used qRT-PCR to examine the expression levels of some PR genes in the leaves of control and drought-stressed tolerant and susceptible genotypes. Experiments were carried out at day six of drought stress, where significant physiological and metabolic changes occurred. First, we could not detect any significant differences between the transcript levels of Chit1b (a basic class I chitinase) and PR-1 genes in control leaves of the two genotypes. However, slight differences were observed regarding transcript levels of Chit4c (an acidic class IV chitinase), PR-2 (a β-1,3-glucanase), and PR-5 (a thaumatin-like) genes between control leaves of MSK and CHR. Chit4c (Fig. 4E) and PR-2 (Fig. 4F) transcript
levels were 2- and 4-fold higher in control MSK than in control CHR leaves, whereas that of PR-5 (Fig. 4G) was 2-fold lower in MSK than in CHR leaves. Examination of these genes under drought conditions showed that expression of Chit1b, Chit4c, and PR-2 genes was significantly induced in the leaves of both genotypes. The transcript levels of Chit4c and PR-2 were 2- and 2.3-fold higher, respectively, in the MSK leaves than in the CHR ones. The most striking difference between the two genotypes was observed in the PR-2 expression which increased by about 70 times more in the MSK leaves compared with its basal level. However, Chit1b mRNA level was more markedly induced in the leaves of susceptible stressed plants compared with tolerant stressed cultivar. In contrast, in both genotypes, mRNA levels of PR-1 did not significantly change in response to drought stress, and expression of PR-5 decreased in water-stressed MSK.

**Drought stress increases susceptibility to pathogen infection, but to a lesser extent in drought-tolerant plants**

To evaluate the effect of drought on grapevine resistance to *B. cinerea*, plants were first exposed to drought stress for 6 days, and then plants or leaf disks from control and drought-stressed plants were inoculated with *B. cinerea* spores. The development of lesions on the leaf disks was followed. Visible disease symptoms were apparent within 3 days post-inoculation (dpi) in leaf disks of control CHR plants, whereas in MSK leaf disks the sizes of the lesions were similar to the size of the drop-inoculum, indicating limited progression of the disease. At 7 dpi, the lesion sizes in control CHR plants were larger than that in control MSK leaf disks (Fig. 5A). The difference

![Fig. 3. Concentrations of free amino acids in the leaves of CHR and MSK genotypes exposed to well-watered and drought stress conditions. Arginine (Arg), ornithine (Orn), glutamate (Glu), glutamine (Gln), γ-aminobutyric acid (GABA), and proline (Pro) concentrations were determined in the leaves of control and drought stressed plants at day six. Other amino acids are presented in Supplementary Table S2. Values are averages of three independent experiments and are shown as means±SDs. Different letters indicate significant differences (Duncan’s multiple range test; P<0.05).](https://academic.oup.com/jxb/article-abstract/66/3/775/478945)

![Fig. 4. Phytoalexin accumulation and transcript levels of defence-related genes in the leaves of CHR and MSK plants exposed to well-watered and drought stress conditions. Plants from CHR (A, B) and MSK (C, D) genotypes were watered (dotted line) or subjected to water stress (solid line) and fully expanded leaves were harvested at the indicated times for determination of resveratrol (A, C), and viniferin (B, D). Each value represents the mean of five replicates and vertical bars represent the standard errors. Different letters indicate significant differences (Duncan’s multiple range test; P<0.05). Transcript level of Chit1 and Chit4c (E), PR-2 (F), and PR-1 and PR-5 (G) genes was determined by qRT-PCR at day zero (open bars) and day 6 in both control (grey bars) and stressed (closed bars) plants. The EF1 and 60SRP genes were used as the internal controls for normalization. Results are expressed as the fold increase in transcript level relative to control leaves of CHR plants at day zero (×1). Values are averages of two experiments repeated twice and are shown as means±SDs.](https://academic.oup.com/jxb/article-abstract/66/3/775/478945)
between genotypes was also striking on a large number of samples taking into account the frequency distribution of disease symptoms (Fig. 5B). Following drought stress, inoculated leaf tissues with *B. cinerea* showed more severe symptoms than the non-stressed plants (Fig. 5A, B). The necrotic lesions formed in CHR leaf disks had an average diameter more than 4-fold higher than those produced in MSK leaf disks (Fig. 5A). We also observed that the number of leaf disks that displayed extensive necrotic lesions was much higher in the drought-stressed CHR than in MSK plants (Fig. 5B). This indicated a possible link between drought-tolerance mechanisms and improved resistance of grapevine to the necrotrophic pathogen.

The previous results indicate that various defence responses induced by drought stress correlate with enhanced diamine and PA oxidation; both were less pronounced in the susceptible genotype. We also showed under non-stressed conditions that following *B. cinerea* infection PA homeostasis was linked to enhanced activity of CuAO and PAO in CHR *in vitro* plantlets (Hatmi et al., 2014). This prompted us to further characterize a relationship between diamine and PA oxidation and immune response in CHR and MSK genotypes exposed to water deficit. For this purpose, we used aminoguanidine (AG), an irreversible inhibitor of the CuAO, and β-hydroxyethyl-hydrazine (HEH), as a competitive inhibitor of the PAO. The ability of AG and HEH to inhibit amine-oxidizing enzymes under control and stress conditions was shown, using Put and Spd as substrates (Fig. 6A, B). CuAO activity induced by water stress was significantly attenuated by AG, but not by HEH in both genotypes (Fig. 6A), whereas PAO activity decreased following pre-treatment with both inhibitors (Fig. 6B). CuAO and PAO seemed more inhibited in MSK plants with AG than in CHR.

The effect of drought stress following pre-treatment of CHR and MSK plants with AG and HEH on induced defence response was evaluated through the analysis of stilbene phytoalexins. Under well-watered conditions, pre-treatment of CHR plants with either AG or HEH resulted in an increase production of resveratrol (Fig. 6C) and ε-viniferin (Fig. 6D), whilst the level of both stilbenes remained unchanged in control MSK plants. However, the drought-stressed plants pre-treated with AG or HEH showed a strong reduction of both resveratrol and ε-viniferin accumulation in both genotypes. Following pre-treatment with AG or HEH, the amount of resveratrol was reduced by about 45% in CHR and by 50–70% in MSK, whereas that of ε-viniferin was reduced by at least 65% in both genotypes compared with the stressed plants. These data point to the drought stress-induced CuAO- and PAO-dependent accumulation of phytoalexins as a major defence reaction in grapevine plants. We also assessed the effect of CuAO and PAO inhibitors on the photosynthetic efficiency as well as on the resistance level towards *B. cinerea*. As shown before, drought stress reduced photosynthetic efficiency (Fig. 6E). Application of AG or HEH under non-stressed conditions can slightly inhibit ΦPSII activity in CHR but not in MSK leaves. However, pre-treatment of plants with AG or HEH before water stress resulted in a strong inhibition of ΦPSII in both genotypes (by about 70–90% in CHR versus 35–60% in MSK) (Fig. 6E). This highlights the importance of PA homeostasis in the regulation of photosynthetic process and probably in drought tolerance mechanisms in grapevine.

To further evaluate whether CuAO and PAO could contribute to the low susceptibility of grapevine leaves to *B. cinerea*, CHR and MSK plants were first treated with AG or HEH before their exposure to drought stress, and then leaf disks were cut and infected with *B. cinerea* conidia. Leaf disks from control plants pre-treated or not with AG or HEH, showed
small necrotic lesions provoked by *B. cinerea* in both genotypes within 3 dpi. However, leaf disks from drought-stressed plants pre-treated with AG or HEH exhibited severe disease symptoms (Fig. 6F). The necrotic lesions formed had an average diameter 1.4- to 2-fold higher than those produced the leaf disks from stressed plants, even though MSK showed lower susceptibility to *B. cinerea* than CHR. Both AG and HEH treatments impaired the ability of the leaves to arrest the pathogen. It can be suggested that oxidation of free PAs under drought stress can affect metabolic defence reactions in grapevine. Thus, modulation of plant PA levels may lead directly or through their oxidation products to significant changes in basal host resistance to pathogen.

**Discussion**

Drought has a profound effect on plant physiology, and adaptation of plants to water stress can influence their ability to resist microbial pathogens. However, the relationship between drought tolerance mechanisms and conditioning of innate immune system remained elusive. Here, we present physiological, molecular, and pharmacological evidence suggesting that drought-induced PA oxidation in grapevine could be involved in both drought-tolerance mechanisms and enhanced immune response towards the pathogen *B. cinerea*. Using two grapevine genotypes differing in their drought tolerance, we show that drought enhanced susceptibility of plants to *B. cinerea*, CHR being more prone to this pathogen than the MSK genotype. Other differences between varieties mainly account for changes in photosynthetic efficiency, ABA synthesis, PA, and PA-related amino acid homeostasis, as well as the level of defence responses such as phytoalexin synthesis and PR-gene expression.

Photosynthesis can provide a sustained energy supply and, therefore, has to be integrated into the stress-tolerance traits. In this study, we show that drought-induced inhibition of photosynthetic efficiency, accompanied by a down-regulation of *PsbP1* gene encoding a photosystem-binding protein 1, was more pronounced in the sensitive plants than in the

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Fig. 6. Effect of aminoguanidine (AG) and β-hydroxyethylhydrazine (HEH), inhibitors of CuAO and PAO, on the drought-induced diamine- and polyamine-oxidase activity, phytoalexin accumulation, photosynthetic efficiency, and leaf susceptibility to *B. cinerea*. Plants from CHR and MSK were watered with 2 mM AG or 1 mM HEH or without inhibitor (Ctrl) 24 h before withholding water. Expanded leaves were harvested on day 6 for CuAO (A) and PAO (B) activity, the phytoalexin resveratrol (C), viniferin (D), and maximum photosynthetic efficiency (ΦPSII) (E) analysis. Leaf disks were excised from the youngest fully expanded leaves and inoculated with *B. cinerea*. The size of the necrotic lesion was determined 3 dpi (F). Data points are means±SDs from 20–30 leaf disks. Different letters indicate significant differences (Duncan’s multiple range test; *P*<0.05).
tolerant one. This can be explained by different photoinhibition of PSII rates, which increased the levels of transcript genes involved in electron transport chain of photosystems (Cramer et al., 2007). The balance between light capture and energy use may be of great relevance for drought tolerance and possibly for immune response (Göhre et al., 2012). Tolerant genotype also exhibited a low enhanced expression of N Ced2 involved in ABA biosynthesis. The level of NC ed2 transcript may be determinant for the ABA homeostasis and thus predispose plants to stress tolerance (Qin and Zeevaart, 1999).

The most significant changes occurred in PA levels under drought condition; particularly an increase in Put and decrease in Spd and Spm are associated with PA synthesis and oxidation indicated by high expression of ADC, CuAO, and PAO2 genes and high activity of CuAO and PAO, as well as Dap accumulation in tolerant plants. The differences in PA levels between genotypes suggest that different PA-regulating mechanisms are involved, such as PA synthesis, catabolism conjugation, and transport (Cona et al., 2006). For instance, soluble and insoluble conjugated PA levels are very low in the leaves of both genotypes (less than 10% of total PAs) and showed similar trends as free forms under drought conditions (data not shown), suggesting that activation of both PA biosynthesis and degradation would be favoured. The increase of Put could also be due to a possible back-conversion of Spd to Put by an unknown grapevine PAO isoform, as reported in Arabidopsis (Moschou et al., 2008a). This suggests that CuAO and PAO may exert a specific role in stress tolerance strategy that minimizes drought-induced cellular damage. It has also been reported in other plant species that the PA synthesis and oxidation pathways are up-regulated following abiotic stress (Alcazar et al., 2010), elicitation (Perez-Amador et al., 2002; Walters et al., 2002), or during pathogen infection (Marina et al., 2008; Gonzalez et al., 2011; Kim et al., 2013). Frequently, stress-tolerant genotypes show enhanced PA biosynthesis in response to abiotic stress compared with stress-sensitive genotypes. In spite of the fact that genetic manipulation of PA biosynthesis leads to enhanced stress tolerance against multiple stresses (Capell et al., 2004; Kasukabe et al., 2004), the role of PA catabolism in this response is obscure (Cona et al., 2006). Yoda et al. (2006) showed the involvement of PAO in programmed cell death induction during the HR-like response. Similarly, Moschou et al. (2008a) showed that salt stress triggers Spd exodus to the apoplast where it is catabolized by PAO, producing H₂O₂ that directs tolerance responses in tobacco. H₂O₂ produced by CuAO and PAO is involved in cell wall maturation and lignification during development as well as in cell wall reinforcement in response to biotic and abiotic stress (Cona et al., 2006). Aminoaldehydes and Dap are also involved in secondary metabolite synthesis and abiotic stress tolerance (Bouchereau et al., 1999). Overall, our data are in accordance with an emerging research for PA catabolism, which seems to contribute to drought stress responses/tolerance (Moschou et al., 2008a).

The cellular PA homeostasis constitutes also an important metabolic node closely connected to plant amino acid metabolism, particularly those of Arg and Orn as the main precursors for Put biosynthesis, and GABA and Pro which can result from Put catabolism (Majumdar et al., 2013). Here, we show that the primary amino acid status of grapevine leaves was profoundly affected by water deficit, and the accumulated levels of most of amino acids were higher in drought tolerant cultivar (Supplementary Table S2). Thus, the adaptation occurred in amino acid level might contribute to PA homeostasis under stress conditions. Looking at PA-related amino acids, we show that drought-tolerant plants exhibit a large accumulation of Arg, Glu, Gln, Pro, and GABA, supporting the idea that drought tolerance of grapevine genotypes is associated with an enhancement of the metabolic flux from Arg to GABA and Pro, whereas drought sensitivity is marked by a general deficiency of these amino acids, which can lead to weakened immune responses against pathogen infection. Under drought conditions, Glu can directly and rapidly be fluxed into Pro and GABA to form the osmolytes for protecting cells from damage (Shelp et al., 1999). Alternatively, increased GABA and Pro contents can provide some indications for oxidative deamination of Put in agreement with hereby reported increases in CuAO and PAO2 gene expression and amine-oxidase activities. Potential roles of these amino acids in protection against pathogen infection were also proposed (Liu et al., 2010; Zeier, 2013).

One important event that is associated to drought tolerance once the grapevine genotypes are exposed to water deficit is the induction of defence mechanisms, including stilbene phytoalexins and the expression of PR-related genes. Here, we found that accumulation of the main stilbenes resveratrol and ε-viniferin is induced following drought stress and amounts of both phytoalexins are generally greater in tolerant plants than in sensitive ones. Correlation between higher protection level and elevated phytoalexin concentrations confirms that these compounds are probably of primary importance for resistance against B. cinerea (Montero et al., 2003; Aziz et al., 2006). Likewise, the expression of some PR genes (Chit1b, Chit4c and PR-2) known to be B. cinerea-responsive allows the clear differentiation of their expression level in drought-tolerant and drought-sensitive genotypes. Chit4c and PR-2 are the only genes that have significantly higher expression in tolerant plants, but the most striking difference between the two varieties was observed in the PR-2 expression. However, we could not detect any significant difference between the transcript levels of PR-1 gene in the two genotypes, whereas the steady state level of PR-5 transcript decreased in tolerant plants. Chit4c and PR-2 encode chitinase and β-1,3-glucanase, respectively. Both enzymes should participate in the plant defence by hydrolysing fungal cell wall components as previously reported (Van Loon and Van Strien, 1999). They also should amplify the plant defence by releasing β-1,3 glucans and chitin oligomers from the pathogen cell walls, both saccharides being well known MAMPs.

Growing evidence indicates that PAs play a role as mediators in defence signalling against pathogens (Takahashi et al., 2004), accumulate during plant–pathogen interactions, and enhance resistance against pathogens (Yamakawa et al., 1998; Cona et al., 2006; Tun et al., 2006). Here, we show
that activation of PA catabolism was correlated to increased immune responses in grapevine leaves under drought conditions, suggesting a possible interconnection between these processes. For instance, the altered PA homeostasis in both genotypes pre-treated with the two inhibitors of CuAO and PAO was enough to influence host physiology and increased susceptibility of leaves to *B. cinerea*. Both inhibitors, AG and HEH, had depressive effects on defence responses, especially on resveratrol and viniferin accumulation, as well as on the photosynthetic efficiency. These data give a first hint that PA oxidation could be crucial, not only in drought tolerance process, but also in regulating grapevine immunity towards the necrotrophic pathogen *B. cinerea*. Thus, modulation of plant PA levels may lead directly or through their oxidation products to significant changes in basal host resistance. It has been reported that PA oxidation ensures recycling of the reduced carbon and nitrogen to Krebs cycle through the formation of Δ1-pyrroline and GABA (Aziz et al., 1998; Moschou et al., 2008a), thus contributing to cellular energy metabolism. PAs could also play a role in the structure and functioning of the photosynthetic apparatus, because of their richness in amine groups and their biosynthesis through amino acid decarboxylases (Walden et al., 1997). PA catabolism by CuAO and PAO leading to the production of H₂O₂ has been implicated in mediating programmed cell death and wall lignification (Walters, 2003). As a signal molecule, H₂O₂ derived from PA oxidation has been shown to mediate the hypersensitive response and the expression of defence genes in tobacco (Yoda et al., 2006; Moschou et al., 2008a). In addition to H₂O₂, GABA has also been shown to play a key role in signal transduction pathways during stress response of many plants (Shelp et al., 1999; Seifi et al., 2013). Our results also support the interpretation that the enhanced susceptibility of grapevine to *B. cinerea* might be due to Spd and Spm interference with ET biosynthesis (Nambeesan et al., 2012), which plays a critical role in imparting resistance against *B. cinerea* (Lloyd et al., 2011).

Fig. 7. Proposed model for the role of polyamine oxidation in drought tolerance and low susceptibility of grapevine to *B. cinerea*. Both drought stress and pathogen are perceived by specific cellular receptors, and then signalling events are activated. The response to both stresses shares polyamine homeostasis/oxidation. As polyamine oxidation increases in drought tolerant genotype, there is an enhanced immune response, low inhibition of photosynthesis, and low susceptibility to *B. cinerea*. Inhibition of drought stress-induced CuAO and PAO activity lowers photosynthetic activity and defence responses and increases susceptibility to the pathogen. (This figure is available in colour at JXB online.)
Overall, our data highlight the influence of water statute on PA homeostasis and immune response and suggest a possible connection between drought tolerance traits and disease resistance mechanisms. In our proposed model (Fig. 7), tolerance to drought stress in grapevine seems to be associated to its capacity to synthesize the PAs through ADC and to express enhanced defence responses and low susceptibility to the necrotrophic pathogen *B. cinerea*. These findings also highlight through a pharmacological approach the importance of drought-stress-induced CuA and PAO pathways not only in the regulation of photosynthetic efficiency, but also in basal immune response and resistance of grapevine plants to *B. cinerea*.

**Supplementary data**

Supplementary data are available at JXB online.

Table S1. Primers sequences used in real time reverse-transcription polymerase chain reaction

Table S2. Free amino acid contents in the leaves of Chardonnay (CHR) versus Meski (MSK) subjected or not to drought stress.

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