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Original Article

Light induces jasmonate-isoleucine conjugation via OsJAR1-dependent and -independent pathways in rice

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ABSTRACT

The bioactive form of jasmonate is the conjugate of the amino acid isoleucine (Ile) with jasmonic acid (JA), which is biosynthesized in a reaction catalysed by the GH3 enzyme JASMONATE RESISTANT 1 (JAR1). We examined the biochemical properties of OsJAR1 and its involvement in photomorphogenesis of rice (*Oryza sativa*). OsJAR1 has a similar substrate specificities as its orthologue in *Arabidopsis*. However, *osjar1* loss-of-function mutants did not show as severe coleoptile phenotypes as the JA-deficient mutants *coleoptile photomorphogenesis 2* (*cpm2*) and *hebiba*, which develop long coleoptiles in all light qualities we examined. Analysis of hormonal contents in the young seedling stage revealed that *osjar1* mutants are still able to synthesize JA-Ile conjugate in response to blue light, suggesting that a redundantly active enzyme can conjugate JA and Ile in rice seedlings. A good candidate for this enzyme is OsJAR2, which was found to be able to catalyse the conjugation of JA with Ile as well as with some additional amino acids. In contrast, if plants in the vegetative stage were mechanically wounded, the content of JA-Ile was severely reduced in *osjar1*, demonstrating that OsJAR1 is the most important JA-Ile conjugating enzyme in the wounding response during the vegetative stage.

Key-words: GH3; JAR1; jasmonate; mechanical wounding; *Oryza sativa* L.; photomorphogenesis.

INTRODUCTION

The original *GRETCHEN HAGEN 3 (GH3)* gene has been discovered in screens for auxin responsive genes. Auxin is causing a rapid induction of several gene families, the so-called early auxin-responsive genes. Among those, *GH3*, *AUX/IAA* and *SAUR* (*SMALL AUXIN UP-REGULATED RNA*) are induced within 5 min after application of exogenous auxin (Guilfoyle 1999). *GH3* was isolated from soybean hypocotyls (Hagen, Kleinschmidt & Guilfoyle 1984), but later it became clear that *GH3* homologs exist in other plants as well (Roux & Perrot-Rechenmann 1997; Roux *et al*. 1998). In the meantime, it is well known that GH3 enzymes are encoded in gene families in each plant species. In rice, 13 *GH3* genes have been identified from the genome sequence (Jain *et al*. 2006; Terol, Domingo & Talon 2006), while the *GH3* family in *Arabidopsis* consists of 19 members and one additional member with a truncated sequence (Hagen & Guilfoyle 2002). GH3 enzymes belong to the acyl adenylate-forming firefly luciferase superfamily whose members can adenylate carboxyl groups in a variety of substrates for further biochemical modifications (Staswick, Tiryaki & Rowe 2002). *Arabidopsis* GH3 enzymes can be divided into three subgroups: (1) enzymes using jasmonate as a substrate; (2) enzymes using auxin or salicylic acid as a substrate; and (3) enzymes using benzoates as substrate (Staswick *et al*. 2002). Among the two members of the jasmonate-conjugating group I in *Arabidopsis*, JASMONATE RESISTANT 1 (JAR1) and AtGH3.10, a biochemical activity on jasmonate could be demonstrated for JAR1; however, for AtGH3.10, no substrate could be identified so far. The group I in rice has three members in total, namely OsGH3.5 (OsJAR1), OsGH3.3 (OsJAR2) and OsGH3.12 (Jain *et al*. 2006; Terol *et al*. 2006). It has been demonstrated that GH3 enzymes in plants conjugate amino acids to plant hormones and either activate (jasmonate, salicylic acid) or inactivate (auxin) them in this way (Staswick & Tiryaki 2004; Staswick *et al*. 2005).

JAR1 is a GH3 family member conjugating isoleucine (Ile) to jasmonic acid (JA) in *Arabidopsis* (Staswick & Tiryaki 2004). By this reaction, the prohormone jasmonate is converted into an active form that can bind effectively to its receptor CORONATINE INSENSITIVE 1 (COI1; Yan *et al*. 2009), which on the other hand interacts with JAZ proteins. In the absence of JA-Ile, JAZ proteins also interact with MYC2, a transcription factor, which positively regulates JA-responsive genes, and repress its function (Chini *et al*. 2007; Thines *et al*. 2007). A specific stereoisomer of JA-Ile, (+)-7-*iso*-JA-Ile, is required (Fonseca *et al*. 2009) to establish a interaction of JAZ proteins with the SCF^{COI1}-complex to induce their ubiquitination and subsequent degradation through the 26S proteasome (Chini *et al*. 2007; Thines *et al*. 2007). As a consequence of JAZ protein degradation, MYC2 will be relieved from the complex and the transcription of JA-responsive genes is activated (for a review, see Wasternack & Kombrink 2010). In addition, the adaptor

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protein NOVEL INTERACTOR OF JAZ (NINJA) is mediating an indirect interaction of JAZ proteins with the transcriptional repressor TOPLESS (TPL; Pauwels *et al.* 2010), which co-represses JA-dependent gene expression. The interaction of COI1 with JAZ proteins is further increased by the presence of inositol-pentakisphosphate (Sheard *et al.* 2010).

One allele of *Arabidopsis jar1*, the *far-red insensitive 219* (*fin219*) mutant, has been isolated in a screen for mutants with reduced sensitivity to far-red (FR) light (Hsieh *et al.* 2000). Initially, it was not clear whether the phenotype of *fin219* was due to a non-functional JAR1 gene because other *jar1* alleles did not show a phenotype in FR light (Staswick *et al.* 2002). However, Chen *et al.* (2007) demonstrated that other *jar1* mutant lines also show a reduced sensitivity to FR light, although not as pronounced as the *fin219* mutant. A recent report by Robson *et al.* (2010) further established a role for JA in photomorphogenesis of *Arabidopsis*. They proved that mutants deficient in JA biosynthesis and signalling are impaired in a number of high-irradiance FR light responses and that FR light-dependent gene expression depends partially on COI1. Additionally, the ectopic expression of *JAZ1* caused constitutive shade avoidance response, which is a phytochrome (phy)-mediated physiological output. As a conclusion, they suggested that JAZ1 proteins are an integration point for JA and phy signalling and that both pathways are required to respond to stress and light. This notion was further substantiated by the observation that plant defence against herbivores is regulated in a phy-dependent manner on the level of JA sensitivity (Moreno *et al.* 2009) and biosynthesis of JA-Ile (Radhika *et al.* 2010).

Rice is an excellent system to study the light dependency of JA responses (Svyatyna & Riemann 2012). The biosynthesis of JA and JA-dependent gene expression is rapidly induced after perception of red light in young seedlings (Riemann *et al.* 2003; Haga & Iino 2004), and seedlings do not perform normal photomorphogenesis if the biosynthesis of JA is disrupted (Riemann *et al.* 2003, 2013). Therefore, JA is a crucial signal for photomorphogenesis of rice.

The present study was motivated by the above-described fact that *osjar1* mutants only show a phenotype in specific light conditions speaking in favour of redundantly active GH3 enzymes (Riemann, Riemann & Takano 2008). Although it has been demonstrated that OsJAR2 can conjugate JA to Ile, so far no physiological function of OsJAR2 was proposed. Therefore, we compared the enzymatic activities of OsJAR1 and OsJAR2 by using recombinant glutathione S-transferase (GST) fusion proteins and demonstrated that they have overlapping substrate specificities. Using *Tos17* insertion lines of *OsJAR1*, we describe physiological functions of this gene in rice photomorphogenesis and in wounding responses.

MATERIALS AND METHODS

Plant material

The seeds used in this study were propagated in the greenhouse of the Botanical Garden of the Karlsruhe Institute of Technology (Karlsruhe, Germany). The mutant lines *osjar1-1* and *osjar1-2* originate from the *Tos17* mutant panel (Miyao *et al.* 2003) and are described by Riemann *et al.* (2008) in more detail. As a wild-type (WT) cultivar, we used *Oryza sativa* L. ssp. *japonica* cv. Nipponbare, the genetic background of *Tos17* lines. The phenotypes we described for *osjar1-1* and *osjar1-2* are conserved in other independent mutant lines we obtained from two resources, the *Tos17* collection (Miyao *et al.* 2003) and the Oryza Tag Line (OTL) collection (Larmande *et al.* 2008). The recessive mutation causes a similar long coleoptile and open husk phenotype in homozygous mutants of different alleles. Supporting Information Table S1 shows the segregation pattern of different alleles obtained in populations of varying sizes.

The mutants *hebiba* and *coleoptile photomorphogenesis 2* (*cpm2*) are described in Riemann *et al.* (2003) and Riemann *et al.* (2013). As a WT control for them, the japonica cultivar Nihonmasari was used.

Plant cultivation

Prior to sowing, seeds were surface sterilized. Firstly, they were shaken in 70% of ethanol for 1 min. Subsequently, they were washed two times with H_2O for 1 min. The washed seeds were then shaken in a hypochlorite solution (Carl Roth, Karlsruhe, Germany) containing 5% of active chlorine for 20 min, and washed with H_2O four times. Twenty seeds per Magenta box (Sigma-Aldrich, St. Louis, MO, USA) were sown on 0.6% of Phytoagar (Duchefa, Haarlem, Netherlands). Seedlings were raised at 25 °C for the times indicated in each experiment. Light irradiation of seedlings was conducted with custom-made LED arrays described in Qiao, Petrasek & Nick (2010) with a light intensity of 10 $\mu mol^{-2}\ s^{-1}$. For wounding experiments, plants were raised as described above for the first 7 d, transferred to soil, and subsequently grown in a phytochamber under short-day conditions (10 h light, 28 °C/14 h darkness, 22 °C). MecWorm treatment was conducted on the uppermost fully expanded leaves of a 6-week-old plant according to Mithöfer, Wanner & Boland (2005) with the following parameters: 30 min wounding, leaves were pierced either every 4 s with an area of 7.5 cm^2, or every 2 s with an area of 22 cm^2, 1 h wounding, every 5 s with an area of 29.5 cm^2; 6 h wounding, every 5 s with an area of 112.5 cm^2. In order to achieve a total time of 6 h, the same region was treated twice.

RNA extraction and gene cloning

Plant material was harvested into liquid nitrogen immediately. The tissue was ground to powder with a TissueLyser (Qiagen, Hilden, Germany), and total RNA was extracted with innuPREP Plant RNA kit (Analytik Jena, Jena, Germany) according to the manufacturer's description. To remove contamination with genomic DNA, the samples were treated with RNase-free DNase (Qiagen).

Using total RNA as a template, cDNA was synthesized with the Superscript III Reverse Transcriptase (Invitrogen, Darmstadt, Germany). We performed the reaction according to standard protocol suggested by the supplier using oligo-dT primers instead of random hexamer primers. This cDNA was

also used to amplify the OsJAR1 protein coding transcripts from WT and mutants and the full-length transcript of OsJAR2.

To test the expression of transcript, the cDNA was diluted 1:50 and 5 μL was applied as template in a PCR reaction. The PCR reaction was performed with Taq Polymerase from New England Biolabs (NEB, Frankfurt, Germany) and ThermoPol buffer (NEB). Sequences of primers are listed in Supporting Information Table S2.

To clone the full-length OsJAR1, cDNA was used as a template and amplified with Phusion Polymerase (Finnzymes, Espoo, Finland). The primers JAR1pGEX.F and JAR1pGEX.R (Supporting Information Table S2) introduce EcoRI and SalI restriction sites at the 5′- and 3′-ends of the cDNA, respectively. Via these restriction sites, the cDNA was transferred to the expression plasmid pGEX-6P-1 (GE Healthcare Europe, München, Germany).

The truncated OsJAR1 transcript from the TOS17 mutants was amplified using the same primer combination as for the WT protein coding sequence in case of *osjar1-2* mutants. For amplification of the truncated *osjar1-1* transcript, a TOS17 specific-primer TOS17.EF (Supporting Information Table S2) was used as reverse primer. Both fragments were cloned to the pGEX-6P1 vector via EcoRI and SalI for OsJAR1-2, and EcoRI and XhoI for OsJAR1-1. The XhoI restriction site is present in the amplified TOS17 sequence and located after the predicted stop codon.

For the amplification of OsJAR2 full-length coding sequence, the primers JAR2.ex.F and JAR2.ex.R (Supporting Information Table S2) were used introducing the restriction sites EcoRI and NotI for following insertion into the pGEX-6P1 vector.

Protein expression and purification

The expression plasmid was transformed into the *Escherichia coli* expression strain BL21. A selected clone was used for a liquid preculture in LB medium (Carl Roth) containing 100 μg mL^{-1} ampicillin and incubated on a rotary shaker at 150 r.p.m. overnight at 37 °C. The next day, a larger volume of LB medium containing 100 μg mL^{-1} ampicillin was inoculated with the preculture in a ratio 1:100 and grown at 37 °C while shaking at 150 r.p.m. until an OD_{600} of 1.0 for OsJAR1, OsJAR1-1, and OsJAR1-2, and OD_{600} of 0.5 for OsJAR2 was reached. The cells were chilled on ice and after 5 min the expression of protein was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Subsequently, the cells were incubated at 18 °C while shaking at a speed of 150 r.p.m.

After 24 h, the expression of protein was stopped by incubating the cells 10 min on ice. The cells were collected by centrifugation for 10 min at 5000 g and 4 °C, and washed with 50 mM Tris-HCl (pH 7.8), containing 300 mM NaCl and 100 mg L^{-1} cholic acid. The volume of washing buffer was half the volume of the liquid culture. Subsequently, the cell pellet was incubated in lysis buffer [50 mM Tris-HCl, pH 7.8; 300 mM NaCl, 100 mg L^{-1} cholic acid, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM dithiothreitol (DTT)] for 1 h on ice. Cells were disrupted using a French Press cell and the suspension centrifuged for 40 min at 20 000 *g* and 4 °C. The supernatant was mixed with three volumes of a saturated ammonium sulphate solution (3.3 M $(NH_4)_2SO_4$, 50 mM Tris, pH 7.8) and incubated at 4 °C overnight. By centrifugation (20 000 *g*) for 20 min at 4 °C, the protein was precipitated. The sediment was dissolved carefully in potassium buffered saline (PBS) and used for purification with Glutathione Sepharose 4B (GE Healthcare) according to the manufacturer's specifications with some modifications. Glutathione Sepharose 4B (1.5 mL) was added to 10 mL of the protein solution and mixed for 2 h by inversion at 4 °C. The suspension was transferred to a closed column. The Sepharose was allowed to settle then the flow-through fraction containing most of the unbound protein was eluted. The column was washed with approximately 10 volumes of PBS. To determine the amount of protein in the washing fraction, the optical density (OD) at 280 nm was determined with a spectrophotometer. If no further protein was washed from the column, the GST fusion protein was eluted with elution buffer. The purity of each fraction was checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and subsequent staining of the gels with Coomassie Brilliant Blue (Carl Roth, Karlsruhe, Germany).

Enzyme activity assay

To check the substrate specificity of each GST fusion protein, the method described by (Staswick & Tiryaki 2004) was used. Each reaction had a total volume of 100 μL containing 2 μg μL^{-1} of purified fusion protein, 3 mM $MgCl_2$, 1 mM (+/−)-JA (Sigma-Aldrich), 1 mM DTT and 1 mM of amino acid in 50 mM Tris-HCl (pH 8.3). All 20 proteinogenic amino acids were tested for their potential to serve as a substrate for the fusion protein. The enzyme reaction was performed at 25 °C for 1 h and stopped by the addition of 10 μL of 0.1 M HCl. The reaction products were extracted with the same volume of ethyl acetate. The extracted reaction product was analysed on TLC plates (DC silica gel 60 F_{254}; Merck, Darmstadt, Germany) developed in 35% chloroform, 55% ethyl acetate and 10% formic acid. The plates were stained with vanillin reagent 6% vanillin and 1% sulphuric acid in ethanol according to Staswick & Tiryaki (2004) subsequently.

Plant hormone analysis

For phytohormone analysis of irradiated seedlings, plant material was frozen in liquid nitrogen immediately after each treatment. After determination of the fresh weight (FW), the tissue was freeze-dried for 48 h. The dried material was ground to fine powder and suspended in hormone extraction buffer (80% acetonitrile, 1% acetic acid, supplemented with internal standards). Plant hormone purification and analysis was conducted as described by Yoshimoto *et al*. (2009). As internal standards to quantify the content of phytohormones in intact plant tissues, ^{13}C-JA-Ile and D_2-JA were used (Tokyo Kasei, Tokyo, Japan).

For phytohormone analysis of MecWorm-treated leaves, the following method was applied. After finishing of single

experiments, plant material was immediately weighed and frozen with liquid nitrogen, and samples were kept at −80 °C until used. For jasmonate analyses, ground plant material was extracted with 1.5 mL of methanol containing 60 ng of 9,10-D_2-9,10-dihydrojasmonic acid and 12 ng of JA-[$^{13}C_6$]Ile conjugate as internal standards. JA-[$^{13}C_6$]Ile conjugate was synthesized as described (Kramell *et al*. 1988) using [$^{13}C_6$]Ile (Sigma-Aldrich). The homogenate was mixed for 30 min on a shaker and centrifuged at 13 000 *g* for 20 min at 4 °C. After the supernatant was collected, the homogenate was re-extracted with 0.5 mL methanol, mixed, and centrifuged, and supernatants were combined. These pooled extracts were dried at 30 °C in a speed vac and re-dissolved in 0.5 mL methanol. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Böblingen, Germany). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 μm; Agilent, Waldbronn, Germany). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was as follows: 0–0.5 min, 5% B; 0.5–9.5 min, 5–42% B; 9.5–9.51 min, 42–100% B; 9.51–12 min, 100% B; 12.1–15 min, 5% B. The mobile phase flow rate was 1.1 mL min^{-1}. The column temperature was maintained at 25 °C, injection volume was 2 μL. An API 5000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source was operated in the negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards, where available. The ion spray voltage was maintained at −4500 eV. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 60 psi, curtain gas at 25 psi, heating gas at 60 psi and collision gas at 7 psi. Multiple reaction monitoring (MRM) was used to monitor the analyte parent ion: *m/z* 209.1 →59.0 [collision energy (CE) −24 V; declustering potential (DP) −35 V] for JA; *m/z* 213.1 →56.0 (CE −24 V; DP −35 V) for 9,10-D_2-9,10-dihydrojasmonic acid; *m/z* 322.2 →130.1 (CE −30 V; DP −50 V) for the JA-Ile conjugate; *m/z* 328.2 →136.1 (CE −30 V; DP −50 V) for the JA-[$^{13}C_6$]Ile conjugate. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionization efficiencies was verified by analysing dilution series of standard mixtures. Jasmonates were quantified relative to the signal of their corresponding internal standard. The peak of the endogenous bioactive form of JA-Ile, (+)-7-*iso*-jasmonoyl-*L*-Ile (Fonseca *et al*. 2009) was used for JA-Ile quantification.

Gene expression analysis

The quality and integrity of RNA that was extracted as described above were analysed using spectrophotometry and agarose gel electrophoresis. First-strand cDNA synthesis was carried out from 1 μg total RNA using DyNAmo cDNA synthesis kit (Thermo Fisher Scientific Inc, Waltham, MA, USA) following the manufacturer's protocol. Primers for real-time PCR (AOC, OsJAR1, OsJAR2) were designed using Primer3 or Primer-BLAST software v4.0 (Rozen & Skaletsky 2000; Ye *et al*. 2012), *OsTIFY10c* primers were adopted from Ye *et al*. (2009), *eEF-1α* from Caldana *et al*. (2007; Supporting Information Table S3), and OsCyclophilin2 from Gutjahr *et al*. (2008). The specificity of the amplification was analysed by melting curve analysis and gel electrophoresis, efficiency was determined by analysis of serial cDNA dilution curves. qPCR analysis was carried out in 20 μL reactions containing in final concentration 200 nM of each primer, 200 nM of each dNTP, 1X GoTaq colourless buffer, 2.5 mM additional MgCl, 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 Gutjahr *et al*. (2008). Three technical replicates were performed for each sample. The relative expression of each gene was calculated with the delta delta C_t method (Livak & Schmittgen 2001) using *eEF-1α* and *OsUBI5* as endogenous control for normalization (Caldana *et al*. 2007; Hellemans *et al*. 2007). Each experiment was repeated for three biological replicates and the mean fold change was calculated and plotted along with corresponding standard deviation values.

RESULTS

Mutation of OsJAR1 partially impairs the response to light

The responses of rice seedlings to light involve *OsJAR1*. If *osjar1* mutant seedlings are raised in continuous light, especially blue (B) or FR light, they develop significantly longer coleoptiles compared with the WT (Riemann *et al*. 2008; Supporting Information Fig. S1). More detailed analysis revealed that coleoptiles of *osjar1* mutants get longer compared with the WT in continuous red (R) light as well (Supporting Information Fig. S1), although this difference was less obvious, probably due to a generally stronger growth repression in this light quality. However, compared with the JA-deficient mutant *hebiba*, the phenotype appeared to be comparatively weak in all light qualities. Therefore, we revisited this effect systematically and compared the seedling phenotypes of two *osjar1* mutants, *osjar1-1* and *osjar1-2*, to that of the rice *ALLENE OXIDE CYCLASE* (*OsAOC*) mutants *hebiba* and *cpm2*, and their respective WTs (Fig. 1; *cpm2* shown as representative example). Although coleoptiles of *osjar1* mutants were longer compared with the WT under the conditions tested, they were clearly shorter compared with *osaoc* mutants. Therefore, we performed a quantitative analysis of the final seedling length in continuous B light in the different genotypes, the light quality resulting in the largest difference in the coleoptile length of WT and *osjar1*. As the mutants have different WT backgrounds, we included two different WTs, Nihonmasari (background of *hebiba* and *cpm2*) and Nipponbare (background of *osjar1-1* and *osjar1-2*), into our analysis. Both WTs showed a similar mean final seedling length (Nipponbare: 6.8 mm, Nihonmasari: 7.2 mm; Fig. 2a,b), while *osjar1* mutants were almost double as long (*osjar1-1*: 13.7 mm, *osjar1-2*: 12.9 mm; Fig. 2a). Both *osaoc* mutants, however, developed even longer seedlings (*hebiba*: 26.4 mm, *cpm2*: 22.5 mm; Fig. 2b). In summary, the *osjar1* mutants show only partial impairment in light-induced inhibition of coleoptile elongation growth in continuous light of all qualities tested with the strongest effect observed in blue light.

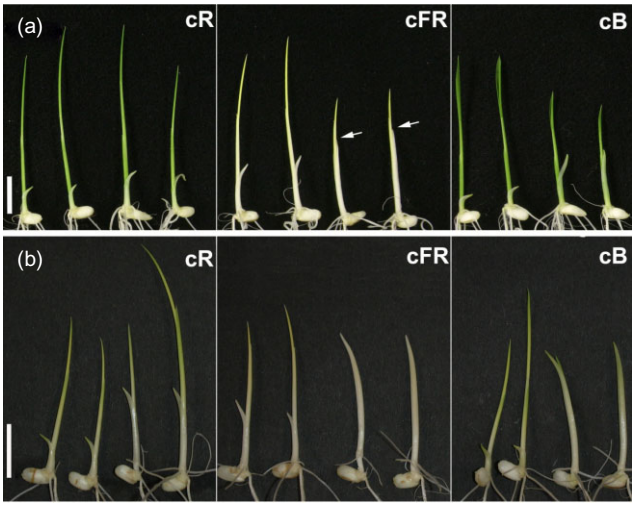

Figure 1. Seedling phenotypes of *osjar1* and *cpm2* mutants. (a) Two seedlings of the wild type (Nipponbare) are depicted next to one seedlings of *osjar1-1* and *osjar1-2*, respectively. They were raised either in continuous red (cR), far-red (cFR) or cB light for 5 d. Arrows indicate the coleoptile tip of seedlings grown in FR light. (b) Two seedlings of the wild type (Nihonmasari) are depicted next to two seedlings of the mutant *cpm2*. They were raised either in cR, cFR or cB light for 5 d. Light intensity: 10 μmol m^{-2} s^{-1}; bars = 10 mm.

OsJAR1 and OsJAR2 have overlapping substrate specificities

It was reported that OsJAR1 and OsJAR2 are able to conjugate JA to Ile (Wakuta *et al*. 2011). In order to investigate the full amino acid substrate spectrum which can be used by OsJAR1 and OsJAR2, we expressed the proteins recombinantly as GST fusion proteins in *E. coli* and purified them for subsequent enzyme activity assays. We could confirm that both proteins are able to generate the JA-Ile conjugate (Fig. 3a,b). The amino acid substrate spectrum of OsJAR1 was similar to that described for AtJAR1 (Staswick & Tiryaki 2004) with additional JA-conjugating activity for the amino acids aspartate, glutamate and proline. Interestingly, OsJAR2 could accept only a subset of those amino acids to form the conjugate with JA. Compared with OsJAR1, no additional amino acid was conjugated to JA. Thus, OsJAR2 can only partly complement OsJAR1 function for the conjugation of JA to certain amino acids, however, including Ile.

One possibility to explain the weaker phenotype of *osjar1* mutants compared with *osaoc* mutants is that the mutants express partially functional proteins, which could lead to a reduced production of JA-Ile. Indeed, we could amplify modified *OsJAR1* transcripts from both *osjar1-1* and *osjar1-2* mutants (Supporting Information Fig. S2). Therefore, we expressed mutant proteins based on the transcript information we obtained in our experiments, and applied the same enzyme activity assay as for the WT protein. However, both mutant proteins were not able to conjugate any amino acid to JA (Supporting Information Fig. S3). Hence, the mutants *osjar1-1* and *osjar1-2* can be regarded as complete loss-of-function mutants.

Biosynthesis of JA-Ile is not impaired in *osjar1* mutants during photomorphogenesis

As we observed a clear phenotype in *osjar1* mutants after raising them in continuous blue light, we examined the contents of JA and JA-Ile in the WT and mutants. Under the applied conditions, the first leaves already pierced the coleoptiles and we measured the hormones in both tissues separately. In WT, JA levels were similar both in coleoptiles (15.2 ng g^{-1} FW; Fig. 4a) and leaves (16.2 ng g^{-1} FW; Fig. 4b). In mutant coleoptiles, however, much higher levels of JA were measured (91.4 ng g^{-1} FW in *osjar1-1* and 124.9 ng g^{-1} FW in *osjar1-2*; Fig. 4a), while we measured levels comparable to those in the WT in leaves (20.9 ng g^{-1} FW in *osjar1-1* and 21.9 ng g^{-1} FW in *osjar1-2*; Fig. 4b). The results for JA-Ile measurements were surprising: In all the cases, JA-Ile levels were either similar or even higher in *osjar1* mutants. In coleoptiles, JA-Ile levels were 4.2, 4.8 and 6.3 ng g^{-1} FW for the WT, *osjar1-1* and *osjar1-2*, respectively (Fig. 4c). In leaves, the mutants even contained more JA-Ile in this stage (5.6 ng g^{-1} FW in *osjar1-1* and 6.8 ng g^{-1} FW in *osjar1-2* compared with 3.5 ng g^{-1} FW in the WT; Fig. 4d). The conclusion from these results was that rice seedlings are able to synthesize JA-Ile even if OsJAR1 function is impaired.

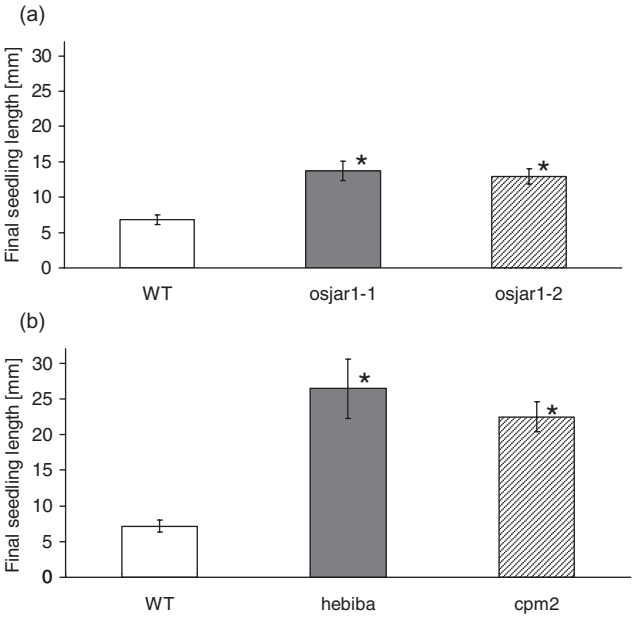

Figure 2. Final length of seedlings raised in continuous blue light. (a) Length of seedlings of Nipponbare [wild type (WT); white bar; $n = 68$], *osjar1-1* (grey bar; $n = 72$) and *osjar1-2* (striped bar; $n = 71$) raised in continuous blue light (10 μmol m^{-2} s^{-1}) was determined after 5 d. The average ± SD is depicted. (b) Length of seedlings of Nihonmasari (WT; white bar), *hebiba* (grey bar) and cpm2 (striped bar) raised under the same conditions as described for (a). The average of 10 seedlings ± SD is depicted. An asterisk indicates a significant difference from the wild-type control in Student's *t*-test at $P < 0.01$.

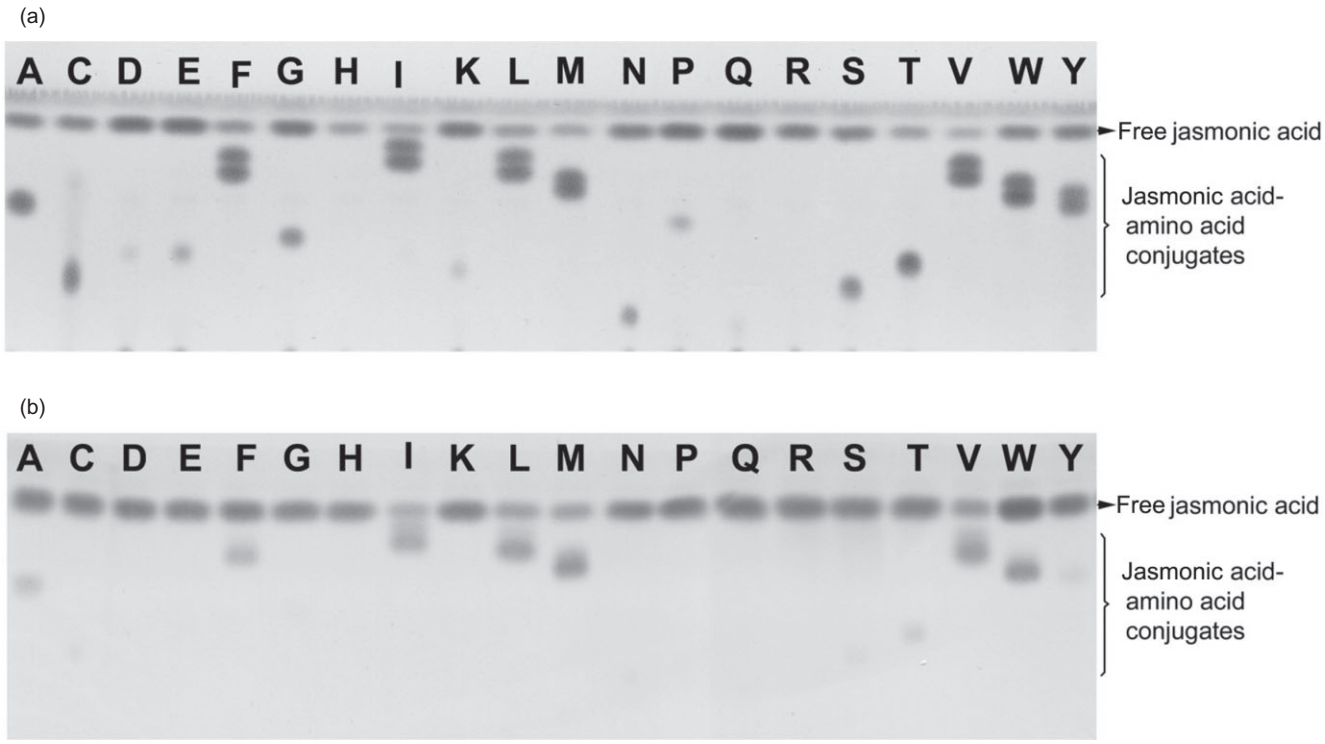

Figure 3. Activity of GST fusion proteins of OsJAR1 and OsJAR2. GST-OsJAR1 (a) and GST-OsJAR2 (b) were incubated with jasmonic acid (JA) and each amino acid (indicated by single-letter code), and reaction products analysed by thin layer chromatography.

In the experimental setting applied for those measurements described above, we could not exclude that the differences observed in WT and the mutants were caused by an altered developmental programme. Therefore, we conducted an experiment using different conditions. We raised seedlings in complete darkness, irradiated them for either 1 or 6 h with blue light, and analysed their JA and JA-Ile levels thereafter. The level of JA was increased 25-fold in the WT upon irradiation with blue light (Fig. 5a). Furthermore, under these conditions, *osjar1-1* and *osjar1-2* accumulated significantly more JA compared with the WT (152.2 ng g^{-1} FW in osjar1-1, 161.2 ng g^{-1} FW in *osjar1-2*, and 53.7 ng g^{-1} FW in WT after 6 h of blue light). Like JA, its amino acid conjugate JA-Ile was induced by blue light as well, 14-fold in the WT after 6 h (Fig. 5b). Similar to the experiment shown in Fig. 4, *osjar1* mutants were able to synthesize JA-Ile, although the synthesis was slightly delayed as after 1 h of blue light the WT contained 2.4 ng g^{-1} FW of JA-Ile, while *osjar1-1* and *osjar1-2* contained only 1.2 and 1.9 ng g^{-1} FW, respectively. However, after 6 h JA-Ile levels were similar in all the three genotypes. From our experiments, it can be hypothesized that JA-Ile contributes to blue light-induced growth regulation. Furthermore, we confirmed that JA-Ile synthesis is not impaired in the mutants during the young seedling stage, suggesting the presence of a redundantly active enzyme.

OsJAR1 is required for the response to wounding

Because we had no evidence that OsJAR1 is absolutely required for the biosynthesis of JA-Ile, we performed a treatment in a different developmental stage. The MecWorm system was used where plants can be mechanically wounded in a highly reproducible manner (Mithöfer *et al*. 2005). In the experiments, we used WT and *osjar1-2* mutant plants to determine JA and JA-Ile levels. In both cases, JA levels increased due to mechanical wounding compared with a non-wounded control plant (Fig. 6a). The relative change due to continuous wounding was 10- to 60-fold in both genotypes after 30 and 360 min, respectively. In contrast, the biosynthesis of JA-Ile was severely compromised in *osjar1-2* mutants (Fig. 6b). While in the WT JA-Ile levels reached up to 30 ng g^{-1} FW in all time points examined, *osjar1-2* mutants produced only 1 ng g^{-1} FW. Although the synthesis of JA-Ile was not completely impaired in the mutants, the relative change in JA-Ile content was only three- to sevenfold, while in the WT the relative change was 100- to 190-fold. Thus, the function of OsJAR1 in response to continuous mechanical wounding cannot be compensated efficiently by other GH3 enzymes.

The expression of JA-inducible genes is altered in *osjar1* mutants

The production of bioactive jasmonates causing the transcription of JA-responsive genes is inducible (for a recent review, see Wasternack & Hause 2013). Among the JA-responsive genes, those encoding biosynthetic enzymes of the JA pathway, and those encoding JAZ repressor proteins are most readily induced. We therefore investigated the regulation of *OsAOC*, *OsJAR1*, *OsJAR2* and *OsTIFY10c/OsJAZ8* (Yamada *et al*. 2012) in response to blue light (Fig. 7) and

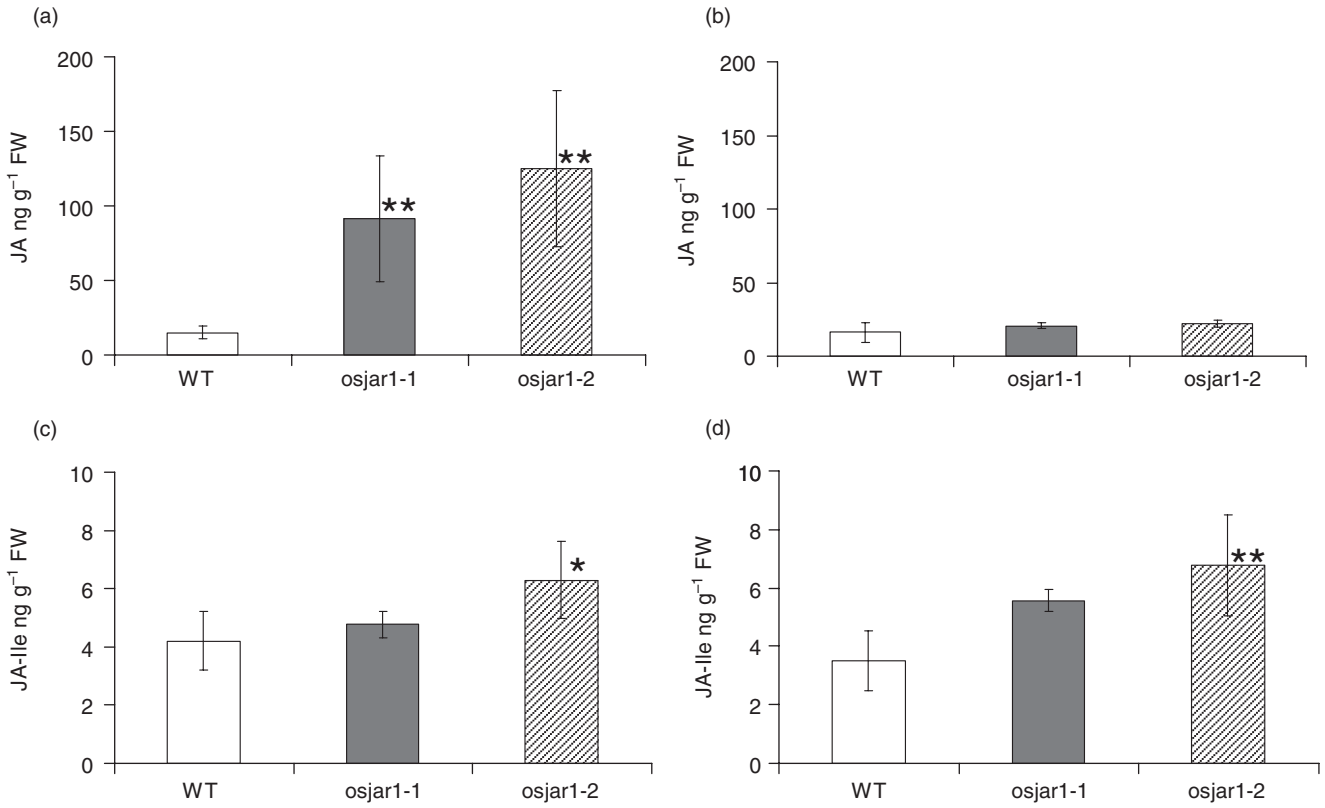

Figure 4. Jasmonic acid (JA) and jasmonic acid-isoleucine (JA-Ile) levels in seedlings raised in continuous blue light. The levels of JA (a, b) and JA-Ile (c, d) in 5-day-old seedlings raised in continuous blue light (10 μmol m^2 s^{-1}) of wild type (white bar), *osjar1-1* (grey bar) and *osjar1-2* (striped bar). Levels in coleoptiles (a, c) and leaves (b, d) are shown, respectively. The results represent the average of three independent experiments ± SD. Significant differences to the wild type in a Student's *t*-test are indicated by one ($P < 0.1$) or two ($P < 0.01$) asterisks.

MecWorm treatment (Fig. 8). As the number of seeds available for the experiments was limited, we used *osjar1-1* for blue light experiments and *osjar1-2* for MecWorm experiments, respectively. In the WT, blue light irradiation caused increases relative to a dark control for the transcripts of *OsAOC* (fivefold), *OsJAR1* (sixfold) and *OsJAZ8* (27-fold) peaking after 2 h of blue light irradiation (Fig. 7). In *osjar1-2*, the induction of *OsAOC* and *OsJAZ8* transcript was significantly reduced (twofold and ninefold induction, respectively), and *OsJAR1* transcripts were not induced at all. *OsJAR2* transcription was not induced during the entire experimental period, neither in the WT nor in the mutant. Only after 6 h a slight relative increase could be observed, which was less than twofold in both genotypes. In contrast to blue light, MecWorm treatment did not cause strong changes in the transcript levels of *OsAOC*, *OsJAR1* and *OsJAR2* (Fig. 8). The largest relative change (twofold) was observed in the expression of *OsJAR1* in the WT after 6 h of MecWorm treatment. In contrast, *OsJAZ8* was induced after 1 h (twofold) and the transcript level further increased to 19-fold relative to the control after 6 h of MecWorm treatment. In *osjar1-1* mutants, the response of *OsJAZ8* was quenched: while the induction in the first hour of wounding was in the same range as in the WT after 6 h, only a fourfold induction was reached. It is noteworthy that the abundance of *OsJAR2* transcripts was severely reduced in leaves compared with seedlings in both WT and *osjar1* (Supporting Information Fig. S4).

DISCUSSION

OsJAR1 is not the only functional JA-Ile conjugating enzyme in rice plants

GH3 enzymes have important functions in plant hormone metabolism and are encoded by a gene family in plants. In *Arabidopsis*, JAR1 has been identified as the enzyme conjugating Ile to JA. The high level of conservation of the protein sequence between OsJAR1 and its orthologue in *Arabidopsis* (61% identities on the amino acid level) suggested that both enzymes have similar functions. In fact, we could demonstrate that AtJAR1 and OsJAR1 have similar substrate specificities *in vitro* (Fig. 3a,d). However, there are differences in the phenotypes of knockout mutants of both plant species demonstrating a different physiological function of JAR1 in rice and *Arabidopsis*. Most clearly, *osjar1* mutants are severely affected in their fertility while *Arabidopsis* mutants are normally fertile (Staswick *et al*. 2002).

We found that in *osjar1* mutants only some of the photomorphogenic phenotypes observed in JA-deficient rice

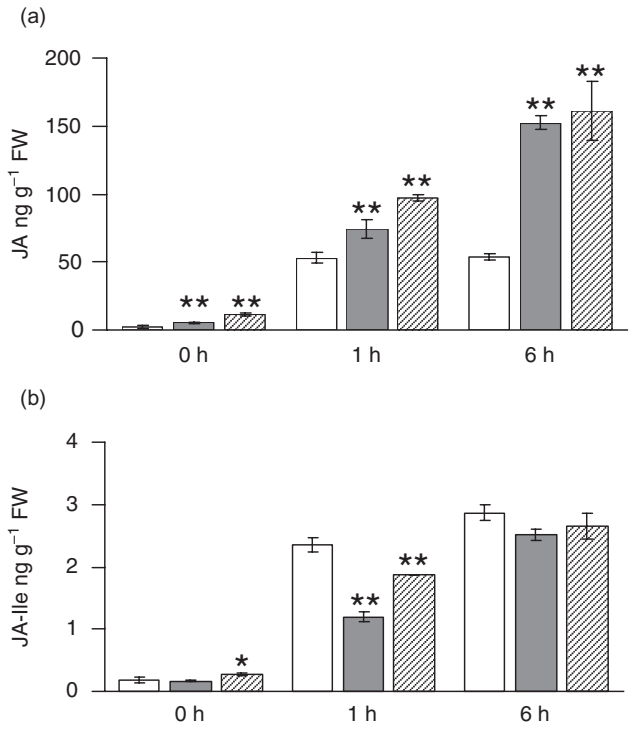

Figure 5. Jasmonic acid (JA) and jasmonic acid-isoleucine (JA-Ile) levels in etiolated seedlings irradiated with blue light. The levels of JA (a) and JA-Ile (b) in 5-day-old seedlings raised in complete darkness and irradiated with blue light (10 μmol m^2 s^{-1}) for the times indicated in the wild type (white bar), *osjar1-1* (grey bar) and *osjar1-2* (striped bar). The results represent the average of three independent experiments ± SD. Significant differences to the wild type in a Student's *t*-test are indicated by one ($P < 0.1$) or two ($P < 0.01$) asterisks, respectively.

mutants such as *hebiba* could be detected (Riemann *et al.* 2003, 2008). One possibility to explain this fact was that *osjar1* mutants still can synthesize JA-Ile due to residual activity of truncated proteins expressed in the insertion mutants. Indeed, we detected partial *OsJAR1* transcripts in *osjar1-1* and *osjar1-2* mutant lines (Supporting Information Fig. S2), and it seemed conceivable that partially functional proteins are synthesized, which account for some JA-Ile production even in the mutants. Such a scenario could have explained why *osjar1* mutants show a less severe photomorphogenic phenotype compared with *hebiba*. Therefore, we examined the enzymatic activity of proteins based on transcript sequences detected in the mutants. However, those proteins were neither able to catalyse the conjugation of Ile to JA nor of any other amino acid tested (Supporting Information Fig. S3).

Previously, it has been found that both OsJAR1 and OsJAR2 are able to generate JA-Ile (Wakuta *et al.* 2011), which could be confirmed by our results (Fig. 3). While in *Nicotiana attenuata* a physiological function of two JAR1 orthologues, JAR4 and JAR6, has been proven (Wang *et al.* 2007), a function for OsJAR2 has remained elusive. In the present study, we could provide evidence that a JA-amino acid conjugating GH3 enzyme different from OsJAR1 has a physiological function during seedling development of rice as the formation of JA-Ile was not *OsJAR1* dependent in this stage (Figs 4 & 5). OsJAR2 is a likely candidate for this enzymatic function, and future studies will explore this possibility. The enzymatic activity of the product of a third GH3 gene in rice belonging to group I (OsGH3.12) was not analysed by us because we did not find any experimental evidence for its expression.

Mutations in OsJAR1 protein impair enzyme activity

Two recent publications describing the crystal structures of three different GH3 proteins from *Arabidopsis thaliana* (Westfall *et al.* 2012; PBS3 and JAR1) and *Vitis vinifera* (Peat *et al.* 2012; GH3-1) deliver insight into the molecular mechanism of the conjugation reaction. GH3 enzymes catalyse a two-step reaction of their specific acyl acid substrates. In the first step of the reaction the acyl acid is adenylated, and in the subsequent reaction an amino acid is transferred leading to

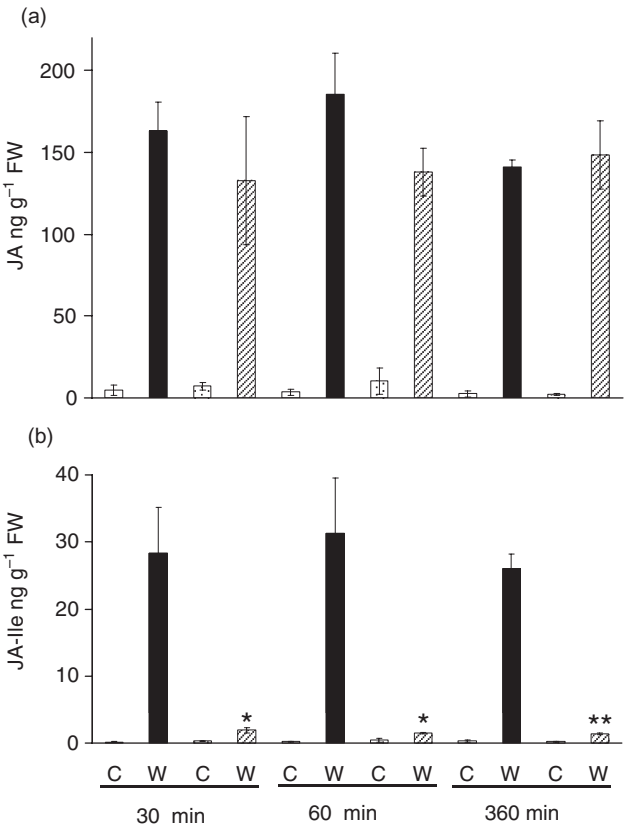

Figure 6. Wound-induced regulation of jasmonate levels. Levels of jasmonic acid JA (a) and jasmonic acid-isoleucine (JA-Ile) (b) in the wild-type (white and black bars) and *osjar1-2* mutant (dotted and striped bars) plants wounded continuously by MecWorm for the periods indicated were compared with control plants in the same photoperiod. The results represent the average of three independent experiments ± SE. c = control, w = wounding. Significant differences to the wild type in a Student's *t*-test are indicated by one ($P < 0.1$) or two ($P < 0.01$) asterisks, respectively.

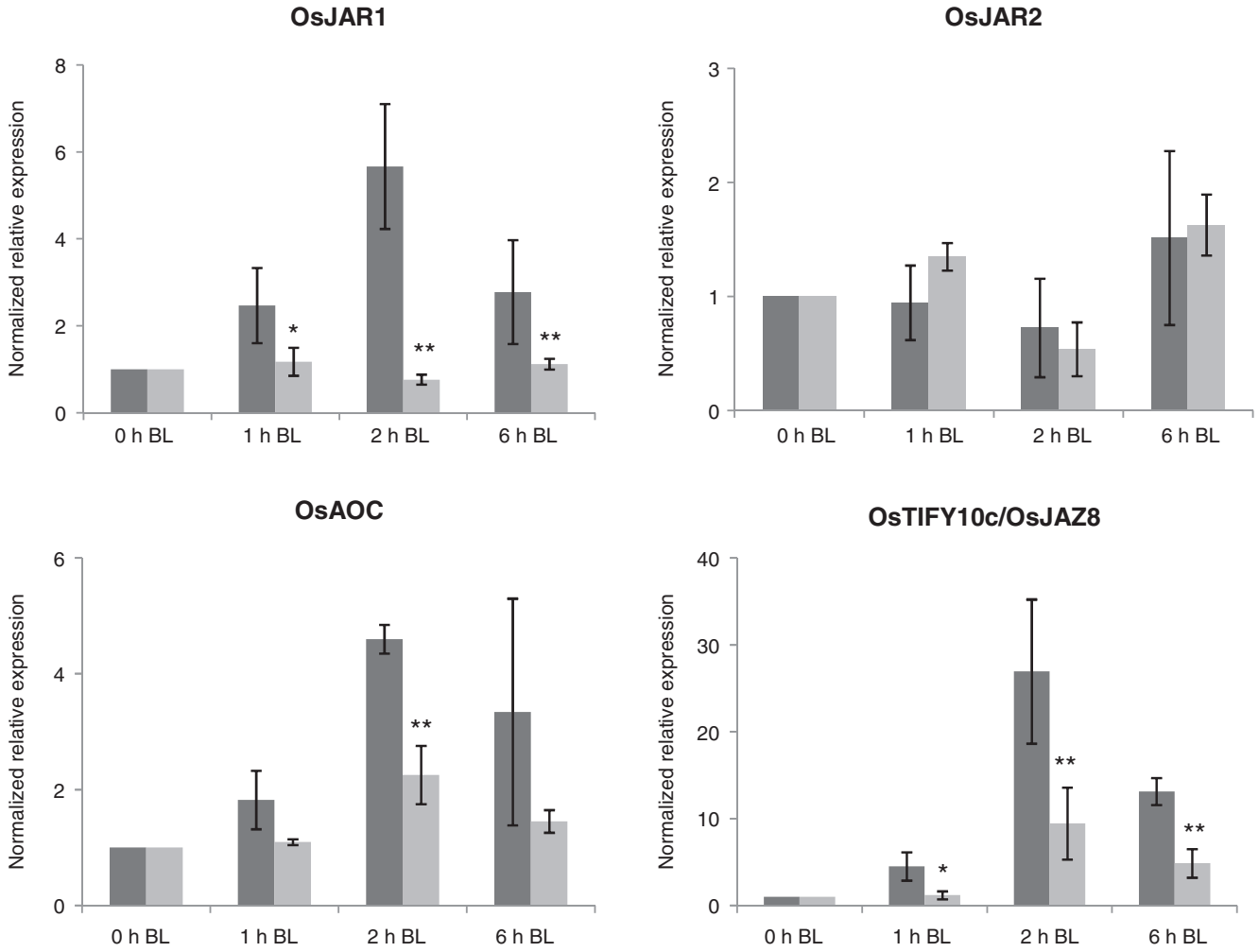

Figure 7. Transcriptional regulation of selected biosynthesis and signalling genes in response to blue light. Wild-type (dark grey bars) and *osjar1-1* (light grey bars) seedlings have been irradiated with blue light for the times indicated. The transcription levels of the genes *OsJAR1*, *OsJAR2*, *OsAOC and OsTIFY10c/OsJAZ8* were quantified relative to etiolated control plants after normalization with three housekeeping genes: *eF1α*, *OsUBI10* and *OsUBI5*. Error bars indicate SD, one and two asterisks a significant difference in a Student's *t*-test at $P < 0.05$ and $P < 0.01$, respectively. $n = 3$.

formation of the acyl acid conjugate. GH3 proteins are supposed to act as monomers composed of a large N-terminal domain harbouring the three previously described motives for ATP/AMP binding, and a smaller C-terminal domain connected through a flexible hinge loop. The catalytically active site is located between the two domains, and residues from both domains contribute to the adenylating step of the conjugation reaction. Upon the adenylation reaction and the release of pyrophosphate, the C-terminal domain rotates along the hinge region and is capping the active site for the subsequent transferase reaction.

The mutated proteins used in the enzyme activity assays are altered in these important regions. The truncated protein encoded by *OsJAR1-1* transcripts lacks some essential residues located in the C-terminal domain, namely residue 511 (valine), which was found to be involved in amino acid binding, and residue 550 (lysine) interacting with β/γ-phosphate in the adenylation reaction. It is also possible that the additional 38 aa residues encoded by the *Tos17* sequence somehow block the entrance of the substrate to the active site leading to the total inactivity. In *osjar1-2* mutants, the TOS17 retrotransposon was spliced out along with 42 base pairs in front of the insertion site and the fourth intron. The resulting transcript carries a premature stop codon in the beginning of the fourth exon so the predicted protein lacks the entire C-terminal domain and the third motif for ATP/AMP binding. As the mutated OsJAR1-1 and OsJAR1-2 proteins were not able to conjugate amino acids to JA, the importance of the respective domains was confirmed by our observations.

Functions of OsJAR1 during photo- and skotomorphogenesis, and wounding

JA-deficient rice mutants show obvious phenotypes in photo- and skotomorphogenesis (Riemann *et al*. 2003; Svyatyna &

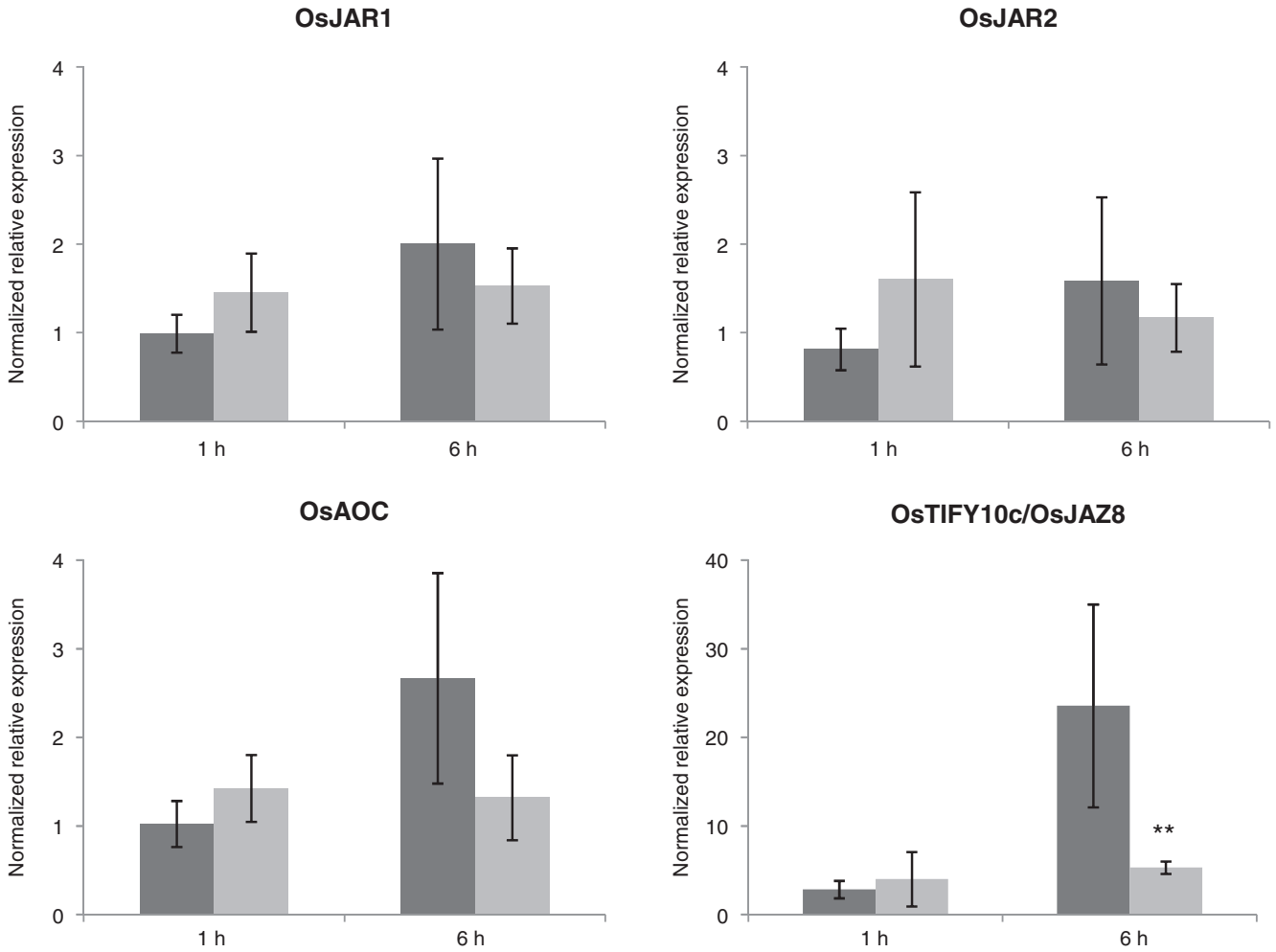

Figure 8. Transcriptional regulation of selected biosynthesis and signalling genes in response to continuous mechanical wounding. Wild-type (dark grey bars) and *osjar1-2* (light grey bars) leaves were wounded by MecWorm for the times indicated. The transcription levels of the genes *OsJAR1*, *OsJAR2*, *OsAOC* and *OsTIFY10c/OsJAZ8* were quantified relative to a non-treated control plant in the same photophase after normalization with two housekeeping genes: *eF1α* and *OsUBI5*. Error bars indicate SD, two asterisks a significant difference in a Student's *t*-test at $P < 0.01$. $n = 3$.

Riemann 2012). Although *OsJAR1* transcripts are induced in the WT by either red, blue or FR light, a pronounced mutant phenotype was reported only in continuous FR and blue light (Riemann *et al*. 2008). Using a larger amount of homozygous seedlings, we were able to demonstrate in this study that *osjar1* mutants show a mild phenotype in continuous red light as well (Supporting Information Fig. S1). In contrast, in the rice *aoc* mutants, *hebiba* and *cpm2*, an obvious phenotype can be observed in all the light conditions (Fig. 1b), and the phenotype in continuous blue light is more severe compared with *osjar1* (Fig. 2a,b).

There are different possibilities to explain this phenotypic discrepancy. The response to some light qualities such as red light, which is mediated by phyA and phyB redundantly (Riemann *et al*. 2008; Fig. 9), might be mediated by amino acid conjugates different from JA-Ile or other JA derivatives, which are synthesized via an *OsJAR1*-independent pathway. The high irradiance response to blue light and FR light is not mediated by phyB, but by phyA. In these light qualities, *osjar1* mutants develop longer coleoptiles, although they accumulate JA-Ile levels similar to the WT (Figs 1, 4 & 5). A comparison with Fig. 7 shows that this similar accumulation of JA-Ile that coexists with a reduced (but not absent) induction of JA-responsive genes shows that JA-Ile is not sufficient for the induction of these genes. However, this does not imply that the induction of these genes is independent of JA-Ile. It merely means that there are additional factors involved. These might be other amino acid conjugates, but this might be as well other jasmonate derivates, whose level is altered by the absence of OsJAR1 (e.g. because more jasmonates are available for conversion by other enzymes). We conclude that also under these conditions other amino acid conjugates of JA are relevant. These alternative conjugates would then be preferentially, although not exclusively, synthesized through OsJAR1 (Riemann *et al*. 2008; Fig. 9a). In order to explore this possibility, further JA derivatives, especially JA amino acid conjugates, have to be analysed in the future in the seedling stage.

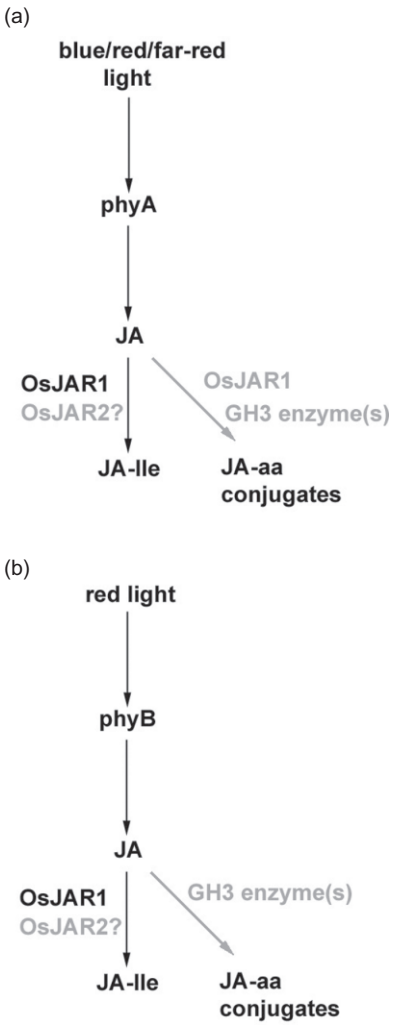

Figure 9. Working model for the light-dependent regulation of GH3 enzymes. (a) Blue, red or far-red light induces the biosynthesis of jasmonic acid (JA) via the photoreceptor phyA. OsJAR1 and another GH3 enzyme, presumably OsJAR2, conjugate JA to isoleucine (Ile). (b) Red light perceived via phyB induces the biosynthesis of JA. Subsequently, JA-Ile is synthesized by OsJAR1 and another GH3 enzyme, presumably OsJAR2. In addition, further JA-amino acid conjugates may be formed in a reaction catalysed primarily by OsJAR1 in (a) and by other GH3 enzymes not yet known in (b). Parts of the pathway depicted in grey are not yet proven experimentally.

Although OsJAR1 conjugates JA and Ile and the photomorphogenic response might partially be mediated by JA-Ile, the existence of alternative and redundant pathways that are *OsJAR1* independent is not excluded. At least OsJAR2 is able to catalyse the same reaction *in vitro* (Fig. 9). Therefore, we examined JA-Ile levels in *osjar1* seedlings. By adjusting irradiation conditions, we were able to generate in etiolated mutant coleoptiles levels of JA-Ile that are similar to the WT (despite with some difference in the velocity of conjugation), and it is noteworthy that in continuous light, even *osjar1* coleoptiles are able to produce JA-Ile (Figs 4 & 5).

We conclude that other GH3 enzymes must be present and active, which might also explain the lack of a skotomorphogenic phenotype in *osjar1*. Hence, we provide evidence that enzymes different from OsJAR1 contribute to the production of JA-Ile. However, although *osjar1* mutants still produce JA-Ile to almost normal levels, this does not imply that in the WT, OsJAR1 does not participate in the conjugation of JA and Ile in blue light because the two OsJAR genes (or another member of the GH3 family) might be functionally redundant. To test for functional redundancy, mutants of redundantly active enzymes such as OsJAR2 are required, which are, at present, not available.

The results of JA-Ile measurements in young seedlings suggested that OsJAR1 may not be the major JA-Ile conjugating enzyme in leaves. The levels of this hormone in *osjar1* seedlings irradiated with blue light were significantly increased compared with etiolated seedlings and reached similar values as those of irradiated WT after extended irradiation times (Fig. 5b). Therefore, we conducted experiments in which leaves of plants in the vegetative stage were wounded, a treatment known to induce the synthesis of JA and JA-Ile. In this developmental stage, biosynthesis of JA-Ile is mainly catalysed by OsJAR1 as the levels of JA-Ile were significantly reduced in *osjar1-2* mutants (Fig. 6b). Even in this experiment, the mutant could still produce some Ile conjugates, but it could be concluded that the activity of other GH3 enzymes in this response is minor.

Investigation of the expression of representative genes in the JA biosynthesis and signalling pathways revealed a major difference between blue light and MecWorm treatment: The transcription of biosynthesis genes (*OsAOC* and *OsJAR1*) was up-regulated in response to blue light in the WT (Fig. 7), while MecWorm treatment did not result in an induction of these genes relative to a control plant (Fig. 8), although the production of JA and JA-Ile was enhanced in response to both treatments (Figs 5 & 6). This discrepancy between phytohormone content and activation of biosynthetic enzymes indicates that the ground level of the biosynthetic enzymes in the developmental stage used for the MecWorm experiments is sufficient to trigger the increase of JA and JA-Ile, and that induction of the encoding genes is a secondary step required to sustain elevated biosynthesis of JA and JA-Ile. Whether enzymatic activity is regulated by a post-translational mechanism or merely controlled by availability of the substrate remains to be elucidated. Intriguingly, we observed a striking difference in the basal expression levels of *OsJAR1* and *OsJAZ8* in leaves sampled in the morning (used as controls for 1 h wounding treatments) compared with those sampled towards the end of the day (as control for the 6 h wounding treatment; Supporting Information Fig. S4). Based on this observation, we suggest that rice activates the transcription of those genes depending on the photophase. Hence, the change of those transcripts relative to a non-treated control plant in response to wounding appears to be comparatively small because the transcript level in control plants is high due to the photophase.

A previous report by Wakuta *et al.* (2011) suggested that *OsJAR1* and *OsJAR2* transcripts are induced by mechanical wounding. To avoid unspecific aspects of wounding related to

mechanical damage rather than to wounding, we employed the MecWorm as strategy to mimic the mechanical aspects of herbivore wounding in a standardized manner. Our results are consistent with published work (Bricchi *et al*. 2010) demonstrating that the biochemical output from MecWorm treatment differs from conventional wounding treatments (Wakuta *et al*. 2011; Fukumoto *et al*. 2013, Shimizu *et al*. 2013), which should be more appropriately referred to as mechanical damage.

Although we found that *OsJAR2* transcripts are less abundant in leaves compared with seedlings (Supporting Information Fig. S4), *OsJAR2* transcripts were, if at all, only marginally affected by both treatments (Figs 7 & 8). Hence, JA-Ile conjugation in blue light (Fig. 5) and residual JA-Ile conjugation during MecWorm treatment (Fig. 6) cannot entirely be explained by transcriptional regulation of the enzymes catalysing this step. However, the observed difference in inducibility of JA-Ile levels could be explained by a different substrate availability in combination with the abundance of OsJAR2 protein. We demonstrated that *osjar1* seedlings accumulated high levels of free JA in continuous blue light (Fig. 5). Because sufficient levels of substrate are present in the mutants, even a default level of OsJAR2 is expected to result in increased levels of JA-Ile. Consistent with this interpretation, in leaves of MecWorm-treated mutant plants, where we did not observe an overproduction of free JA (in contrast to blue light irradiation), the accumulation of JA-Ile was severely reduced (Fig. 6). In order to verify this possibility, it will be essential to study the abundance of OsJAR1 and OsJAR2 on the protein level in the respective tissues.

In summary, our results suggest that the synthesis of JA-Ile (and probably of other JA-amino acid conjugates) is catalysed by multiple GH3 enzymes in rice. Depending on the environmental conditions and developmental stages, those enzymes can be active redundantly or almost exclusively. However, we did not find conditions or stages in which the impairment of OsJAR1 function causes complete loss of JA-Ile production, suggesting that rice plants have established a system of redundancy for this important biochemical step.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Final length of coleoptiles in continuous light. Seedlings of the wild type (Nipponbare, white bars), *osjar1-1* (grey bars) and *osjar1-2* (striped bars) were raised in continuous light (10 μmol m^{-2} s^{-1}) of different qualities (cBL = blue light, cRL = red light, cFRL = far-red light) until the leaves pierced the coleoptiles. From left to right $n = 47, 21, 24, 46, 24, 25, 25, 23$ and 25, respectively. Error bars indicate ± SD, an asterisk significant difference in a Student's *t*-test at $P < 0.01$.

Figure S2. Gene and predicted protein structure of the wild type, *osjar1-1* and *osjar1-2* based on transcript information. The gene structure is depicted above the expected protein structure. The protein structure is deduced from the respective transcripts amplified from each genotype. Untranslated regions (UTRs) are shown as blue boxes and exons in orange. Introns are represented by black lines. The proteins are depicted in yellow bars, red boxes indicate motive I-III, which have been described to be important for the catalytic activity of GH3 enzymes. Green boxes in the protein sequence depict parts encoded by the Tos17 transposon. aa = amino acid.

Figure S3. Activity of GST fusion proteins of OsJAR1-1 and OsJAR1-2. GST-OsJAR1-1 (a) and GST-OsJAR1-2 (b) were incubated with JA and each amino acid (indicated by single-letter code), and reaction products were analysed by thin layer chromatography.

Figure S4. Comparison of transcript levels relative to the expression in an etiolated wild-type seedling. The transcript levels of *OsJAR1*, *OsJAR2*, *OsAOC* and *OsTIFY10c/OsJAZ8* in etiolated seedlings and leaves in the morning and evening, respectively, relative to the expression level in a selected sample of an etiolated wild type. Wild type is shown in dark grey bars, *osjar1-1* (seedlings) and *osjar1-2* (leaves) in light grey bars. For normalization, two housekeeping genes, *OsCyclophilin2* and *eF1*α, were used. Error bars indicate SD, two asterisks a significant difference in a Student's *t*-test at $P < 0.01$. $n = 3$.

Table S1. Analysis of different *osjar1* alleles and their segregation patterns. The columns for WT, Ht and Hm indicate the number of wild-type, heterozygous and homozygous mutant plants in the population, respectively. T-DNA lines originate from the OTL collection, TOS17 from the Tos17 collection. fw = forward; rev = reverse.

Table S2. Sequences of primers used for cloning. Restriction sites are depicted in red.

Table S3. Sequences of primers used for qPCR analysis.