Identification of rice *Allene Oxide Cyclase* mutants and the function of jasmonate for defence against *Magnaporthe oryzae*

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Received 16 November 2012; revised 17 December 2012; accepted 2 January 2013; published online 24 January 2013.

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SUMMARY

Two photomorphogenic mutants of rice, *coleoptile photomorphogenesis 2* (*cpm2*) and *hebiba*, were found to be defective in the gene encoding allene oxide cyclase (*OsAOC*) by map-based cloning and complementation assays. Examination of the enzymatic activity of recombinant GST–*OsAOC* indicated that *OsAOC* is a functional enzyme that is involved in the biosynthesis of jasmonic acid and related compounds. The level of jasmonate was extremely low in both mutants, in agreement with the fact that rice has only one gene encoding allene oxide cyclase. Several flower-related mutant phenotypes were observed, including morphological abnormalities of the flower and early flowering. We used these mutants to investigate the function of jasmonate in the defence response to the blast fungus *Magnaporthe oryzae*. Inoculation assays with fungal spores revealed that both mutants are more susceptible than wild-type to an incompatible strain of *M. oryzae*, in such a way that hyphal growth was enhanced in mutant tissues. The level of jasmonate isoleucine, a bioactive form of jasmonate, increased in response to blast infection. Furthermore, blast-induced accumulation of phytoalexins, especially that of the flavonoid sakuranetin, was found to be severely impaired in *cpm2* and *hebiba*. Together, the present study demonstrates that, in rice, jasmonate mediates the defence response against blast fungus.

Keywords: jasmonate, blast disease, *Oryza sativa*, *Magnaporthe oryzae*, phytoalexin, sterility.

INTRODUCTION

An increasing body of evidence supports a major role of jasmonates (jasmonic acid (JA) and related compounds) as a hormone in plant development and in plant responses to environment. For instance, the role of jasmonate in wound signalling has been characterized in great detail (Koo and Howe, 2009). In some plant species, jasmonate has been shown to function in developmental processes such as anther dehiscence (Ishiguro et al., 2001), tendril coiling (Falkenstein et al., 1991) and sex determination (Acosta et al., 2009; Yan et al., 2012). Jasmonate is an important
Jasmonate has been shown to participate in the defence against fungal infection by inducing JA and JA-dependent transcripts, leading to the defense response to the fungal pathogen <i>M. oryzae</i> (Rakwal et al., 1992; Dillon et al., 1997; Grayer and Kokubun, 2001), and the synthesis of several phytoalexins has been shown to be induced by exogenous JA (Rakwal et al., 1996).

The analysis of jasmonate function in the response to fungal infection was limited by the fact that well-characterized JA-deficient mutants were not available. The mutant <i>hebiba</i> was found to be deficient in JA, but the causative mutation was not known (Riemann et al., 2003). We isolated another mutant, <i>cpm2</i>, showing phenotypes very similar to that of <i>hebiba</i>. In the present work, we found that AOC is responsible for the overlapping phenotypes of the two mutants. Subsequently, we made use of these mutants to investigate the function of jasmonate in the defense against fungal pathogens using <i>M. oryzae</i> as a model.

**RESULTS**

**Isolation of the <i>cpm2</i> mutant and its comparison with <i>hebiba</i>**

The <i>cpm2</i> mutant was isolated from a γ-ray-mutagenized <i>M. oryzae</i> population of <i>japonica</i> cv. Nihonmasari (Biswas et al., 2003). In addition, the mutant exhibited a long mesocotyl in etiolated seedlings. Furthermore, the mutant produced at most only a few fertile seeds in each panicle and is almost sterile. As the mutant was successfully pollinated by the wild-type, this feature is apparently caused by a reduction in male fertility.

All of these traits have been observed in the mutant <i>hebiba</i> (Riemann et al., 2003). Hence we compared the two mutants under identical conditions to quantify their long-coleoptile phenotype. Because it was difficult to obtain homozygous mutant seeds in sufficient quantity due to male sterility, the experiments were performed using heterozygous populations. Seedlings were raised under continuous red light for several days until the first leaf emerged from the coleoptile to determine their final coleoptile length. Within heterozygous populations of the two mutants, a 3:1 segregation pattern characteristic of a recessive mutation was observed: a quarter of the plants showed long coleoptiles (<i>cpm2</i> 27.9%, <i>hebiba</i> 23.2%), while the other plants showed short coleoptiles similar to the wild-type Nihonmasari (Figure 1). The wild-type had a mean final coleoptile length of 6.6 mm, but <i>cpm2</i> and <i>hebiba</i> developed significantly longer coleoptiles (16.0 and 23.4 mm for <i>cpm2</i> and <i>hebiba</i>, respectively). Thus, both mutants exhibit the long-coleoptile phenotype, but with different amplitude.

**Identification of <i>CPM2</i> and <i>HEBIBA</i> loci by map-based analysis**

We used a map-based cloning strategy to identify the mutations responsible for the <i>cpm2</i> and <i>hebiba</i> phenotypes.
The cpm2 mutant was crossed with the indica cultivar Habataki, which showed a final coleoptile length similar to that of Nihonmasari (Figure S1). Approximately 4000 F2 seedlings were screened for a long-coleoptile phenotype under continuous red light. Selected seedlings (738) were transferred to soil and raised further. Part of the second leaf was harvested from each plant, frozen, and subjected to DNA extraction. The DNA samples were used for subsequent analysis. We were able to confirm sterility in all of the plants grown to maturity.

After rough mapping using a DNA sample from ten individuals, the mutation was found to be located on chromosome 3 between the SSR markers RM15224 and RM3513 (Table S1). Subsequently, DNA samples of all individuals were used for fine mapping analysis, and the identified region containing the CPM2 locus was narrowed to between the SSR markers AC114896–1 and AC093312–1, which are located close to the publicly available markers RM15253 and RM15326, respectively (Figure 2a and Table S1). The marked region encompassed approximately 2.8 Mbp. We attempted to narrow the candidate region further without a success due to the highly repetitive sequences found between markers AC097367a and AC093312–1. Furthermore, the recombination rate around the mapped region was apparently very low, probably due to the presence of the centromere between the markers AC106887–1 and RM15326.

By analysing individuals of a hebiba x Kasalath population displaying a clear long-coleoptile phenotype, we found that some SSR markers within the CPM2 candidate region (AC106887–1, AC147803d and AC0907367a, Figure 2a and Table S1) could not be amplified, suggesting that hebiba has a genomic deletion in the region containing these markers. We determined the sequences flanking the deletion by inverse PCR using genomic DNA of hebiba as a template, and found a deletion of approximately 170 kb (Figure 2a). The same deletion was identified by a novel sequencing method independently of us (Nordström et al., 2013).

Within the deleted region, the gene OsAOC [Os03 g0438100 according to the Rice Annotation Project database (http://rapdb.dna.affrc.go.jp/); LOC_Os03 g32314.1 according to the Michigan State University Rice Genome Annotation Project (http://rice.plantbiology.msu.edu)], which encodes a key enzyme for JA biosynthesis, was identified. We sequenced OsAOC of cpm2 and detected a deletion of 11 bp in the first exon of this gene (Figure 2b). Therefore, OsAOC appears to be the most likely candidate gene causing the common phenotypes of the two mutants.

**Complementation of cpm2 and hebiba by OsAOC**

Based on the above analysis, the cpm2 mutant was transformed with a genomic fragment containing the OsAOC promoter and the coding region. The T1 generation of complemented cpm2 plants was raised under continuous red light to determine the final coleoptile length. Approximately three-quarters of T1 plants had short coleoptiles comparable to those of wild-type, indicating that cpm2 was complemented by OsAOC. Some T1 seedlings were grown to maturity to harvest T2 caryopses. From those caryopses, we obtained homozygous T2 populations, all individuals of which carry the transgene and show the wild-type phenotype with regard to the final coleoptile length (Figure 3a,b). Furthermore, it was found that the T1 plants determined to be homozygous transformants show a wild-type level of fertility, demonstrating that the sterility of the mutant was also rescued by the
transgene (Figure 3c). We also transformed the hebiba mutant with a genomic fragment containing the OsAOC promoter and coding region. As shown in Figure 3(d), approximately three-quarters of the T1 seedlings had short coleoptiles comparable to wild-type coleoptiles. The plants with short coleoptiles were determined to be transgenic, while those with a long coleoptile were not. Thus, hebiba was also complemented by OsAOC. From

Figure 2. Map-based analysis of the genomic loci responsible for cpm2 and hebiba mutations.
(a) A region of chromosome 3 in which cpm2 and hebiba were found to be mutated. Map-based analysis indicated that the locus responsible for the cpm2 mutation resides between the markers AC114896-1 and AC093312-1. Within this region, hebiba was found to have a deletion of 170 kb that contains the rice allene oxide cyclase gene (OsAOC). The border sequences of the deleted region are shown; sequences in grey indicate the deleted edges. Black bars represent BAC clones. The numbers in parentheses indicate the number of recombinants. Horizontal arrows indicate the positions of genes annotated by the Rice Annotation Project database (http://rapdb.dna.affrc.go.jp/).
(b) Mutation of OsAOC found in cpm2. The first exon was found to have a deletion of 11 bp, which is shown in grey.

Figure 3. Complementation of cpm2 and hebiba mutants.
(a) Final coleoptile length in seedlings of the wild-type (WT), cpm2 and two complemented cpm2 lines (T2 generation) raised under continuous red light. Values are means ± SE of 19-34 seedlings.
(b) Representative seedlings of WT, cpm2 and two complemented cpm2 lines raised as described above. The arrows indicate the tips of the coleoptiles.
(c) Fertility of wild-type (WT), cpm2 and complemented cpm2 plants. The complemented plans used were of the T1 generation, and were determined to be homozygous transformants by analysis of T2 seedlings.
(d) Distribution of the final coleoptile length in complemented hebiba seedlings (T1 generation) raised under continuous red light. A total of 162 caryopses harvested from four independent T0 transformants were used to obtain the data.
these results, we conclude that the lack of functional OsAOC is responsible for the common mutant phenotypes in cpm2 and hebiba.

**OsAOC encodes a functional AOC**

In order to verify the presumed AOC activity of the product encoded by OsAOC, we used a recombinantly expressed GST fusion protein in combination with recombinantly expressed GST–OsAOS1, as described by Stenzel et al. (2003) and Zerbe et al. (2007), to perform a coupled enzyme assay. OsAOS1 was confirmed to be a functional AOS by a spectrophotometric enzyme assay (K.H. and M.I., unpublished results). Both proteins were expressed without the chloroplast target signal. After purification by affinity chromatography (Figure S2), protein samples were subjected to an enzyme activity assay. The substrate of AOS, 13-hydroperoxy-octadecatrienoic acid (13–HPOT), was metabolized, presumably into allene oxide, when GST–OsAOS1 was present in the reaction mixture, but remained unaltered in the absence of GST–OsAOS1 (Figure 4a). In the presence of both GST–AOS1 and GST–OsAOC, cis–(+)-OPDA was preferentially synthesized. This OPDA enantiomer is the precursor of the naturally occurring JA (Stintzi et al., 2001; Taki et al., 2005; Bottcher and Pollmann, 2009). In the presence of GST–OsAOS1 only, racemic OPDA was produced (Figure 4b and Figure S3), and no OPDA (not even in racemic form) was detectable when only GST–OsAOC or neither of the two enzymes was present in the reaction mixture (Figure 4b). This result indicates that OsAOC encodes a functional AOC.

**Jasmonate levels are severely reduced in cpm2 and hebiba**

In order to test whether OsAOC is required for the biosynthesis of jasmonate, we compared endogenous levels of JA and JA–Ile in the fourth leaf sheaths of 3-week-old plants of the wild-type, cpm2 and hebiba. JA and JA–Ile were extracted from the plant materials and quantified by HPLC-ESI-MS/MS using a chiral column that enabled the separation of natural and unnatural enantiomers of JA. Endogenous levels of natural JA in the wild-type, cpm2 and hebiba were 6.0, 0.5 and 0.5 ng g⁻¹ fresh weight (FW) (Figure 5a,c), and those of natural JA–Ile were 0.5, 0.03 and 0.05 ng g⁻¹ FW, respectively (Figure 5b,d), demonstrating that jasmonate biosynthesis is severely suppressed in cpm2 and hebiba. A low level of natural JA (approximately one-tenth of the wild-type) was detected in both cpm2 and hebiba. A comparable level of unnatural JA was also detected in the two mutants, but was not detected at all in the wild-type (Figure 5a,c). These results indicate that the wild-type exclusively biosynthesizes the natural JA enantiomer, whereas the mutants produce low levels of racemic JA from allene oxide, most probably in a non-enzymatic manner.

As it has been well established that jasmonate biosynthesis is transiently induced by wounding in rice leaf blades (Kiribuchi et al., 2005), we compared the levels of JA and JA–Ile in wounded leaf blades of the wild-type, cpm2 and hebiba. In the wild-type, accumulation of JA and JA–Ile was initiated within 30 min, peaking at 1 h (JA, 379 ng g⁻¹ FW) or 30 min (JA–Ile, 107 ng g⁻¹ FW) after wounding (Figure S4). Such wound-induced accumulation of JA and JA–Ile was undetectable in cpm2 and hebiba, confirming that JA biosynthesis does not operate in the mutants.

**Altered flower morphology and heading time in JA biosynthesis mutants**

Abnormal anthesis was observed in cpm1, a mutant of OsAOS1 (Haga and Iino, 2004), but the overall flower morphology remained unaltered (Figure 6a) (Biswas et al., 2003). In contrast, cpm2 and hebiba flowers showed clear morphological differences from wild-type flowers. The
sterile lemmas of most mutant flowers were longer compared to the wild-type flowers, and some mutant flowers showed an elongated palea (Figure 6a and Figure S5) (see Yoshida and Nagato, 2011; for rice flower morphology). Occasionally additional bract-like organs developed in the mutants between the sterile lemma and palea (Figure 6a, indicated by arrows), or between the lemma and palea, as well as additional anthers (up to seven) and pistils (up to three).

Previously, hebiba was found to show early heading (Riemann et al., 2003). We therefore compared heading times of the wild-type, cpm2 and hebiba under short-day conditions (10 h light/14 h darkness, Figure 6b). Under these conditions, the inflorescences of wild-type plants appeared after 50 days. This period was significantly shorter in all tested mutants (cpm1, 45.8 days; cpm2, 44.7 days; hebiba, 42.5 days). Even under long-day conditions, cpm2 and hebiba showed significantly earlier heading.

Jasmonate-dependent defence response to blast disease

We used cpm2 and hebiba to investigate a possible involvement of jasmonate in the resistance to fungal pathogens. Excised leaf sheaths were inoculated with spores of M. oryzae, and the growth of M. oryzae hyphae in the epidermis was investigated microscopically. The experiments were performed in the absence or presence of exogenous JA. The extent of infection was separated into three categories: ‘infected’ (more than one cell was infected with hyphae originating from the observed appressorium), ‘one cell infected’ (only one cell beneath the observed appressorium was infected by hyphae), or ‘non-infected’ (no cell was infected by the observed appressorium) (Figure 7a). We first compared the susceptibility to a compatible strain (Ina86–137) between the wild-type and cpm2, but found no significant difference (Figure S6a). However, application of JA caused a significant reduction in susceptibility (a
under short-day conditions (10 h light/14 h dark). Values are means ± SE of six plants. Asterisks indicate statistically significant differences from the wild-type (Student’s t test; *P < 0.05 and **P < 0.01).

We performed subsequent experiments with an incompatible strain (P91–15B). In the wild-type control in the absence of exogenous JA, the proportion of the appressoria causing the category ‘infected’ category was 1.4%, whereas the corresponding proportions in cpm2 and hebiba were 14.6% and 13.3%, respectively (Figure 7b, –JA). The results indicated that resistance against hyphal growth is reduced in jasmonate-deficient mutants. In the presence of exogenous JA, the proportion of appressoria causing the ‘infected’ category was significantly lower in cpm2 and hebiba compared to that without JA (Figure 7b, +JA). In all three genotypes, treatment with JA reduced the proportion of the appressoria causing the categories ‘infected’ and ‘one cell-infected’ categories below that for the control without exogenous JA. These observations indicate that exogenous JA rescues the resistance of the mutants, and that resistance is enhanced in the wild-type by application of JA. Together these results demonstrate that jasmonate plays a significant role in the resistance to fungal infection.

We additionally observed that infected leaf sheaths of the mutants develop tissue browning, indicating accumulation of brownish substances. This tissue browning was probably caused by fungal infection, as reported previously for pathogen-treated rice (Chi et al., 2009), and Sorghum bicolor (Nicholson et al., 1987). Tissue browning was not observed in the wild-type; furthermore, the tissue browning observed in the mutants was reduced by JA application (Figure 7c). Our result provides genetic and physiological evidence that the blast-induced tissue browning was caused by the lack of jasmonate.

Subsequently, we examined the effect of Magnaporthe oryzae infection on accumulation of jasmonate levels in the wild-type, and compared them to the levels in mock-treated plants. The levels of JA and its active form JA-Ile in the fourth leaf sheaths were quantified at the time of inoculation (initial), and 1 and 2 days post-inoculation (dpi). Fungal infection did not have an effect on the level of JA. On the other hand, the level of JA-Ile was significantly enhanced by inoculation, and this increase did not occur in
the mock-treated control (Figure 8). The result indicated that JA-Ile is related to disease resistance.

Jasmonate-dependent production of phytoalexins upon *M. oryzae* infection

Phytoalexins are produced in response to pathogen infection. We investigated the effect of *M. oryzae* infection on production of the flavonoid phytoalexin sakuranetin and the major diterpenoid phytoalexins momilactones and phytocassanes.

As shown in Figure 9(a), the level of sakuranetin was increased at 1 and 2 dpi (with a maximum of 2.5 μg g⁻¹ FW at 2 dpi) in the wild-type, but no significant increase was detected in the mutants. However, blast-induced accumulation was observed for momilactones and phytocassanes. Momilactones accumulated to a concentration of 4.3 μg g⁻¹ FW at 2 dpi in the wild-type. This response was partially suppressed in *cpm2* and *hebiba*, in which momilactone levels reached 2.4 and 1.6 μg g⁻¹ FW at 2 dpi, respectively (Figure 9b and Figure S7). For the pathogen-induced increase of phytocassanes, no significant differences were observed among the wild-type, *cpm2* and *hebiba* (Figure 9c and Figure S7). These results demonstrate that the pathogen-induced accumulation of sakuranetin, and partially that of momilactones, is mediated by jasmonate, but jasmonate is dispensable for synthesis of phytocassanes.

DISCUSSION

Severe jasmonate deficiency in rice *AOC* mutants impairs development

Research on jasmonate biosynthesis and signalling in rice has been limited by the fact that well-characterized biosynthetic mutants were unavailable. We succeeded in isolating two jasmonate-deficient mutants, *cpm2* and *hebiba*. In this study, we demonstrate that mutations in *OsAOC*, which
encodes a key enzyme of JA biosynthesis, are responsible for the phenotypes of cpm2 and hebiba. OsAOC is a single-copy gene, which explains the severe jasmonate deficiency of the mutants. This is in contrast to Arabidopsis, which has four copies of AOC (Stenzel et al., 2003). Thus the mutants are excellent tools to study the function of jasmonate in rice.

In addition to the photomorphogenic phenotype of seedlings (Figure 1) (Riemann et al., 2003), the two mutants showed developmental phenotypes such as reduced fertility, early flowering and altered flower architecture (Figures 3c and 6, and Figure S5). We confirmed that the open-husk phenotype, as described for osjar1 mutants (Riemann et al., 2008), is a jasmonate-dependent phenotype. It will be interesting to investigate the role of jasmonate in husk closure in relation to genes such as OsCRINKLY4 (Pu et al., 2012) that have been shown to participate in this process. We further observed other abnormalities in the morphology of rice flowers that have not been described elsewhere, such as elongated sterile lemma (Figure 6a). In addition, we observed an early-flowering phenotype of the jasmonate-deficient mutants (Figure 6b).

In rice, phytochrome B regulates expression of the florigen Hd3a (Tamaki et al., 2003) via Heading date 1 (Ishikawa et al., 2011), and it has been shown that phytochrome-deficient mutants show an early-flowering phenotype (Izawa et al., 2002; Takano et al., 2005). Under short-day conditions, phyB mutants flowered approximately 10 days earlier compared to the wild-type (Takano et al., 2005). Under similar short-day conditions, the jasmonate-deficient mutants flowered 5-8 days earlier than the wild-type. Because jasmonate biosynthesis is likely to be regulated by phytochrome, which becomes obvious in the phenotype of cpm1, cpm2 and hebiba (Biswas et al., 2003; Riemann et al., 2003; Haga and lino, 2004; present work), it is an intriguing possibility that phyB regulation of florigen expression is mediated by jasmonate. This possibility warrants further study on the function of jasmonate in the regulation of heading time.

Both cpm2 and hebiba develop long coleoptiles under continuous red light (Figure 1), although the phenotype is more severe in hebiba compared to cpm2. While cpm2 is a specific OsAOC mutant with a deletion of 11 bp in the first exon, a 170 kb region is missing in hebiba, which includes OsAOC and other genes. A possibility that explains the difference in severity of phenotypes of these mutants is that hebiba is a complete loss-of-function mutant, while cpm2 produces a truncated, partially functional enzyme. However, this possibility is unlikely because both mutants are similarly incapable of synthesizing JA (Figure 5). Therefore, it is more likely that the difference in phenotype is caused by other genes deleted in hebiba (Table S2). One or more of those genes may mediate jasmonate-dependent growth inhibition. As the phenotypes of hebiba were complemented well using the OsAOC gene, the contribution of such genes may be minor.

**Jasmonate mediates resistance to M. oryzae**

The role played by jasmonate in the response to M. oryzae is under debate. It has been reported that the levels of JA and JA-Ile increase after blast infection of intact rice plants (Wakuta et al., 2011; Peng et al., 2012). However, Yara et al. (2008) did not find any clear change in blast susceptibility in transgenic rice plants in which expression of OsAOC or OsOPR7 was suppressed by RNAi.

Using the two jasmonate-deficient mutants cpm2 and hebiba, we were able to demonstrate that fungal hyphae of an incompatible strain of M. oryzae grow more easily within the tissue (Figure 7). The results indicate that the jasmonate present in the wild-type mediates at least a part of the defence response to M. oryzae. An increase in M. oryzae susceptibility of the mutants was observed with an incompatible strain but not with a compatible strain (Figure 7 and Figure S6). This may mean that endogenous jasmonate in the wild-type is not sufficient to defend against virulent, compatible strains, and that a further decrease in jasmonate in the mutants does not result in an apparent increase in infection. On the other hand, we showed that exogenously applied JA increases resistance to the compatible strain. This result suggests that, although not effective, jasmonate-mediated defence signalling also operates in a compatible interaction, although not in an effective manner.

The question is whether or not jasmonate levels are enhanced in leaves of wild-type rice in response to blast infection. Our investigations showed that the levels of JA and JA-Ile increase upon blast infection (see above). We observed blast-induced accumulation of JA-Ile but not free JA (Figure 8). This result suggests that OsJAR1, the rice enzyme catalysing conjugation of JA to Ile (Figure 8), is the primary metabolic target of blast infection. However, the partial disagreement with previous reports warrants further investigation (Yara et al., 2008; Wakuta et al., 2011; Peng et al., 2012).

**Blast-induced production of phytoalexins is mediated by jasmonate**

Several diterpenoid phytoalexins and a flavonoid phytoalexin, sakuranetin, have been identified in blast-infected rice leaves, with sakuranetin being the most abundant (Kodama et al., 1992; Koga et al., 1995 Grayer and Kookun, 2001). In fact, phytoalexins have been shown to be induced in rice leaves in response to M. oryzae infection (Kodama et al., 1992; Dillon et al., 1997). Furthermore, application of exogenous JA induced accumulation of momilactones and sakuranetin (Rakwal et al., 1996), and expression of naringenin-7-O-methyltransferase, a key enzyme of sakuranetin biosynthesis, was also found to be
induced by exogenous JA (Tamogami et al., 1997; Shimizu et al., 2012). These results suggest that blast-induced accumulation of phytoalexins is mediated by jasmonate. However, due to the lack of jasmonate-deficient mutants, it has been difficult to demonstrate whether endogenous jasmonate is involved in blast-induced phytoalexin accumulation.

In this paper, we demonstrate that blast-induced accumulation of sakuranetin and momilactones is impaired in jasmonate-deficient mutants, although accumulation of phytoalexins occurred normally in the mutants (Figure 9). It is notable that the induction of sakuranetin was almost completely abolished in the mutants. The result provides clear genetic evidence for the involvement of jasmonate in blast-induced accumulation of sakuranetin and momilactones. The results also indicate that a jasmonate-independent pathway operates for blast-induced accumulation of phytoalexins and partially for accumulation of momilactones. It is thus plausible that jasmonate-mediated phytoalexin accumulation contributes to the defence against blast fungus infection. Our results encourage further investigation into jasmonate-mediated phytoalexin accumulation as a defence mechanism against pathogenic micro-organisms.

EXPERIMENTAL PROCEDURES

Plant material

A japonica type rice, cv. Nihonmasari, was used as wild-type. This is also the genetic background of the mutants hebiba (Riemann et al., 2003) and cpm1 (Biswas et al., 2003). The cpm2 mutant was isolated from -ray-mutagenized M lines of Nihonmasari as described by Biswas et al. (2003).

For experiments determining the final coleoptile length, surface-sterilized caryopses were sown on 0.7% agar in clear plastic containers. Seedlings were raised at 25 °C under continuous far-red light (Riemann et al., 2008), and individuals with long coleoptiles were selected. SSR markers (Table S1) were applied to narrow down the genomic region deleted in hebiba. By inverse PCR, a product of 1800 bp was obtained using the primers 5- TGCTGACACTTCATGCGTACAG and 5- GCCGCCAACATTGTCG-3'. Subsequently, the flanking border sequences of hebiba were amplified directly from genomic hebiba DNA by PCR using the primers 5-GGCGGGTTAAATCTAGAGG-3' and 5'-CTGATGCCAACATAAGGATC-3'.

Complementation

cpm2 and hebiba were transformed with a genomic fragment containing OsAOC and a region of (primer sequences: 5-GGAAAGCTTCAAGAACACCATATAACGCT CTC-3' and 5-GCGGTACACACCATACATGCGTACAGCTGGA-3'). The genomic region was cloned into plG121-Hm-8 (http:// lifesciencedb.jp/dbdbj-e/card.cgi?project_id=725283) via HindIII and KpnI. The mutants were transformed with this plasmid using Agrobacterium. For each mutant, several lines of the T1 generation were assayed using the following approach. Coleoptyes were germinated under continuous red light. The final coleoptile length of each individual was recorded before transplantation into soil. Genomic DNA was extracted to examine the presence of the transgene and the mutation using the primers 5-GCTGACATT GCATTC-3' and 5- TACACAGGCATGCGTACAGA-3' for the transgene and 5- AGGACATCTCTCGACACCT-3' and 5'-CTG CGAGTCCGTGCA-3' for the cpm2 mutation. Analysis was performed by electrophoresis on a 4% agarose gel (NuSieve agarose, Lonza, http://www.lonza.com/). The presence of the hebiba mutation was examined by PCR using the primers 5'-CATGCGCATA TG-AAGATC-3' and 5'-GGCGGGTTAATCTAGAGG-3'. For cpm2 complementation, two selected T1 lines were assayed as described for the T1 generation. Additionally, the fertility of each plant was examined by counting empty flowers and flowers containing caryopses.

Expression and purification of GST fusion proteins

A partial sequence of OsAOC lacking the chloroplast transit peptide was amplified from Nihonmasari cDNA using Phusion DNA polymerase (Finzymes, http://www.thermoscientificbio.com/finzymes/) and the primers 5'-GGATCCCTCGAGTCCTGGGGATCC-3' and 5'-CTGAGAGCTATCGGTCCAAGA-3'. The PCR product was cloned into pGEX-6P-1 (GE Healthcare, http:// www3.gehealthcare.com/en/Global_Gateway) using BamHI and Xhol (New England Biolabs, https://www.neb.com/). Competent BL21 Escherichia coli were transformed with either pGEX-6-P1 plasmid containing OsAOC or OsAOS1 (K.H. and M.I. unpublished results).

For expression of GST-OsAOs1, the cells were cultivated in Luria-Bertani (LB) medium until an absorbance at 600 nm of 0.75-0.85 prior to induction of protein expression by isopropyl thio-/-o-galactoside (0.1 mM). To express GST-OsAOC, the culture medium was supplemented with isopropyl thio-/-o-galactoside (0.5 mM) directly. Both cultures were incubated for 2 days (18°C, 200 rounds per minute). The culture medium was removed by centrifugation (10 000 g, 4°C, 10 min) and resuspended in 50 mM Tris (pH 7.8) containing 300 mM NaCl, 100 mg/L cholic acid, 5 mM

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EDTA and 10 mM dithiothreitol. The sedimented cells were suspen-
ded in fresh buffer for washing and centrifuged (10 000 g, 4 °C, 10 min).
The supernatant was removed and the cells were sus-
pended in lysis buffer (50 mM Tris (pH 7.8), 300 mM NaCl, 100 mg
mL⁻¹ cholic acid, 5 mM EDTA and 10 mM DTT). After an incubation
of 1 hour on ice cells were disrupted in a French pressure cell, and
centrifuged (20 000 g, 4 °C, 40 min). The proteins were precipitated
by addition of an equal volume of 3.3 M ammonium sulfate con-
taining 50 mM Tris (pH 7.8). This suspension was centrifuged
(20 000 g, 4 °C, 10 min) and the sediment was resuspended in phos-
phate-buffered saline. Subsequently, the reaction products were
purified using glutathione Sepharose 4B (GE Healthcare) accord-
ing to the manufacturer's instruction. Fractions of purified proteins
were analysed by SDS–PAGE and Coomassie staining (Figure S2).

Coupled enzyme activity assay
The enzyme reaction for OsAOC was performed as described by
Stenzel et al. (2003) with minor modifications. Affinity-purified
GST–OsAOS1 (10 μg) and GST–OsAOC (30 μg) were incubated with
25 μM of 13-HPOT in 50 mM sodium phosphate buffer (pH 7,
25 °C, 1 h). Subsequently, the reaction products were extracted
with two volumes of ethyl acetate, and subjected to capillary GC–
MS after treatment with ethereal diazomethane. The enantiomer
preference of OsAOC was examined by chiral GC-MS using a
JMS-T01000 K9 system (JEOL, http://www.jiel.com/), fitted with a
wall-coated open tubular column fused-silica chemically bonded
capillary Chirasil-DEX column (length 10 m, 0.25 mm internal
 diameter, 0.25 μm thick; Varian, www.varianinc.com). The identifi-
cation of reaction products was performed as described previ-
ously (Tani et al., 2008).

Extraction and determination of jasmonates
Plant tissues (40–200 mg FW) were homogenized with a Multi
c.co.jp/) and suspended in 2 ml of 80% v/v methanol containing 1%
v/v acetic acid. After addition of 10 ng [1H₂]-JA and [13C₆]-JA
(JMS, Ltd, http://www.ssc-jp.com/) or a Sumichiral OA
www.home.agilent.com) equipped with a PEGASIL ODS column
www.home.agilent.com) or a PEGASIL ODS column (Shimizu
et al., 1999) and suspended in 2 ml of 80% v/v methanol containing
1% v/v acetic acid. After incubation (4 °C, 15 min, 800 g), the supernatant
was infected by the observed appressorium), as described by Tan-
abe et al. (2006). To supplement the hydroponic culture medium
with exogenous JA, (±)-JA was added (final concentration 100 μM)
24 h before inoculation.

Extraction and determination of phytoalexins
Plant tissue (40–200 mg FW) was homogenized using a Multi
Beads Shocker and suspended in 2 ml of 80% v/v methanol con-
taining 1% v/v acetic acid. The sample was centrifuged (4 °C,
15 min, 800 g). The supernatant was collected and subjected to
phytoalexin analysis by LC-ESI-MS/MS, comprising an API-3000
spectrometer with an electrosprey ion source and an Agilent 1100
HPLC instrument equipped with a Pegasil ODS column (Shimizu
et al., 2008). Phytoalexin levels were determined using combina-
tions of precursor and product ions (m/z 317/299 for phytocass-
anes A, D and E; m/z 335/317 for phytocassanc B; m/z 315/271 for
phytocassane C; m/z 315/271 for momilactone A; m/z 331/269 for
momilactone B; m/z 287/167 for sakuranetin) in MRM mode. The
retention times (in min) for phytocassanes A, B, C, D and E,
momilactones A and B, and sakuranetin were 4.8, 4.2, 3.8, 5.9, 5.3,
4.9 and 3.3, respectively.

ACKNOWLEDGEMENTS
This work was supported by the Japan Society for the Promotion of
Science (KAKENHI grant number 16370027) and the Ministry of
Education, Culture, Sports, Science and Technology (KAKENHI
grant number 23120524) to M.I., and by the Programme for Pro-
motion of Basic and Applied Researches for Innovations in Bio-orien-
ted Industries to K.O. and H.Y. T.S. was supported by the Japan
Society for the Promotion of Science as a research fellow. M.R.
was supported by a postdoctoral fellowship from the Japan Soci-
it for the Promotion of Science and the Alexander von Humboldt
Foundation. We would like to thank Y. Iguchi and Y. Mitsubayashi
for technical assistance.
SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Segregation of the long-coleoptile phenotype in the F2 mapping population.

Figure S2. Coomassie staining of affinity-purified GST-OsAOS1 and GST-OsAOC.

Figure S3. Cyclization of allene oxide to (+)-cis-OPDA by OsAOC.

Figure S4. Effect of wounding on accumulation of jasmonates in leaf blades.

Figure S5. Impaired husk closure in cpm2 and hebiba.

Figure S6. Magnaporthe oryzae (Ina86–137) inoculation assay with excised leaf sheaths.

Figure S7. Effect of fungal inoculation on accumulation of each component of the major diterpenoid phytoalexins of rice in wild-type, cpm2 and hebiba.

Table S1. Primer sequences of SSR markers.

Table S2. Genes deleted in hebiba.

REFERENCES


