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Jasmonate is required for the response to osmotic stress in rice

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ABSTRACT

Plants have the ability to alleviate the harmful effects caused by abiotic and biotic stress. Phytohormones play a very important role in the acclimation to these stresses. To study the role of jasmonate in the acclimation to osmotic stress, an ALLENE OXIDE CYCLASE (AOC) mutant of rice (*cpm2*), disrupted in the biosynthesis of jasmonic acid (JA), and its wild type (WT) background were employed to investigate their responses to osmotic stress caused by treatment with polyethylene glycol (PEG) 6000. WT showed tolerance to osmotic stress, correlated with a fast transient increase of JA and JA-isoleucine (JA-Ile) in the shoots prior to an increase in abscisic acid (ABA), followed by a second increase in jasmonates when exposing to osmotic stress during 24 h. In roots, the pattern of hormonal increase was similar, but the response appeared to be faster, and remained transient, also with respect to low levels of jasmonates upon continuing osmotic stress. The mutant, which was containing extremely low levels of jasmonates, was hypersensitive to the stress. However, ABA accumulated in both, shoots and roots of *cpm2*, to similar (but not equal) levels as those seen in the WT, demonstrating that the biosynthesis or catabolism of ABA in response to osmotic stress is at least partially independent of JA, but can be modulated by JA. Our results suggest that jasmonates operate in parallel, presumably synergistically, to ABA, and are indispensable for osmotic stress tolerance in rice.

1. Introduction

Throughout their life cycle, plants are often exposed to various environmental challenges, including different kinds of biotic and abiotic stresses (Yan and Xie, 2015). However, due to their sessile life style, it is not possible for plants to escape from inhospitable places (Yan and Xie, 2015). To protect themselves from harmful factors, plants have evolved adaptive strategies to environmental variation at the morphological, physiological, cellular, and molecular levels (Yoshida et al., 2015). Such strategies include mechanisms for activation of diverse developmental processes, as well as activation of signalling networks to deploy stress acclimation (Jones and Dangl, 2006; Dar et al., 2015; Yan and Xie, 2015). Osmotic stress is associated with diverse stresses including drought, salinity, and freezing stress, and is considered to be the common component in different types of water-related stress (Hazman et al., 2016). In addition, osmotic stress is probably the most serious factor limiting crop quality and productivity worldwide (Riemann et al., 2015; Wang et al., 2015). Therefore, it is necessary to understand how plants respond to osmotic stress for enhancing the acclimation of plants to such diverse stresses (Fujita et al., 2013; Wang et al., 2015).

Since osmotic stress is found to activate several signalling pathways which results in changes of physiological metabolism and gene expression (Wang et al., 2016), it has long been considered that phytohormones play a critical role in plant defence to stresses. Plant hormones contribute to regulation of multiple life processes and signalling networks when plants are exposed to biotic and abiotic stresses (Dar et al., 2015). Among the phytohormones, abscisic acid (ABA), ethylene (ET), jasmonic acid (JA), and salicylic acid (SA) were identified as four major hormones regulating plant stress acclimation (Kumar and Klessig, 2000; Asselbergh et al., 2008). Abscisic acid plays a critical role for the acclimation to osmotic stresses, such as the regulation of stomatal movement in guard cells, as well as the expression of genes related to osmotic stress resistance (Finkelstein et al., 2002; Himmelbach et al., 2003; Cutler et al., 2010; Ding et al., 2014). Adaptive gene expression in response to osmotic stress can, however, also be mediated by ABA-independent signalling pathways (Yoshida et al., 2014). In the ABA-dependent pathway, bZIP-type transcription factors, such as ABFs/AREBs (ABRE-binding or responsive factors) (Choi et al., 2000; Yoshida et al., 2010; Xu et al., 2013), AP2/ERF (Mizoi et al., 2012), MYB, MYC, NAC, and bHLH were identified as positive regulators (Abe et al., 2003; Ding et al., 2014; Nakashima et al., 2007). In contrast, the DREB2 protein, a member of the AP2/ERF family (Sakuma et al., 2006; Yoshida et al., 2014), is able to activate the expression of target genes encoding

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proteins involved in osmoprotection and metabolism independently of ABA (Abe et al., 2003; Ding et al., 2014; Nakashima et al., 2007). In addition, also the SNAC1-regulated protein phosphatase gene OsPP18 mediates osmotic and oxidative stress responses through the ABA-independent pathway (You et al., 2014, 2012).

In higher plants, ABA is synthesised via a C_{40} carotenoid intermediate, followed by a two-step conversion of the intermediate xanthoxin to ABA through ABA aldehyde (Xiong and Zhu, 2003). So far, ABA-deficient mutants, enzymes and genes have mainly been studied in *Arabidopsis* (Schwartz et al., 2003). Besides ABA, jasmonates (JA and its oxylipin derivatives) act as important signals which regulate various aspects of plant development, e.g. stamen maturation, root growth, fertility, sex determination, tendril coiling, and leaf senescence (Santino et al., 2013). In addition, jasmonates also mediate plant defence against various biotic stresses, such as bacterial and fungal pathogens, but also the response to abiotic signals, such as light (Wasternack and Hause, 2013). Jasmonates participate as well in the regulation of biosynthetic pathways of various secondary metabolites, such as terpenoids, indole alkaloids, nicotine, flavonoids, ginsenosides, benzophenanthridine alkaloids, and glucosinolates (Wasternack, 2014). Previous studies have led to the conclusion that jasmonic acid is needed for ABA accumulation in *Arabidopsis thaliana* and *Citrus* (Ollas et al., 2013, 2015). However, it is not clear, how jasmonic acid regulates ABA synthesis.

JA is synthesized from α-linolenic acid (α-LeA/18:3) through the octadecanoid pathway partitioned between plastids and peroxisomes (Wasternack and Hause, 2013). An alternative way of jasmonate biosynthesis, independent of OPDA REDUCTASE 3 has been found recently (Chini et al., 2018). JA is then conjugated with amino acids, preferentially isoleucine, to form bioactive (+)-7-*iso*-JA-Ile, catalysed by the jasmonoyl amino acid conjugate synthase JASMONATE RESISTANT 1 (JAR1) (Staswick et al., 2002; Staswick and Tiryaki, 2004). The product, (+)-7-*iso*-JA-Ile, is the most bioactive form of jasmonates (Fonseca et al., 2009) and binds to its cognate co-receptor complex consisting of the F-box protein CORONATINE INSENSITIVE 1 (COI1; Xie et al., 1998; Sheard et al., 2010), and a jasmonate ZIM-domain (JAZ) protein (Chini et al., 2007; Thines et al., 2007). Upon binding to the SCF^{COI1}-complex, the JAZ proteins are poly-ubiquitinated and degraded via the 26S proteasome pathway, thereby releasing transcription factors, such as MYC2 (Lorenzo et al., 2004; Fernández-Calvo et al., 2011), that then activate downstream transcriptional cascades to modulate various physiological processes. Besides being a positive regulator of JA responses, MYC2 also is a positive regulator of ABA signalling, illustrating, how complex the interaction of these two hormones is (Kazan and Manners, 2013).

In rice, jasmonates can mediate many developmental processes and stress responses (Svyatyna and Riemann, 2012; Liu et al., 2015; Dhakarey et al., 2017; Trang Nguyen et al., 2019). Our previous investigation showed that in OPDA- and JA-deficient rice *allene oxide cyclase (aoc)* mutants, salt stress tolerance was increased (Hazman et al., 2015). A similar phenotype was found in plants overexpressing a jasmonate inactivating enzyme (Kurotani et al., 2015). Similarly, the AOC mutant *coleoptile photomorphogenesis 2 (cpm2)* showed more tolerance for drought stress (Dhakarey et al., 2017). The lack of jasmonate in these mutants is accompanied by differential accumulation of reactive oxygen species (ROS; Hazman et al., 2015; Dhakarey et al., 2017), and altered cell wall architecture (Dhakarey et al., 2017). Is this indicative of a better stress tolerance or of a higher damage in jasmonate deficient mutants?

In these studies (Hazman et al., 2015; Dhakarey et al., 2017) osmotic stress was always acting in combination with other stress factors: For instance, salt stress is composed of ionic, and osmotic stress, and single stress factors may, in combination, act in a non-additive way. In fact, a comparative study of osmotic, salt, and alkalinity stress (Hazman et al., 2016) showed clearly that the combination of individual stress factors is perceived as a new stress quality. Likewise, under natural conditions, drought stress is accompanied by mechanic stress (soil impedance stress). To dissect osmotic stress from these accompanying factors, in this study, we used polyethylene glycol (PEG) 6000 in a hydroponic system to compare the AOC mutant *cpm2* (Riemann et al., 2013) with WT plants. We report that *cpm2* plants, under these conditions, are less tolerant to osmotic stress, suggesting that JA plays a positive role for the acclimation to osmotic stress, if not accompanied by soil impedance stress. We show that, in rice, jasmonates accumulate more rapidly than ABA. We demonstrate further that the ABA accumulation in response to osmotic stress is independent of JA. However, JA positively modulates ABA accumulation in the shoot. We propose a working model, where jasmonates act in parallel to ABA during osmotic acclimation in rice.

2. Materials and methods

2.1. Plant materials, growth, and stress conditions

Oryza sativa L. *ssp. japonica* cv. Nihonmasari and the jasmonate deficient mutant *cpm2* (Riemann et al., 2013) were employed for the current study. This mutant, carrying an 11-bp deletion in the first exon of *OsAOC*, had been generated by γ-ray irradiation and has been backcrossed to Nihonmasari 6 times to eliminate possible side mutations. The rice seeds were dehusked and surface-sterilised by incubating them in 70 % ethanol for 1 min followed by brief washes with double-distilled water. Ethanol incubation and washing were repeated for 3 times. Subsequently, the seeds were incubated in a sodium hypochlorite solution containing ~5 % of active chlorine for 20 min followed by three washing steps in sterilised double-distilled water. The seeds were sown on 0.4 % phytoagar medium (Duchefa, The Netherlands), which contained 0.344 g/L Murashige and Skoog Basal Salt Mixture (MS). The seeds were germinated in a culture room (at 25 °C, continuous white light with 120 μmol $m^{-2}{\cdot}s^{-1}$ of photosynthetically available radiation, Neon tube TLD 36 W/25, Philips, Hamburg, Germany) for 7 d, and then transferred to custom-made sterilised floating racks and moved to a glass container containing double-distilled water and 0.344 g/L MS for 1–2 d acclimation. Subsequently, half of the seedling population was transferred to a 25 % solution of PEG 6000 to induce osmotic stress and the other half was transferred to water as a control. Shoots and roots were harvested separately, frozen in liquid nitrogen, and stored at −80 °C until use for phytohormone or gene expression analyses.

2.2. Measurement of stomatal conductance (Gs)

Plants were cultured as described above, but raised for additional 21 d on 0.344 g/L MS solution to measure leaf stomatal conductance using a leaf porometer (SC-1, Decagon Devices, Pullman, Washington USA).

2.3. Measurement of relative water content (RWC) of shoots

For all treatments, shoots were used for the determination of RWC. First, fresh mass (FM) was determined. The leaves were then placed in distilled water inside a closed 50 mL CELLSTAR® Polypropylene Tube. After 12 h, samples were gently wiped with tissue paper to remove the water from the leaf surface and to determine the turgid mass (TM). In the last step, samples were placed in a drying oven at 70 °C for 72 h followed by determination of dry mass (DM). RWC was calculated according to the equation: (RWC in %) = [(FM - DM)/(TM - DM)] * 100. Five biological replicates were used for each treatment.

2.4. Endogenous levels of ABA, OPDA, JA, and JA-Ile

OPDA, JA, JA-Ile, and ABA were quantified simultaneously from about 50 mg of tissue using a standardised ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)-based method according to Balcke et al. (2012). [$^2H_5$]OPDA, [$^2H_6$]JA, [$^2H_2$] JA-Ile, and [$_2H_6$] ABA (50 ng each) were added as internal standards.

2.5. RNA extraction and quantitative real-time PCR

The InnuPrep plant RNA kit (Analytika Jena RNA kit) was used to isolate total RNA from the shoots of control and plants subjected to osmotic stress, whereas the Spectrum Plant Total RNA kit (Sigma-Aldrich RNA kit) was used to isolate total RNA from roots. In both cases, the RNA was purified according to the protocol of the respective manufacturer, an 1 µg of total RNA supplied with RNase inhibitor (New England Biolabs; Frankfurt/M., Germany) was used for cDNA synthesis using the M-MuLV cDNA Synthesis Kit (New England Biolabs) according to the instructions of the manufacturer.

Real-time PCR analysis was carried out in 20 µL reaction tubes containing 200 nM of each primer, 1X GoTaq colourless buffer, 200 nM of each dNTP, 2.5 mM $MgCl_2$, 0.5 U GoTaq polymerase (Promega, Mannheim, Germany), 1x SYBR green I (Invitrogen, Darmstadt, Germany), and 1 µL of a 1:10 cDNA dilution according to Svyatyna et al. (2014). The primer sequences for the genes of interest and the housekeeping gene OsUbiquitin5 are listed in Supplementary Table S1. The measured C_t values were then used to estimate the steady-state level of the respective transcript using the $2^{-\Delta\Delta}C_t$ method (Livak and Schmittgen, 2001).

2.6. Measurement of malone dialdehyde (MDA)

To estimate the degree of lipid peroxidation as readout for oxidative stress, levels of malone dialdehyde (MDA) in shoots and roots were determined using the thiobarbituric acid (TBA) method as described by Hodgson and Raison (1991) with minor modifications. Briefly, shoots and roots were collected in 2 mL reaction tubes, their fresh masses determined, and then shock-frozen in liquid nitrogen. After addition of a sterilised steel bead (5 mm) to the tube, the frozen tissue was ground twice in the Tissuelyser (Qiagen, Hilden, Germany) at 23 Hz for 45 s. One ml of 0.1 M phosphate buffer (pH 7.4) was then added to the tube followed by centrifugation at 8000 × g for 10 min, and 200 µl of the supernatant were transferred to a mixture containing 750 µl of 20 % (v/v) acetic acid, 750 µl of 0.8 % (w/v) TBA, 200 µl of Milli-Q water, and 100 µl of 8.1 % (w/v) sodium dodecyl sulfate (SDS). As blank, 200 µl of supernatant were replaced by 200 µl volume of 0.1 M phosphate buffer. Subsequently, reaction mixtures were incubated at 98 °C for 1 h. After cooling down to room temperature, the absorbance at 535 nm was measured in a UV–vis Spectrophotometer (Uvikon XS, Goebel Instrumentelle Analytik GmbH, Germany) and corrected against the absorbance at 600 nm monitoring unspecific background. Lipid peroxidation could then be calculated as µM g FW^{-1} MDA from A_{535} to A_{600} using an extinction coefficient of 155 mM^{-1} cm^{-1}. Data represent mean values and standard errors from four independent experimental series.

2.7. Measurement of superoxide

Rice shoots and roots used for superoxide measurements were 10 d old and treated with 25 % PEG 6000 for 24 h. O_2^- in rice shoots and roots was estimated using the nitroblue tetrazolium (NBT) method as described by Vacca et al. (2004) with minor modifications. Samples were collected in 2 mL reaction tubes, frozen in liquid nitrogen and homogenised as described above. After adding 100 mM sodium-phosphate buffer (pH 7.2) containing 1 mM diethyl dithiocarbamate to the reaction tubes to inhibit SOD activity, samples were centrifuged at 12,000× g for 20 min, and the concentrations of superoxide anions (O_2^-) were determined spectrometrically in the supernatant. The A_{530} in the supernatant was measured in the spectrophotometer. The concentration of superoxide was calculated using the formula $c = A_{530}/KL$, with k the extinction coefficient of 12.8 mM^{-1} cm^{-1} (Murphy et al., 1998), L the thickness of cuvette (1 cm), and c the concentration of superoxide.

2.8. Data analysis

The significance of the difference between control and PEG 6000 treatment were analysed by a Student's *t*-test using PASW Statistics 18.0 software (Macintosh, SPSS Inc., Chicago, IL, USA). Some of the data (ABA, OsLEA3, Gs) were analysed by simultaneously comparing the differences between control and treatment, and WT and *cpm2* using one-way analysis of variance (ANOVA). Once a significant difference was detected, Tukey's Honestly Significant Difference (HSD) tests at $p < 0.05$ was used to identify the statistically significant differences. The results shown in the graphs are presented as the mean value ± standard error.

3. Results

3.1. The jasmonate biosynthesis mutant cpm2 is more sensitive to osmotic stress

Rice seedlings (10 d old) were subjected to stringent osmotic stress (incubation with 25 % PEG 6000) for a short time (18 h or 24 h). In response to the treatment, obvious phenotypic differences between WT and JA-deficient *cpm2* mutant plants were observed. WT leaves looked almost entirely healthy and turgescent, showing modest rolling just at the tip of third leaf (Fig. 1A). In contrast, *cpm2* leaves were totally rolled in response to osmotic stress, the second leaves even showed a bit of necrosis. After 24 h, leaves of both genotypes showed rolling, but *cpm2* leaves were affected more severely (Fig. 1B). In addition, the second leaves of *cpm2* showed necrosis at the tip and appeared to have shrunken, probably due to loss of water (Fig. 1B). Moreover, the upper one third of the third leaf in WT was rolled after incubation of the plant for 24 h in PEG 6000, whereas the third leaves in *cpm2* were entirely wilted (Fig. 1B). To quantify the responses, which were observed phenotypically, we measured the relative water content (RWC) of rice shoots in each treatment. In control treatments, both WT and *cpm2* exhibited a RWC of around 95 % (Fig. 1C). When 10 d old plants were exposed to a strong osmotic stress, RWC changed differentially in both the genotypes. After treatment for 24 h, RWC in WT leaves still maintained a high level similar to that of control plants, while in *cpm2*, RWC decreased to 27 % (Fig. 1C).

Besides RWC, we also investigated stomatal conductance (G_s) as G_s is closely related to the water balance in plants, especially directly to transpiration (Caird et al., 2007). In order to assess the status of stomata in WT and *cpm2* when exposed to osmotic stress, we measured G_s in plants of both genotypes after 3 h of the PEG treatment. In controls, the G_s values were almost identical between WT and *cpm2* (Fig. 1D). After exposure to osmotic stress for 3 h, G_s in both, WT and *cpm2*, decreased significantly, however, the decline was more pronounced in WT than in *cpm2* (Fig. 1D). Hence, *cpm2* plants failed to reduce stomatal conductance as efficiently as the WT, which most likely contributes to a faster loss of water. Summarising these phenotypical and physiological observations, the JA biosynthesis mutant *cpm2* can be considered to be more sensitive to osmotic stress than the WT.

3.2. The jasmonate biosynthesis mutant cpm2 accumulates more ROS compared to WT

Plants accumulate reactive oxygen species (ROS) when subjected to osmotic stress (Borsani et al., 2001). Rice seedlings (age 10 d) were treated with 25 % PEG 6000 for 24 h. In shoots of the WT shoots, superoxide levels increased almost threefold in response to osmotic stress. However, this increase was strongly enhanced (to more than tenfold compared to the control) in *cpm2* shoots (Supplementary data Fig. S1A). In roots, $O_2^{\cdot -}$ levels did not show any significant change ($p > 0.05$) neither in WT nor in *cpm2* (Supplementary data Fig. S1B).

Malondialdehyde (MDA) as marker for lipid peroxidation, increased in response to osmotic stress in both, shoots and roots. This increase

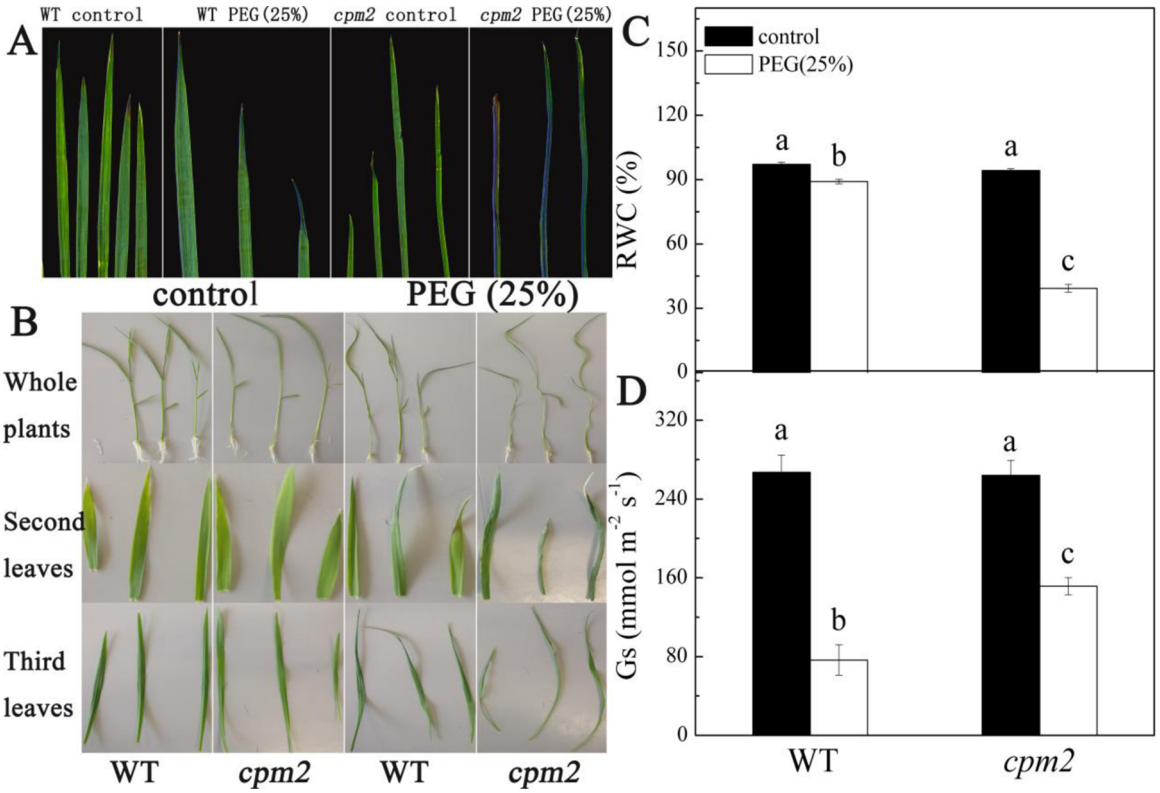

Fig. 1. Effects of osmotic stress performed by application of 25 % PEG6000 on morphology, RWC and stomatal conductance of rice wild type (WT) and a JA biosynthesis mutant (*cpm2*) at day 10 after sowing. (A) Phenotype of representative plants after osmotic stress for 18 h. (B) Phenotype of plant and individual leaves after osmotic stress for 24 h. (C) Relative water content (RWC) subjected to osmotic stress for 24 h. (D) Stomatal conductance (Gs) of plants subjected to osmotic stress for 3 h three weeks after sowing. Values of RWC and Gs are means ± SE of eight plants from 3 experimental series in Fig. 1D and 1C, respectively. Different letters indicate a significant difference, according to Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$).

was more pronounced in the mutant, especially in leaves, less in the shoot (Supplementary data Fig. S1C, D). Hence, PEG 6000 caused oxidative stress, and the mutant was impaired in maintaining oxidative homeostasis as compared to WT.

3.3. Increase in jasmonate levels preceded ABA increase during the osmotic stress response in rice seedlings

In order to obtain insight into the temporal pattern of the accumulation of jasmonates and ABA during the response to osmotic stress, we conducted time-course experiments in both, WT rice shoots and roots. For each time point of the control experiments (roots submerged in H_2O), OPDA, JA, JA-Ile, and ABA contents were very low, demonstrating that the experimental manipulation of plants by itself did not cause any significant modulation of hormonal levels (Fig. 2). In the shoot, JA and JA-Ile had increased already at 30 min after the onset of the PEG treatment and reached a peak at 1 h. After 3 h of treatment, the levels of these two compounds were lower again, but not as low as the control. After 24 h, a second clear increase was observed (Fig. 2C, E). In roots, amounts of JA and JA-Ile had increased significantly already after 30 min of the PEG treatment, and subsequently returned to the levels seen in control plants. JA and JA-Ile levels were even lower than those in the control plants at 6 and 24 h of treatment, respectively (Fig. 2D, F). Interestingly, OPDA content in shoots showed a pronounced increase at later time points (3 h–24 h, Fig. 2A) as compared to the minor increase seen for JA and JA-Ile JA-Ile (Fig. 2A, B, and C) indicating that only a part of the OPDA was channelled to JA synthesis. For the root OPDA levels did not show any response, except a minute transient increase at 30 min of PEG treatment; in addition, the steady-state level of OPDA in roots was generally much lower than in shoots, indicating that OPDA was quickly and efficiently converted to JA and JA-Ile as their levels were extremely high at this time-point. Overall, the steady-state levels of jasmonates increased first in roots, and only later in shoots. Conversely, this transient increase vanished in roots earlier as compared with shoots. With some delay, the time course patterns of ABA content were similar to those seen for the jasmonates. For the shoot ABA increased after 3 h and remained very high at 6 h. 24 h after exposure to PEG it decreased but still remained higher than in control (Fig. 2G). For the root (Fig. 2H), the ABA accumulation was much less pronounced, but still, ABA content in the PEG treated seedlings was slightly, but statistically significant higher as compared to the control (Fig. 2H). It is noteworthy to mention that levels of JA and JA-Ile changed faster than that of ABA in both rice shoots and roots, which would be consistent with a potential role of JA and JA-during the early response to osmotic stress.

3.4. Accumulation of ABA accumulation in response to osmotic stress is more pronounced in the WT as compared to cpm2

In order to examine the role of JA for osmotic-stress induced ABA accumulation, we then compared hormonal levels in WT and *cpm2* after 3 h of osmotic stress, the time-point, where ABA levels had reached saturation. As expected, jasmonate levels were extremely low in roots and shoots of the JA-deficient mutant, and they did not change in response to the stress treatment (Fig. 3 A–F), not even in the shoots, where, in the WT, the levels of OPDA, JA and JA-Ile were higher in stressed plants as compared to the water-treated controls (see Fig. 3A, C, E). However, the levels of ABA increased significantly ($p < 0.01$) in response to osmotic stress in both WT and mutant roots and shoots, respectively (Fig. 3 G and H). While the response quality was maintained in the mutant, the amplitude clearly differed: In the WT, ABA accumulated around twofold (5252 pmol g^{-1}) compared to the mutant

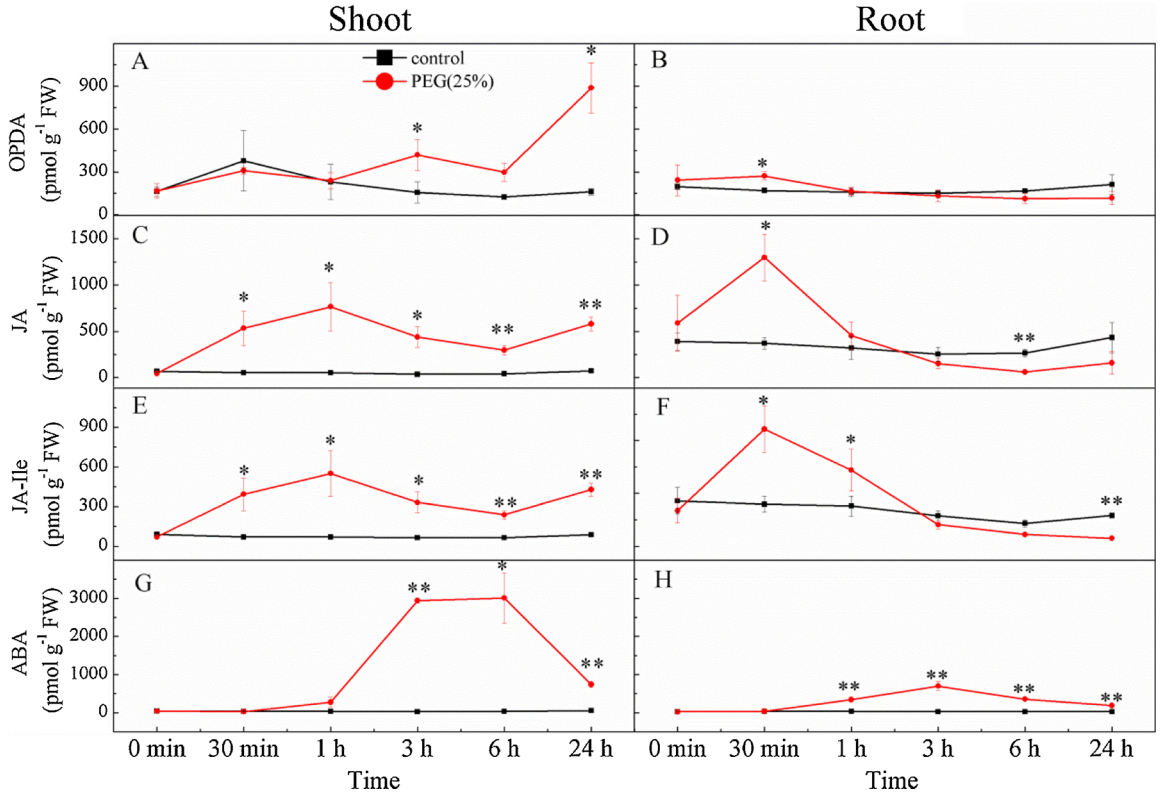

Fig. 2. Time course of phytohormone levels in rice (WT) shoots (A, C, E, G) and roots (B, D, F, H) in response to osmotic stress (25 % PEG 6000). (A, B) 12-*cis*-oxophytodienoic acid (OPDA), (C, D) jasmonic acid (JA), (E, F) JA-isoleucine (JA-Ile), and (G, H) abscisic acid. Control plants were incubated in H_2O for the times indicated. Values represent the mean of three independent experiments ± SE. Means followed by asterisks indicate statistically significant differences between the control and PEG 6000 treatment at each time point (Student's t test; *P < 0.05 and **P < 0.01).

(2197 pmol g^{-1}) (Fig. 3G). In contrast, the response in the root was inversed, here, the levels in the WT were reduced by more than a third (434 pmol g^{-1}) as compared to the WT (669 pmol g^{-1}) (Fig. 3H).

3.5. Response of genes involved in JA synthesis and signaling to osmotic stress is altered in cpm2

Analogous to the levels of plant hormones, we assessed the expression of JA-synthesis genes (*OsAOS1*, *OsAOS2*, *OsAOC*, *OsOPR7*, and *OsJAR1*), JA-response genes (*OsJAZ1*, *OsJAZ4*, and *OsJAZ11*), and a marker gene for ABA responses (*OsLEA3*). Samples used for gene expression measurement were collected at 1 h and 3 h after the onset of the PEG treatment. To compare the results, steady-state transcript levels were compared to those found for the WT without PEG treatment (control) at 1 h as reference. In the WT, both genes encoding for ALLENE OXIDE SYNTHASE, the enzyme acting before AOC, were affected by osmotic stress treatment. *OsAOS1* was found to be upregulated in both rice shoots and roots (~10-fold) in osmotic stress conditions after 1 h (Fig. 4A, B). At 3 h, *OsAOS1* transcripts were still induced in shoots, but the level was a bit lower (~4-fold) (Fig. 4A). In contrast, in roots, *OsAOS1* did not show any difference between the control and PEG6000 treatment (Fig. 4B). For *cpm2* roots, *OsAOS1* was upregulated much less (only a third) in response to osmotic stress as compared to the WT (Fig. 4B). Besides *OsAOS1*, *OsAOS2* also showed a similar result. It was upregulated (~3-fold) in both, WT shoots and roots, 1 h after the PEG6000 treatment, but not at the 3 h time point (Supplementary data Fig. S2).

When exposed to osmotic stress, relative expression of shoot *OsAOC* was upregulated around 5-fold in WT at the two time points we investigated. However, in roots, *OsAOC* was only upregulated at the 1-h time point. Osmotic stress also induced the expression of *OsOPR7* in WT shoots and roots, but was just about 2-fold (Fig. 4E, F). In *cpm2*, expression of *OsAOC* and *OsOPR7* was not sensitive to osmotic stress, and the relative expression of both genes was very low in the mutant (Fig. 4C-F). *OsJAR1* did not respond to osmotic stress, when rice seedlings (both genotypes) were treated with PEG6000 not for any time point (Fig.4 G, H).

The expression of two JA-response genes (*OsJAZ4* and *OsJAZ11*) was induced by osmotic stress in WT leaves (Fig. 5A, C), while in roots, *OsJAZ1* was investigated instead of *OsJAZ4* as it was regulated by osmotic stress more clearly (Fig. 5B) being upregulated (~3-fold) at 1 h of PEG6000 treatment, but not at 3 h. At 1 h of treatment, *OsJAZ11* was upregulated in both WT shoots and roots. In addition, when WT was exposed to osmotic stress for 3 h, *OsJAZ11* was also upregulated in shoots and in roots. As expected, in the *cpm2* mutant, all the *OsJAZ* genes (*OsJAZ1*, *OsJAZ4*, and *OsJAZ11*) were expressed at very low levels and did not respond to osmotic *cpm2* (Fig. 5A-D).

In order to investigate, how ABA-dependent signalling is affected by impaired accumulation of jasmonates, we measured the transcript accumulation of the *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) gene *OsLEA3*. LEA genes are induced by stresses through both ABA-dependent and ABA-independent pathways (Kaur et al., 2014; Kobayashi et al., 2004), and *OsLEA3* has been shown to be regulated depending on ABA (Lu et al., 2009; Kim et al., 2009). In rice shoots, the *OsLEA3* transcript was upregulated in both, WT and *cpm2*, at two time points, with the strongest upregulation detected at 3 h (Fig. 6A). For Interestingly, at both time points, WT showed a higher upregulation compared to *cpm2*. In roots, the situation was different to shoots: 1 h after PEG6000 treatment, both WT roots and *cpm2* roots showed a similar upregulation (~30-fold) (Fig. 6B).while at 3 h after onset of the PEG6000 treatment, *cpm2* roots showed a higher (~400-fold) upregulation of *OsLEA3* than in the WT (~300-fold) (Fig. 6B). Thus, the regulation of *OsLEA3* correlated well with the changes in ABA levels, but the amplitude in gene induction was stronger than changes in ABA

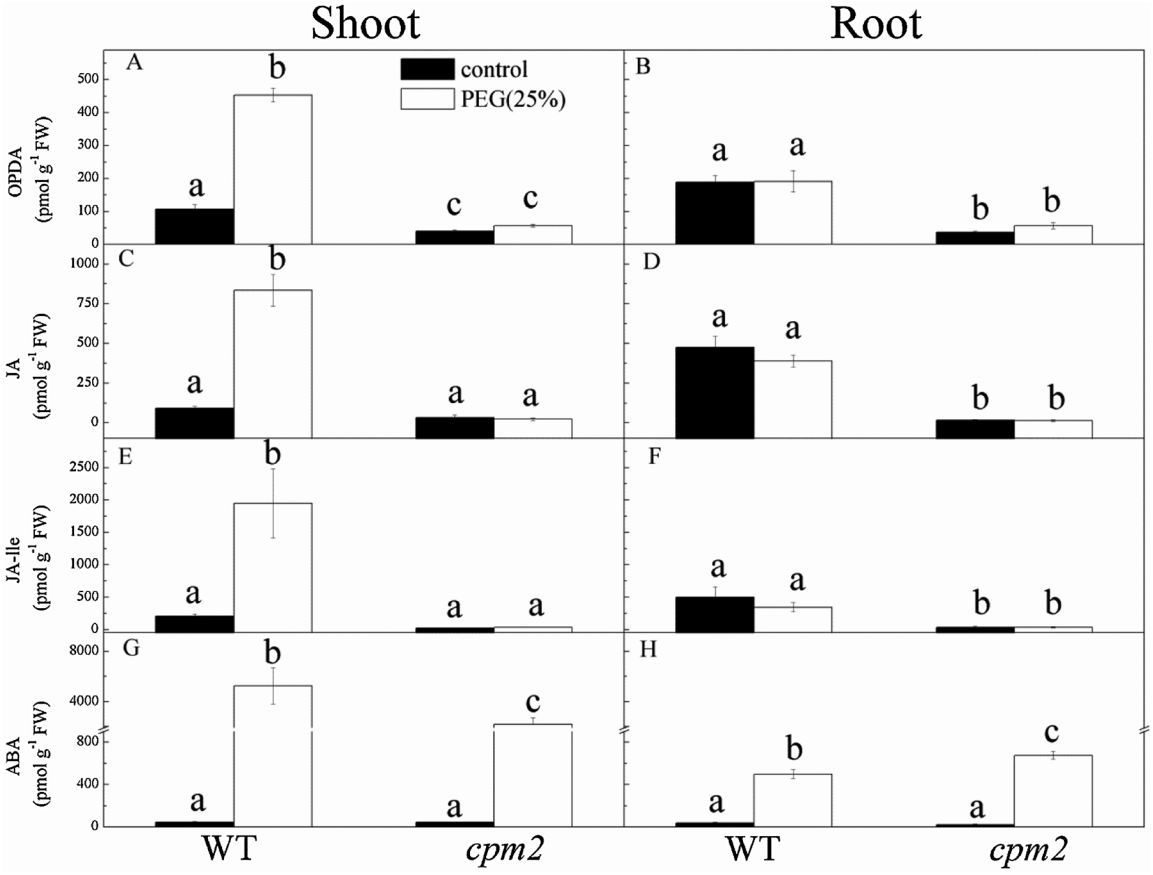

Fig. 3. The level of phytohormones in shoots (A, C, E, G) and roots (B, D, F, H)of wild type (WT) and the JA biosynthesis mutant (*cpm2*) under control conditions (black bars) and after 3 h of osmotic stress (25 % PEG 6000, white bars). (A, B) OPDA, (C, D) JA, (E, F) JA-Ile, and (G, H) ABA. Values represent the mean of at least four independent experiments ± SE. Results for the control and treatment are indicated by black and white bars, respectively. Significant differences for OPDA, JA, JA-Ile and ABA among different treatments or genotypes are indicated by different letters, according to Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$).

levels (Fig. 3H).

4. Discussion

JA plays an important role in the acclimation to abiotic stress, however, the function of JA in diverse stresses is still under debate As outlined in the introduction often stresses such as drought or salinity are composed of several factors. In the present study, we wanted to break down the complexity of drought stress down, and revisited the contribution of osmotic stress as a central component. We, therefore asked, whether JA is required for the response to osmotic stress, and to what extent JA is involved in the regulation of ABA biosynthesis and signalling.

4.1. JA is necessary for efficient osmotic acclimation

JA contributes to the defence in biotic and abiotic stress in most cases, and generally functions as a positive regulator of stress tolerance (Creelman and Mullet, 1995; Walia et al., 2007). In the present work, we employed the AOC mutant *cpm2*, and the corresponding WT *Nihonmasari*, and observed the response of these two genotypes to osmotic stress imposed by a chemical compound in a hydroponic system. In contrast to the situation under drought stress (Dhakarey et al., 2017), we found that *cpm2* seedlings were stronger affected by osmotic stress (Fig. 1A-C). This shows that JA is a positive regulator of osmotic acclimation. Since young leaves were more vulnerable to osmotic stress, this function of JA seems to be more relevant in young, developing leaves, while mature leaves had acquired already a higher innate resistance (Fig. 1), which might be linked with more developed cuticles or other morphological acclimations that constrain transpiration. The lower tolerance of *cpm2* to osmotic stress is not only in contrast to the situation under drought (Dhakarey et al., 2017), but also differs from other complex derivatives of osmotic stress, such as salt stress. For instance, the AOC mutants (*hebiba* and *cpm2*) were found to cope better with salt stress compared to the wild type (Hazman et al., 2015). Along the same line, plants overexpressing a JA-Ile inactivating enzyme also showed more salt stress tolerance than the wild type (Kurotani et al., 2015). The discrepancy with the results obtained from drought stress (Dhakarey et al., 2017) can be resolved: In that study, plants were raised in soil, and a part of the better performance of the jasmonate synthesis mutants could be explained by increased cell wall modification in *cpm2* that may have enhanced the resistance to hard soil, an aspect which is not relevant for osmotic stress in a hydroponic system used in the current study. Also the difference with the pattern observed under salt stress, can be understood as salinity is composed of ionic and osmotic stress. Differences between osmotic stress and salinity are, therefore, not surprising, e.g. differential gene expression in response to both stresses cannot only be observed for genes functional in ion detoxification, but also for central signalling genes such as nitrate reductase (NR) which is involved in the generation of nitric oxide (Hazman et al., 2016). By comparing equivalent stringencies of osmotic stress, salt stress, and alkaline stress, these authors could show that the combination of individual stress components is perceived as a new stress quality and not as a mere addition of the responses to the individual components. The current finding that the behaviour of the mutants differs between hydroponically administered osmotic stress, and drought stress as complex syndrome provides further evidence for the holistic processing of stresses, hence when transferring conclusions

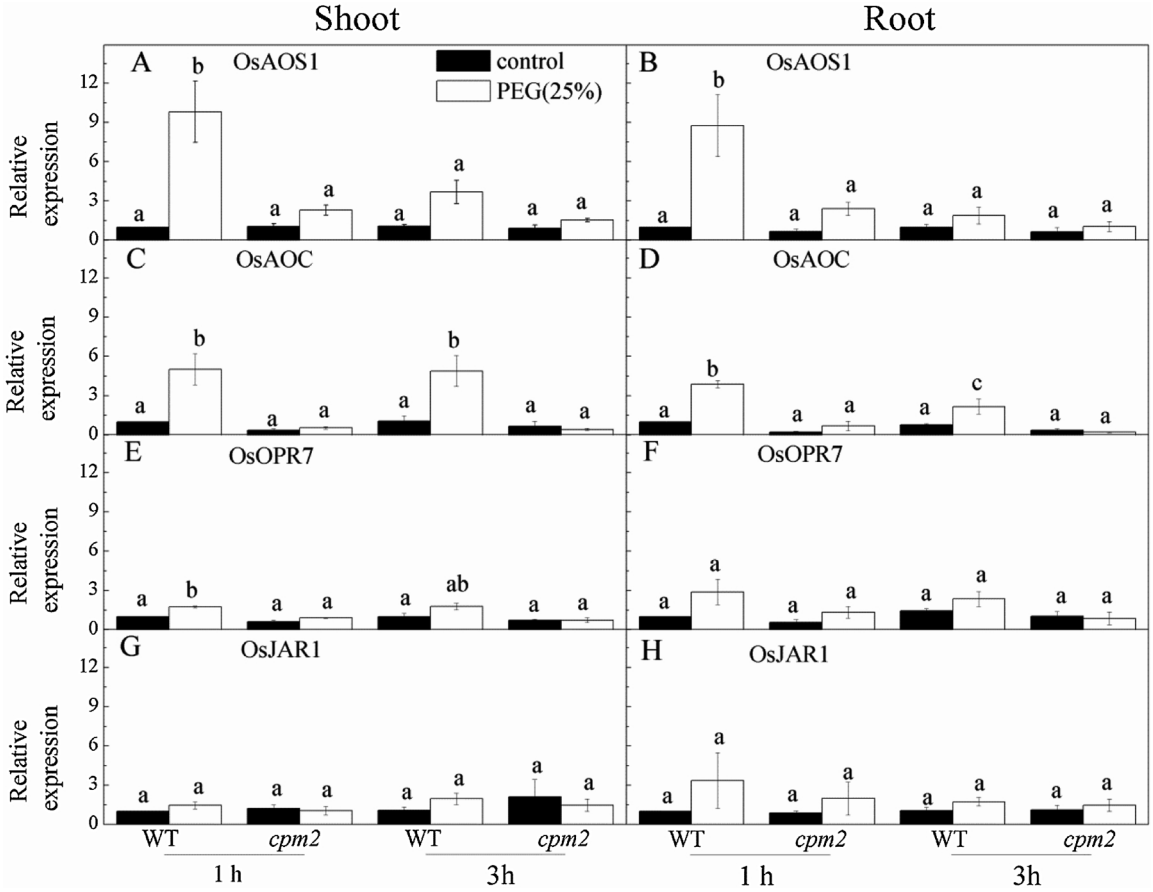

Fig. 4. Transcriptional regulation of selected JA biosynthesis genes in response to osmotic stress (25 % PEG 6000) in shoots and roots of wild type (WT) and *cpm2* seedlings.

(A, B) *OsAOS1*, (C, D) *OsAOC*, (E, F) *OsOPR7*, and (G, H) *OsJAR1*.

10-day-old seedlings were exposed to water (control, black bars) or osmotic stress (25 % PEG 6000) for 1 h and 3 h. The transcript levels were quantified relative to control plants after normalization with *OsUBQ5* as housekeeping genes. Values represent the mean ± SE of three independent experiments with three technical replicas per experiment. Significant differences among different treatments or genotypes are indicated by different letters, according to Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$).

from one experimental system to the other, the details of stress treatment really matter.

4.2. JA improves oxidative balance under osmotic stress

When suffering from abiotic and biotic stress, one of the most common responses in plants is the accumulation of reactive oxygen species (ROS). These by-products of aerobic metabolism are toxic to plant cells (Yamamoto et al., 2002), but, on the other hand, ROS also induce some stress defence genes and are acknowledged as important signals in stress acclimation (Mittler et al., 2011). One of the events linked with damage by ROS is the peroxidation of membrane lipids, triggering a chain reaction accumulating malondialdehyde (MDA), which can be measured as readout for oxidative damage (De Maria et al., 1996). In the present study, both, MDA, and $O_2^{\cdot -}$ accumulated more in *cpm2* shoots (Supplementary data Fig. S1), while, in the roots, there were no significant differences between wild type and mutant (Supplementary data Fig. S1). This might indicate that the mitigating role of jasmonate in the shoot is related to photosynthesis. However, a deeper kinetic study on the devleopment of ROS in the different tissues would be required in order to support such a scenario. How JA improves the buffering of these ROS, remains to be elucidated as well – one possibility would be the accumulation of non-enzymatic antioxidants, alternatively, the activity of superoxide dismutase might be stimulated. A third potential mechanism would be binding of the jasmonic-acid precursor OPDA to cyclophilin 20 – 3 (CYP20 – 3), which will activate a cysteine synthase complex resulting in accumulation of thiol compounds that will then buffer reactive oxygen species (Park et al., 2013). In the mutant, no OPDA can be formed, such that this CYP20 – 3 dependent redox regulation would remain inactive. No matter, which of these three mechanisms is relevant here, the reduced stomatal conductance in the mutant would accentuate oxidative stress further, because in *cpm2* CO_2 uptake is decreased, leading to an accumulation of reduction equivalents (NADPH), which will feed back to electron transport, such that the plastoquinone pool becomes saturated and the electrons from water splitting will be accepted by oxygen, leading to further superoxide accumulation.

4.3. Differential increase of jasmonates in roots and in shoots

When WT seedlings were treated with 25 % PEG, jasmonates were strongly induced and accumulated very quickly in both, the shoots and the roots (Fig. 2), whereby the jasmonate accumulation in roots preceded that in shoots, and was transient, whereas it accumulated over time in shoots. This indicates that there must be a basic difference of JA signalling in the two organs. It is noteworthy that levels of JA and JA-Ile

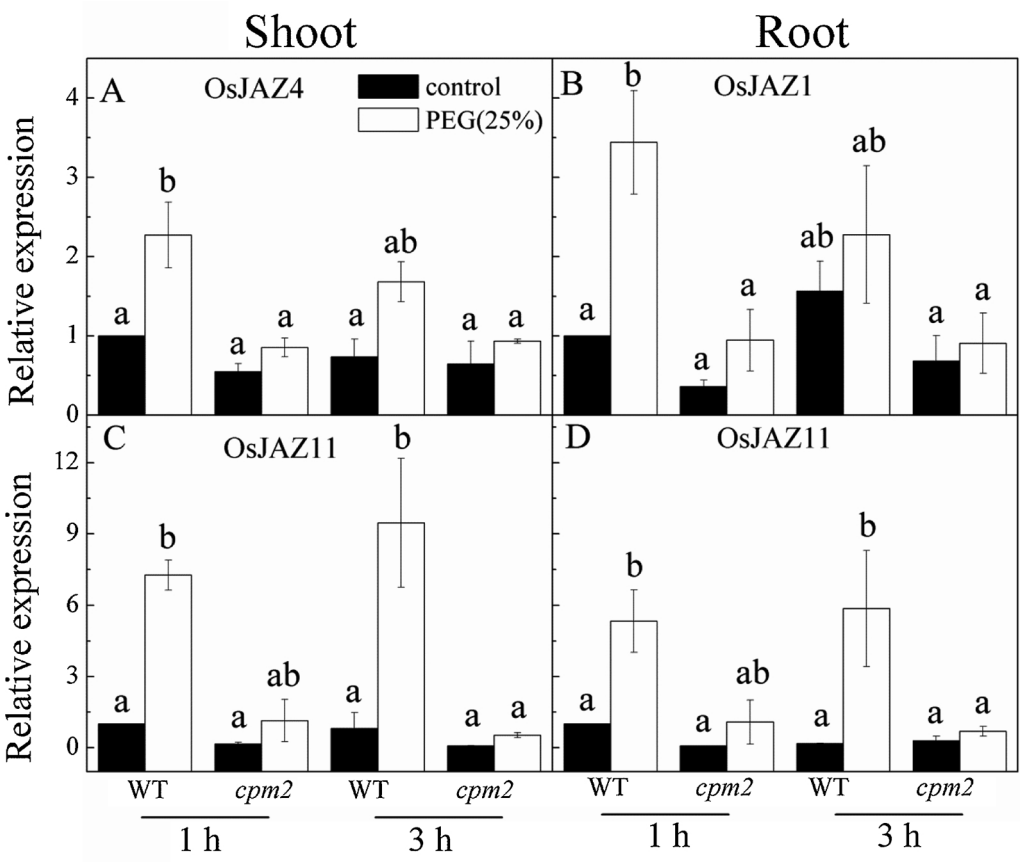

Fig. 5. Transcriptional regulation of selected JA signaling genes in response to osmotic stress (25 % PEG 6000) in shoots and roots of wild type (WT) and *cpm2* seedlings.
(A) Shoot *OsJAZ4*, (B) root *OsJAZ1*, (C) shoot *OsJAZ11*, (D) root *OsJAZ11*.
10-day-old seedlings were exposed to water (control, black bars) and osmotic stress (25 % PEG 6000) for 1 h and 3 h. The transcript levels were quantified relative to control plants after normalization with housekeeping gene *OsUBQ5*. Values represent the mean of three independent experiments ± SE. Significant differences among different treatments at both genotypes and time points (1 h and 3 h) are indicated by different letters, according to Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$).

were not as different as in many other cases in which JA-Ile levels were 10 − 100-fold lower as compared to JA levels. This is quite similar to findings in a different study (Hazman et al., 2019) in which salt stress was applied to rice seedlings of similar stage. Therefore such efficient coupling of isoleucine to JA might be characteristic for this type of abiotic stress response in rice.

The difference in timing and amplitude of jasmonate accumulation between root and shoot is consistent with a model, where the root responds directly to the osmotic challenge, while the shoot responds indirectly. For instance, the lack of adaptive responses in the root should, upon continuing exposure to stress, impair the redox homeostasis in the shoot. Thus, the second rise of jasmonates might be indicative of damage. Especially, the strikingly high levels of OPDA after 24 h of stress in rice leaves are indicative of a damage response (Fig. 2A), consistent with previous results obtained for salinity stress (Hazman et al., 2015, 2019). We propose that the damage that occurred in leaves might be due to dysregulation of photosynthesis which warrants future studies on this aspect. However, the first rise of JA and JA-Ile levels in the shoot, peaking at 3 h after the onset of stress (Fig. 2C, E), are not accompanied by an increase of OPDA (Fig. 2A). The low steady-state levels of OPDA are indicative of efficient conversion of OPDA into JA and JA/Ile, and demand a different explanation compared to the second jasmonate peak at 24 h.

For instance, transcripts for jasmonate synthesis, i.e. *AOS1*, less pronounced also *AOC*, and *OPR7* (Fig. 5A,C, E) increase rapidly in the wild type, but not in the mutant, which tells that jasmonates are required for gene activation. This positive feedback of jasmonate upon its own signalling is consistent with classical findings in *Arabidopsis* (Laudert and Weiler, 1998). Also the four tested *JAZ* transcripts are rapidly upregulated in shoots and roots alike (Fig. 6). These identical time courses of jasmonate-dependent gene expression contrast with the delayed accumulation of JA and JA-Ile (Fig. 3) in the shoot as compared to the root. When the gene expression in the shoot can keep pace with that in the root, although the presumed inducer, JA-Ile accumulates later, there must be a second signal that accelerates gene expression in the shoot. This second signal might be a consequence of oxidative imbalance in the cell itself. However, the difference in the stress response of stomatal conductance at 3 h between WT and mutant (Fig. 1D) is relatively mild and cannot account for the complete absence of transcript induction in the mutant. Thus, such a cell-autonomy model, where the transcripts respond exclusively to the local situation in the photosynthetic cell, fail to explain the observed patterns, necessitating an alternative model:

The root accumulates JA and JA-Ile more rapidly as compared to the shoot, indicative of a direct response. If this jasmonate accumulation would release a systemic signal to the shoot, the accelerated induction of JA-pathway genes on the background of a much slower accumulation of jasmonates themselves, could be explained. In fact, root-born signals to the shoot that convey information on osmotic stress before the shoot runs into damage in consequence of disbalanced photosynthetic electron transport has been reported for rice (Urano et al., 2014). The nature of this signal is not known, but has been shown to activate the α-unit of the trimeric G-protein. The possibility that this signal is JA itself, or its transport form MeJA, is not very likely, because one should then see a faster accumulation of JA in the shoot.

Taken together, the observed pattern of hormonal content and expression of hormone-related genes are consistent with a scenario, where roots use jasmonate signals for immediate adaption, while stress acclimation in the shoots is indirect. Here, the dependence on jasmonates seems to stem from a (systemic?) signal, whose release depends on jasmonate, but is not identical to jasmonate. During continuous stress, this early and adaptive jasmonate response is followed by a hyper-

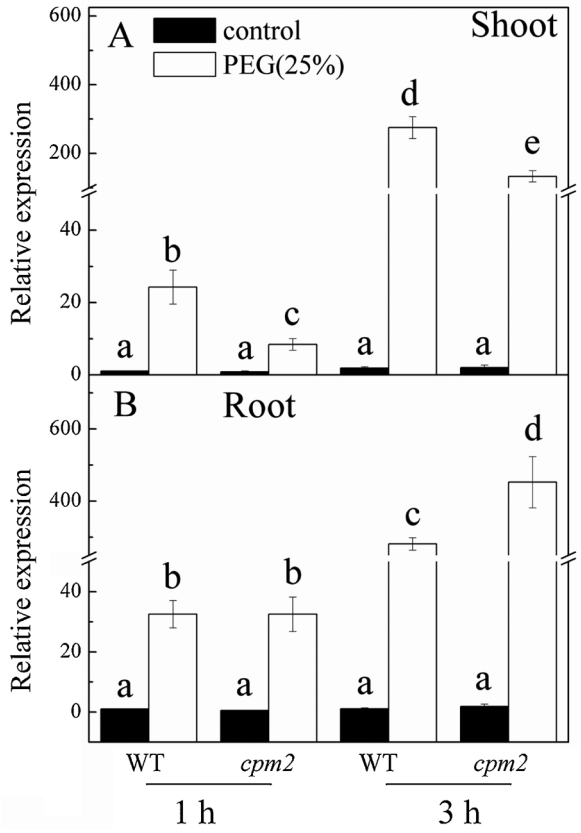

Fig. 6. Transcriptional regulation of *OsLEA3* in response to osmotic stress (25 % PEG 6000) in shoots (A) and roots (B) of wild type (WT) and *cpm2* seedlings. 10-day-old seedlings were exposed to water (control, black bars) and osmotic stress (25 % PEG 6000) for 1 h and 3 h. The transcript levels were quantified relative to control plants after normalization with housekeeping gene *OsUBQ5*. Values represent the mean of three independent experiments ± SE. Significant differences among different treatments or genotypes are indicated by asterisks, according to Tukey's Honestly Significant Difference (HSD) test (*P < 0.05 and **P < 0.01).

accumulation of jasmonates with a different chemical signatures (elevated OPDA levels) indicative of a damage due to sustained severe stress conditions. It will be interesting to challenge this modell by examining additional mutants in the JA biosynthesis and signalling pathways such as *aos1*, *aos2*, *opr7* and *coi1* in future.

4.4. ABA and JA pathways respond to osmotic stress in parallel

ABA is the central signal which controls stomatal aperture and transcriptional activity in response to osmotic stress in plants (Fujita et al., 2011). Similar to jasmonates, the accumulation of ABA was transient, and preceded that in the shoot, which initiated later, but was more persistent. With the same argument as for jasmonates, a signal generated in the roots that were submerged in the PEG solution and therefore sensed the osmotic pressure first, can be inferred. It is tempting, to link these conclusions with the so called root-brain hypothesis (Baluška et al., 2004), suggesting that the root cap may act as a donor for signals that are than conveyed through the vascular tissues for rapid information exchange between belowground (roots) and aboveground (shoots and leaves) organs. The nature of these signals remains to be elucidated. In addition to phytohormones, hydrogen peroxide, but also physical signals, such as hydraulic conductance have been discussed (Urano et al., 2014).

In our study we did not observe a strong dependence of ABA biosynthesis on JA, although in both organs, the peak of ABA content was observed around 2 h later than that of JA/JA-Ile (Fig. 2). Higher levels of ABA were found in shoots of *cpm2* mutants, but less ABA was detected in mutant roots (Fig. 3G, H), supported by the expression patterns of the ABA marker gene *OsLEA3* (Fig. 6). Thus both, the hormone and transcript levels, showed that JA interacted synergistically with ABA in rice shoots, and ABA biosynthesis did not depend on JA (Fig. 7). Previous studies found that transient accumulation of JA was needed for the ABA accumulation in *Arabidopsis* (Ollas et al., 2015) and *Citrus* (Ollas et al., 2013) roots when exposed to drought stress. However, in rice, both hormone biosynthesis pathways seem to be induced by osmotic stress more or less independently, as jasmonates were induced earlier than ABA, and ABA was induced in the absence of JA. We propose, in the system we studied, the JA pathway influences ABA signalling rather than biosynthesis. This is consistent with other studies, where JAZ proteins had been shown to interact with the central ABA signalling component ABI5 (Ju et al., 2019) and to supress ABA signalling (Fu et al., 2017), This possibility will be explored in future experiments by performing gene expression studies in the mutant in time-course experiments. However, it requires more extensive pre-experiments on ABA-responsive genes to obtain a broader image as they are not characterized as well as in *Arabidopsis thaliana.* In contrast, it has been shown recently that ABA responsive plastid lipases are involved in JA biosynthesis (Wang et al., 2018). This feedback mechanism, which might be used during stress acclimation, is worth to be studied in rice as well.

Contributions authors

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J. Ma – acquisition of data, interpretation of data, final approval

B. Hause – measurement of plant hormones, critical revision, final approval

P. Nick – obtaining of funding, conception and design of study, critical revision, final approval

M. Riemann – conception and design of study, analysis and interpretation of data, critical revision, final approval

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G. Tang– conception and design of study, acquisition of data, statistical expertise, analysis and interpretation of data, drafting the article and final approval of the submitted version.

J. Ma – acquisition of data, interpretation of data, final approval

B. Hause – measurement of plant hormones, critical revision, final approval

P. Nick – obtaining of funding, conception and design of study, critical revision, final approval

M. Riemann – conception and design of study, analysis and interpretation of data, critical revision, final approval

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

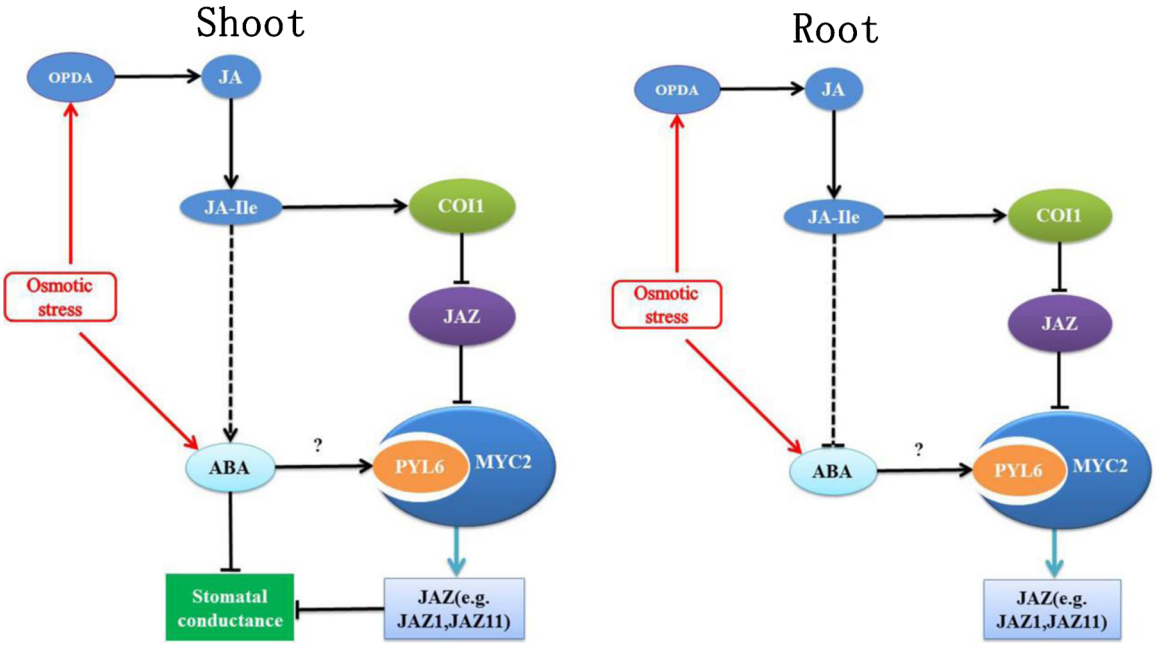

Fig. 7. Proposed working model representing some possible mechanisms for how jasmonates contribute to osmotic stress adaptation. The observed phenotype of increased osmotic stress tolerance in the wild type could be linked to the following possible mechanisms: When suffering from osmotic stress, first rice roots received the stress, later stress arrived in rice shoots. JA and ABA levels increase in response to osmotic stress in roots and shoots, respectively. However, our data indicate that JA promotes ABA accumulation in shoots, while it represses ABA accumulation in roots. In shoots JA and ABA induce stomatal closure, which reduces loss of water. ABA might enhance JA-related signaling through the receptor PYL6 which has been shown to interact with MYC2 physically (Aleman et al., 2016). It is noteworthy that the response in shoots was strong and sustainable, while it was weak and transient in roots. Possibly the smaller amplitude of the response in roots indicates that roots, although directly exposed to the stress, are more robust in order to induce countermeasures of the plant.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2020.104047.

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