Contrasting Effects of Ethylene Biosynthesis on Induced Plant Resistance against a Chewing and a Piercing-Sucking Herbivore in Rice

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ABSTRACT **Ethylene is a stress hormone with contrasting effects on herbivore resistance. However, it remains unknown whether these differences are plant- or herbivore-specific. We cloned a rice 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene, *OsACS2*, whose transcripts were rapidly up-regulated in response to mechanical wounding and infestation by two important pests: the striped stem borer (SSB) *Chilo suppressalis* and the brown planthopper (BPH) *Nilaparvata lugens*. Antisense expression of *OsACS2* (as-*acs*) reduced elicited ethylene emission, SSB-elicited trypsin protease inhibitor (TrypPI) activity, SSB-induced volatile release, and SSB resistance. Exogenous application of ACC restored TrypPI activity and SSB resistance. In contrast to SSB, BPH infestation increased volatile emission in as-*acs* lines. Accordingly, BPH preferred to feed and oviposit on wild-type (WT) plants—an effect that could be attributed to two repellent volatiles, 2-heptanone and 2-heptanol, that were emitted in higher amounts by as-*acs* plants. BPH honeydew excretion was reduced and natural enemy attraction was enhanced in as-*acs* lines, resulting in higher overall resistance to BPH. These results demonstrate that ethylene signaling has contrasting, herbivore-specific effects on rice defense responses and resistance against a chewing and a piercing-sucking insect, and may mediate resistance trade-offs between herbivores of different feeding guilds in rice.**

Key words: rice; *OsACS2;* ethylene; direct defense; indirect defense; herbivore resistance.

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INTRODUCTION

Ethylene is key regulator of plant development and defense. The hormone is elicited by insect attack and has been suggested to play an important role in induced resistance to herbivores (Lu et al., 2006; von Dahl and Baldwin, 2007; Zhou et al., 2011). Ethylene positively regulates the accumulation of defensive proteins and secondary metabolites, including phenolics, alkaloids, and terpenoids, as well as other volatile organic compounds (Horiuchi et al., 2001; Schmelz et al., 2003; Hudgins and Franceschi, 2004; Harfouche et al., 2006), most probably by synergizing jasmonate (JA) signaling (Schmelz et al., 2003; von Dahl and Baldwin, 2007). In accordance with its role as a defensive synergist, ethylene has been found to improve resistance against chewing herbivores. The fall armyworm *Spodoptera frugiperda*, for instance, grew better on maize plants that were treated with ethylene biosynthesis or perception inhibitors (Harfouche et al., 2006). In contrast, ethylene mutants in *Arabidopsis thaliana* were found to be more resistant against *Spodoptera littoralis* (Bodenhausen and Reymond, 2007) and tomato plants impaired in ethylene signaling supported lower potato aphid growth (Mantelin et al., 2009), implying that ethylene signaling can also reduce resistance against herbivores. Of particular importance in this context is that ethylene can also serve as a volatile signal that may alter herbivore behavior and attraction (Robert et al., 2012). It remains to be determined whether the influence of ethylene signaling on plant resistance depends on the plant species, the herbivore feeding

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guild, or the level of herbivore specialization. Molecular approaches may be particularly helpful in this context as, contrary to the use of biochemical inhibitors, they exclude chemical non-target effects on herbivores. Unfortunately, to date, ethylene mutants have only been used in dicot plant–insect models, and the precise role of ethylene signaling in monocot resistance remains largely unknown.

In both mono- and dicots, ethylene is synthesized via two enzymatic steps: the conversion of S-adenosyl methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) by an ACC synthase (ACS) and the oxidative cleavage of ACC by an ACC oxidase (ACO) (Zarembinski and Theologis, 1994; Wang et al., 2002). ACS has been demonstrated to be the rate-limiting enzyme of ethylene biosynthesis (Yang and Hoffman, 1984; Kende, 1993) and is commonly encoded by multi-gene families. In *A. thaliana* and rice (*Oryza sativa*), the ACS family has 12 and 6 members, respectively. According to their C-terminal sequences, ACSs are divided into three types (Iwai et al., 2006; Argueso et al., 2007). ACS transcript levels in plants are regulated in a developmental and tissue-specific manner and can be influenced by various biotic and abiotic stresses like insect attack or water stress (Zarembinski and Theologis, 1997; Van der Straeten et al., 2001; von Dahl et al., 2007; Mantelin et al., 2009). Phytohormones including ethylene, indole acetic acid (IAA), abscisic acid (ABA), and gibberellic acid (GA) also regulate ACS levels (Van der Straeten et al., 2001; Wang et al., 2005). ACS activity can be modulated by posttranslational modifications such as ubiquitination/degradation and protein phosphorylation. Recent studies have established that ACS protein turnover via the ubiquitin/26S proteasome system (UPS) plays an important role in regulating the production of ethylene. So far, several proteins in *Arabidopsis*, such as ETHYLENE OVERPRODUCING-1 (ETO1), ETO1-LIKE 1 (EOL1), and EOL2, members of the broad complex/tramtrack/bric-a-brac (BTB) protein superfamily that form components of Ub–protein ligases (or E3s) and participate in substrate recognition via the UPS, have been found to interact directly with type-2 ACSs and to promote their degradation via a proteasome-dependent pathway which results in a decrease of ethylene production (Chae et al., 2003; Wang et al., 2004; Christians et al., 2009). The phosphorylation of type 1 and type 2 ACS proteins blocks the ability of the ETO1/EOL proteins to bind, thus inhibiting the ubiquitination of these ACS proteins and their degradation by the 26S proteasome. In *Arabidopsis*, ACS2 and ACS6 can be phosphorylated by MPK6, thus leading to an accumulation of ACS proteins, an increase in ACS activity, and augmented ethylene production (Liu and Zhang, 2004; Joo et al., 2008; Han et al., 2010).

Rice, one of the most important food crops in the world, suffers heavily from insect pests (Cheng and He, 1996). Previous studies with rice have shown that herbivore attack induces a variety of plant hormones including jasmonic acid (JA), salicylic acid (SA), and ethylene, which subsequently regulate defensive responses, including the release of herbivore-induced plant volatiles (HIPVs) and the accumulation of trypsin proteinase inhibitors (TrypPIs; Lou et al., 2005a, 2005b, 2006; Lu et al., 2006; Zhou et al., 2009; Lu et al., 2011). Among the rice ACSs, *OsACS3*, *OsACS4*, and *OsACS6* are constitutively expressed, while *OsACS1* and *OsACS2* are induced upon infection by the rice blast fungus *Magnaporthe oryzae* (Iwai et al., 2006). Overexpression of *OsACS2* enhances the levels of pathogen-induced ethylene and defense gene transcripts as well as resistance to necrotrophic and hemibiotrophic fungal pathogens (Helliwell et al., 2011), suggesting that OsACS2 is implicated in the biosynthesis of pathogen-elicited ethylene. Exogenous application of ethephon can elicit the production of TrypPIs (Wang et al., 2011) and the release of volatiles in rice (Lu et al., 2006). However, the precise role of ethylene in herbivore-induced defense responses remains unknown.

To understand the importance of ethylene for rice resistance against herbivores, we isolated *OsACS2* and investigated its role in herbivore-induced defense responses by combining chemical and molecular analyses, reverse genetics, and bioassays with different herbivores. Our results provide new evidence for the role of ethylene in attacker-specific plant resistance to herbivores.

RESULTS

Isolation and Characterization of *OsACS2*

Using reverse transcription-polymerase chain reaction (RT–PCR), the full-length cDNA of *OsACS2* (TIGR ID: Os04g48850), including an open reading frame of 1452 bp, was obtained (Supplemental Figure 1). A phylogenetic tree deduced from the sequence alignment of 29ACSs revealed that OsACS2 belongs to type 2 (Supplemental Figure 2), together with the *A. thaliana* AtACS2 and AtACS6, whose C-terminal contains three conserved Ser residues that are targets for phosphorylation by MPK6 (Liu and Zhang, 2004), as well as a conserved Ser residue that is a phosphorylation site of CDPK (Tatsuki and Mori, 2001; Sebastià et al., 2004). Quantitative real-time PCR (qRT–PCR) analysis revealed low constitutive expression of *OsACS2*. Mechanical wounding and the striped stem borer (SSB) *Chilo suppressalis* caterpillar infestation resulted in a rapid and strong increase in *OsACS2* transcript levels, starting <0.5 h and peaking at 1 h after treatment (Figure 1A and 1B). The rice leaf folder (LF) *Cnaphalocrocis medinalis* and the brown planthopper (BPH) *Nilaparvata lugens* infestation also increased the mRNA levels of *OsACS2*, but the elicitation, especially BPH infestation, was slower than after mechanical wounding and SSB infestation (Figure 1C and 1D). Treatment with JA, SA, or the ethylene precursor ethephon did not strongly enhance the transcript accumulation of *OsACS2* (Figure 1E–1G). The data indicate that *OsACS2* is induced in a JA-, SA-, and ethylene-independent manner by wounding and herbivore attack.

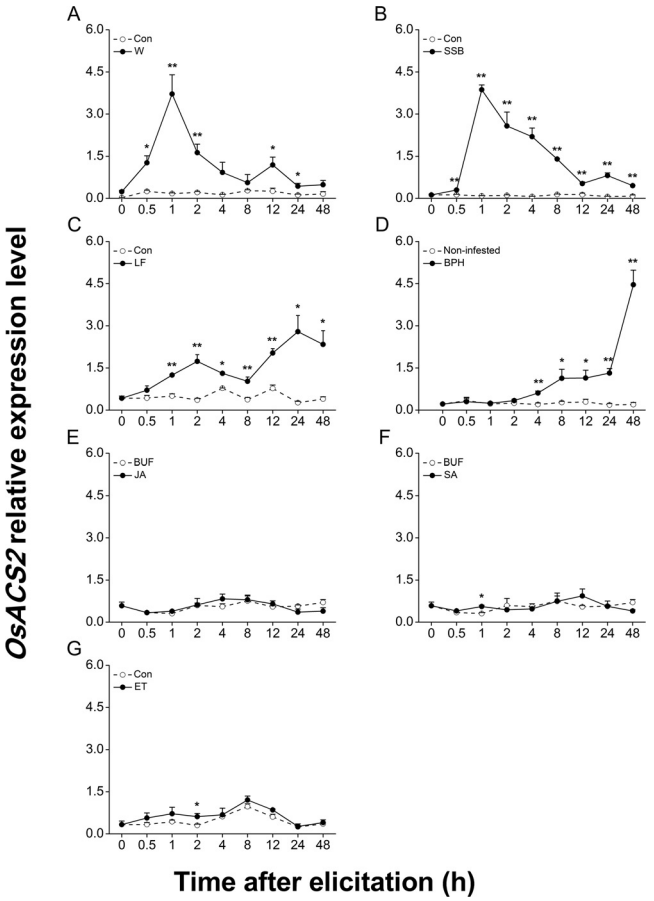

Figure 1 *OsACS2* Transcripts Are Induced upon Herbivore Attack.

Mean transcript levels (+SE, $n = 5$) of *OsACS2* in rice stems that were mechanically wounded **(A)**, infested by the striped stem borer (SSB) **(B)**, the brown planthopper (BPH) **(D)**, or treated with jasmonic acid (JA) **(E)**, salicylic acid (SA) **(F)**, or the ethylene releaser ethephon (ET) **(E)**, and in rice leaves that were infested by rice leaf folder (LF) **(C)**. W, mechanical wounding; BUF, buffer; Con, non-manipulated controls. Asterisks indicate significant differences in transcript levels between treatments and controls (* $P < 0.05$; ** $P < 0.01$, Student's *t*-tests).

OsACS2 Mediates Herbivore-Induced Ethylene Biosynthesis

To understand the role of *OsACS2* in the herbivore-induced ethylene biosynthesis and defense responses, we constructed pCAMBIA–1301 transformation vectors carrying a 421-bp reverse fragment of *OsACS2* (Supplemental Figure 3) and generated transgenic rice plants using *Agrobacterium tumefaciens*-mediated transformation. Using this procedure, we obtained two *OsACS2*-silenced (as-*acs*) T_2 homozygous lines (A1 and A30) with a single T-DNA insertion (Supplemental Figure 4). qRT–PCR analysis revealed that SSB-induced transcripts of *OsACS2* in A1 and A30 lines were only 33.2% and 30.8% of those in wild-type (WT) plants at 1 h after SSB caterpillar infestation (Figure 2A). Considering that *OsACS1* (Os03g51740) has the highest similarity (59.2%) in nucleotide sequence to *OsACS2* (Supplemental Figure 5), we investigated whether antisense expression of OsACS2 co-silences the expression of *OsACS1*. No difference was observed in levels of constitutive and SSB-induced *OsACS1* transcripts between WT and as-*acs* lines (Supplemental Figure 6). Silencing *OsACS2* did not influence growth and development of rice plants (Supplemental Figure 7). Ethylene quantification revealed that silencing *OsACS2* significantly reduced SSB- and BPH-elicited ethylene accumulation: the levels of ethylene in A1 and A30 lines were only 52.7% and 64.1%, and 32.8% and 48.2% of those in WT plants at 24 and 48 h after SSB infestation (Figure 2B); similarly, silencing *OsACS2* also reduced ethylene accumulation by about 45% at 8 and 24 h after BPH attack (Figure 2C). These results suggest that OsACS2 specifically regulates herbivore-induced ethylene biosynthesis. We also investigated whether silencing of *OsACS2* influences JA and SA biosynthesis. Constitutive and SSB-induced JA and SA levels were similar between as-*acs* lines and WT plants (Supplemental Figure 8), showing that ethylene signaling does not influence JA and SA accumulation in rice.

OsACS2 Regulates the Production of Herbivore-Induced TrypPIs and Volatiles

TrypPIs have been demonstrated to be important resistance proteins in rice against lepidopteran herbivores, such as SSB and LF (Zhou et al., 2009; Lu et al., 2011; Qi et al., 2011). No difference in basal TrypPI activity between as-*acs* lines and WT plants was observed. However, when plants were infested by SSB larvae, the TrypPI activity in as-*acs* lines was significantly lower than that in WT plants (Figure 3A). To determine whether the reduction in TrypPI activity in as-*acs* lines is caused by a reduction of ACC, which is produced by ACS from S-adenosylmethionine and then converted to ethylene, we measured TrypPI activity in ACC complemented as-*acs* lines. We found that TrypPI levels in as-*acs* lines that were treated with ACC and SSB were similar to WT plants (Figure 3B).

Herbivore-induced rice volatiles play an important role in host/prey location of natural enemies of herbivores (Lou et al., 2005a, 2005b, 2006; Lu et al., 2006; Qi et al., 2011). Therefore, we collected and analyzed the volatiles emitted from WT and as-*acs* plants when they were infested by SSB or BPH. In total, 20 volatile compounds were identified from the headspace of non-infested and herbivore-infested as-*acs* lines and WT plants (Figure 4 and Supplemental Tables 1 and 2). Herbivore infestation significantly increased the release of volatiles in both WT and as-*acs* plants. While there was no difference in constitutive

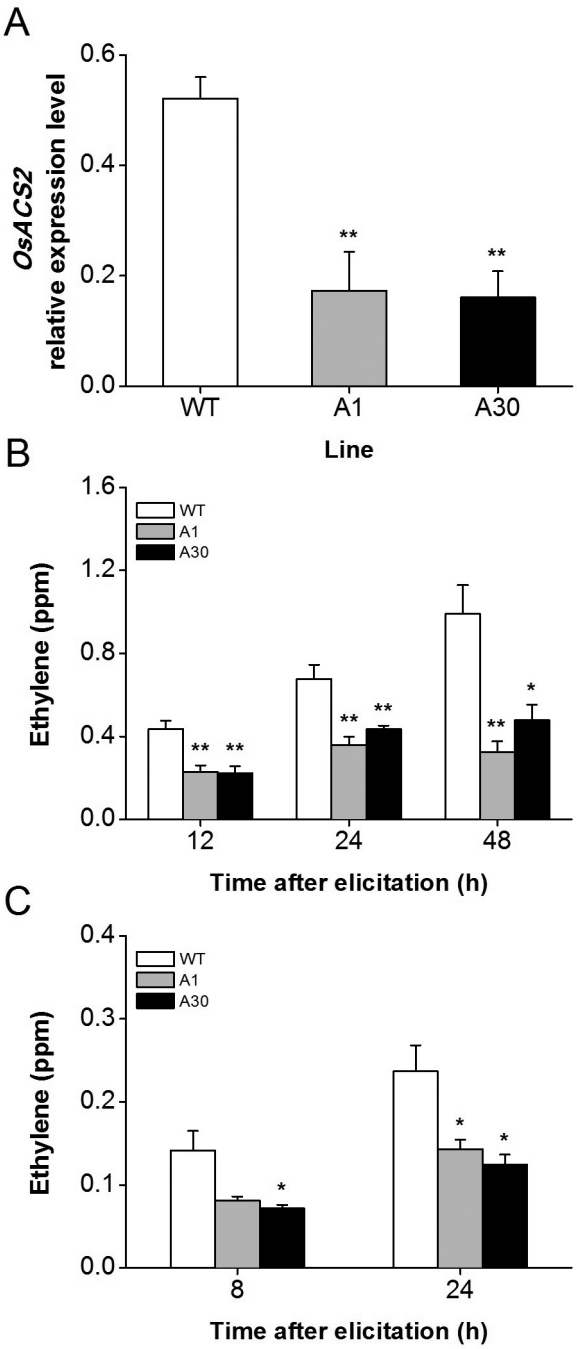

Figure 2 Silencing *OsACS2* Reduces Herbivory-Induced Ethylene Emission.

(A) Mean transcript levels (+SE, $n = 5$) of *OsACS2* in as-*acs* lines and wild-type (WT) plants that were individually infested by SSB for 1 h.

(B) Mean levels (+SE, $n = 5$) of ethylene emitted from WT plants and as-*acs* lines infested with a single third-instar larva of the striped stem borer (SSB).

(C) Mean levels (+SE, $n = 5$) of ethylene emitted from WT plants and as-*acs* lines infested with 12 gravid BPH female adults. Asterisks indicate significant differences between as-*acs* lines and WT plants (* $P < 0.05$; ** $P < 0.01$, Student's *t*-tests).

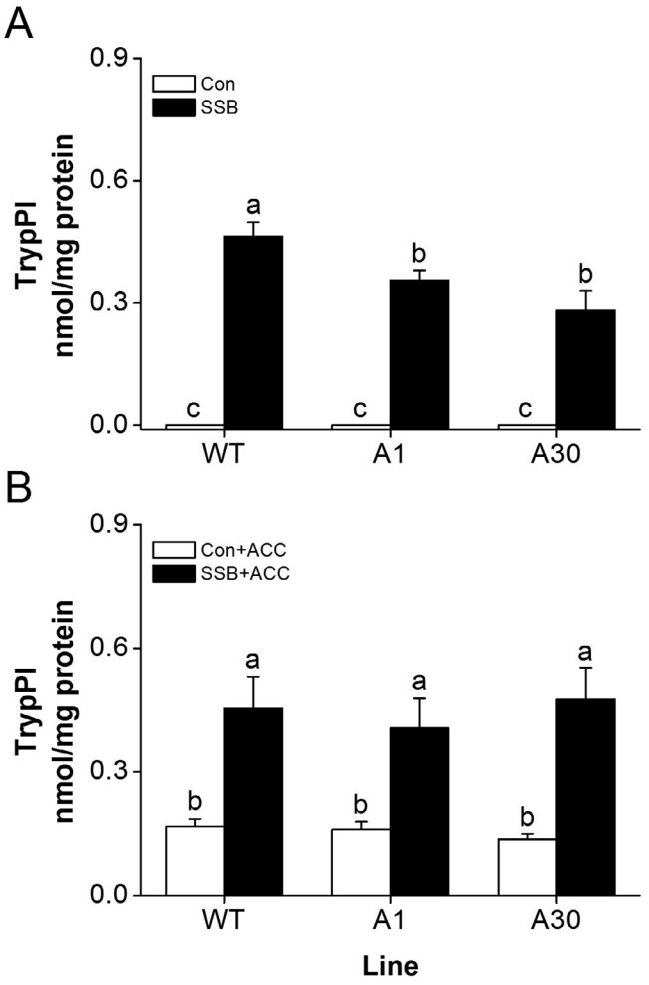

Figure 3 Ethylene Regulates Herbivory-Induced TrypPI Activity.

(A) Mean TrypPI activity (+SE, $n = 5$) in wild-type (WT) and as-*acs* plants that were kept herbivore-free (Con) or where infested by the striped stem borer (SSB) for 3 d.

(B) Mean TrypPI activity (+SE, $n = 5$) in WT and as-*acs* plants that were first treated with ACC for 1 d followed by a third-instar SSB larva for 3 d (SSB+ACC) or treated with ACC only (Con+ACC). Letters indicate significant differences between treatments and lines ($P < 0.05$, Duncan's multiple range test).

volatiles between as-*acs* lines and WT plants, SSB-infested as-*acs* lines emitted significantly lower amounts of volatiles than WT plants (Figure 4 and Supplemental Table 1). The release of 11 compounds was obviously impaired in as-*acs* lines. Surprisingly, when infested by BPH, as-*acs* lines emitted significantly higher amounts of volatiles than WT plants (Figure 4 and Supplemental Table 2): the levels of six compounds, including 2-heptanone, 2-heptanol, α-thujene, (+)-limonene, (E)-linalool oxide, and linalool, were significantly enhanced in as-*acs* lines. Again, ACC complementation of SSB-infested WT and as-*acs* plants attenuated this difference (Supplemental Table 3).

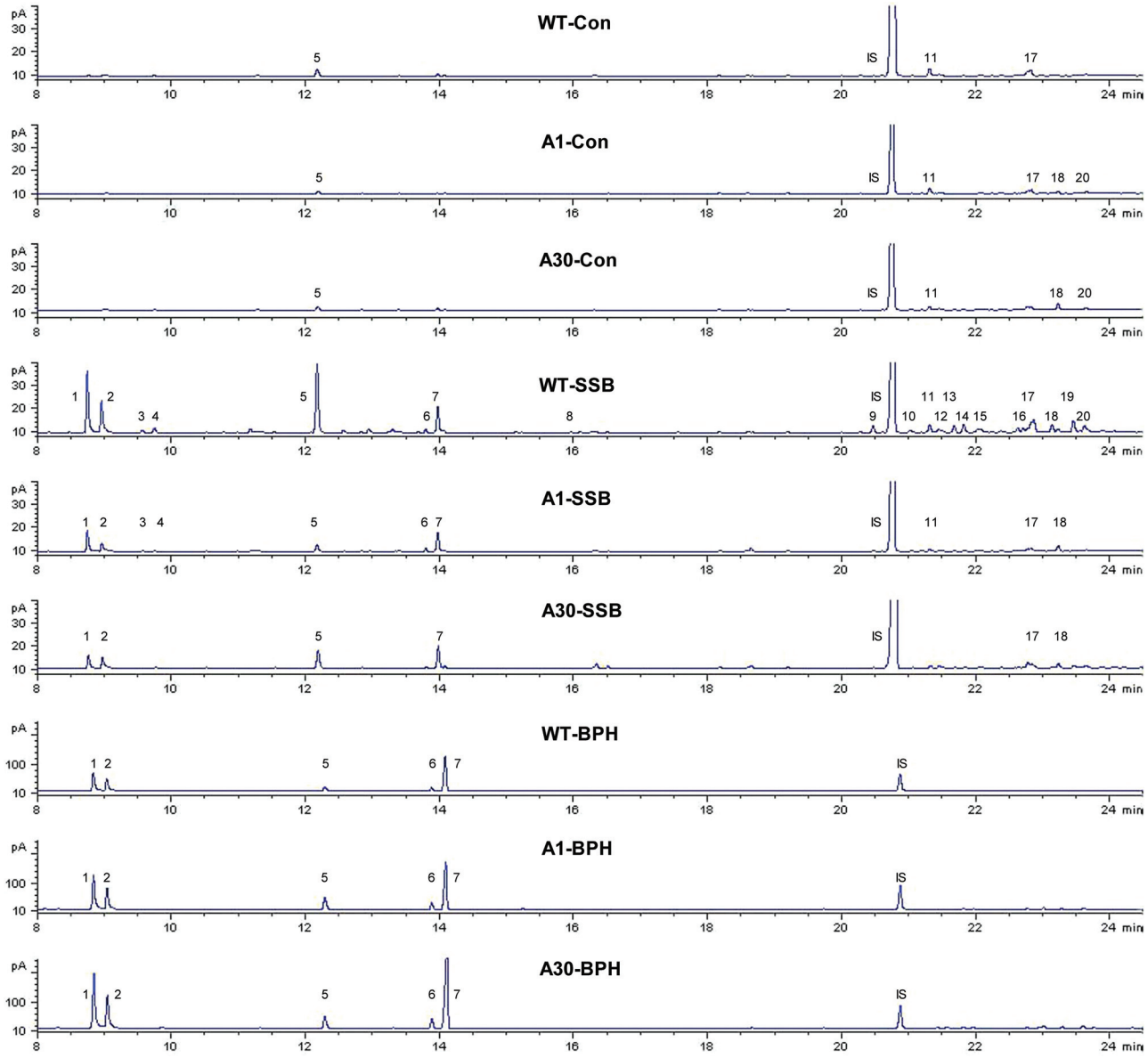

Figure 4 Ethylene Regulates Herbivory-Induced Volatile Emission.

Typical chromatograms obtained by head space collections from non-manipulated (Con), striped stem borer (SSB)-, or brown planthopper (BPH)-infested wild-type and as-*acs* plants. Numbers represent chemicals as described in Supplemental Tables 1–3.

OsACS2 Positively Mediates Rice Resistance to SSB

We assessed the performance of SSB on transgenic lines and WT plants to determine whether ethylene signaling mediates resistance to rice herbivores. SSB caterpillars gained more mass on as-*acs* lines than on WT plants (Figure 5A). By day 12, the masses of SSB caterpillars that fed on A1 and A30 lines were about 1.6-fold higher than those that fed on WT plants. In accordance with this finding, as-*acs* lines were more severely damaged by SSB infestation than WT plants (Figure 5C). However, when plants were treated with ACC, SSB caterpillars that fed on as-*acs* lines gained the same weight as on WT plants (Figure 5B).

OsACS2 Negatively Regulates Direct and Indirect Resistance in Rice to BPH

We also investigated whether antisense expression of *OsACS2* influences BPH preference and performance. Compared to as-*acs* lines, BPH female adults preferred to feed and lay eggs on WT plants (Figure 6A and 6B). The number of BPH eggs on A1 and A30 lines was only 30.6% and 32.3% of those on

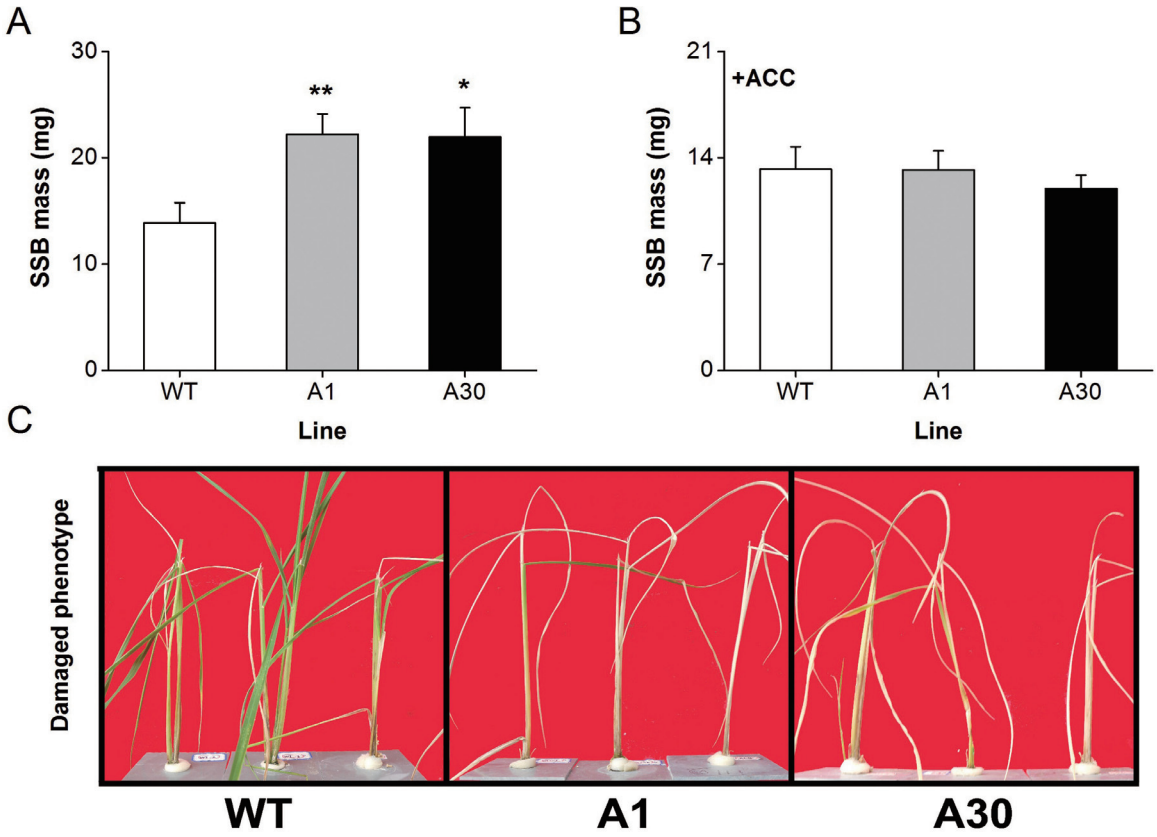

Figure 5 Ethylene Increases Resistance against the Striped Stem Borer (SSB).

(A) Mean larval mass (+SE, $n = 60$) of SSB that fed on wild-type (WT) plants or as-*acs* lines for 12 d.

(B) Mean larval mass (+SE, $n = 60$) of SSB that fed on WT plants or as-*acs* lines that were pretreated with 100 μM ACC. Asterisks indicate significant differences between as-*acs* lines and WT plants (* $P < 0.05$; ** $P < 0.01$, Student's *t*-tests).

(C) Damage phenotypes of as-*acs* lines and WT plants that were individually infested by a third-instar SSB larva for 14 d ($n = 6$).

WT plants, respectively. Consistently with the feeding preference, the amount of honeydew, an indicator of food intake, excreted by BPH female adults fed on A1 and A30 lines were reduced by 33.6% and 26.0%, respectively, compared to WT plants (Figure 6F). However, antisense expression of OsACS2 did not influence the survival rate of BPH nymphs (Figure 6E). Similarly to resistance to SSB, exogenous application of ACC restored feeding and oviposition preference of BPH female adults for as-*acs* lines and WT plants (Figure 6C and 6D). In accordance with the increase in BPH-induced volatiles in as-*acs* lines, the volatiles emitted from BPH-infested as-*acs* lines were more attractive to *Anagrus nilaparvatae*, an egg parasitoid of BPH, than those from BPH-infested WT plants (Figure 6G). Our results therefore suggest that ethylene deficiency increases rice direct and indirect resistance against BPH.

Altered Volatile Chemicals but Not Ethylene in as-*acs* Lines Influence the Feeding and Oviposition Preference of BPH

To investigate whether the reduced preference of BPH for as-*acs* lines resulted from the change in induced volatiles or the reduced ethylene emissions, we investigated the influence of synthetic ethylene, 2-hepenone, and 2-heptanol, all of which were dramatically induced by BPH infestation and changed in as-*acs* lines, on the behavior and host preference of female BPH adults. Linalool also influenced by as-*acs* silencing, but was not tested, as its role as a BPH repellent is well established (Xiao et al., 2012). Our data revealed that ethylene by itself did not influence the behavioral response of female BPH adults (Supplemental Figure 9). Two-hepenone and 2-heptanol, on the other hand, had a repellent role to BPH: BPH female adults preferred to feed and oviposit on control plants over plants that were treated with 2-hepenone or 2-heptanol (Figure 7).

DISCUSSION

In this study, we combined chemical and molecular analyses, reverse genetics, and bioassays to elucidate the role of ethylene in rice resistance against different pest insects. We found that the transcript levels of *OsACS2* were strongly increased after plants were wounded or infested by insects (Figure 1). Antisense expression of *OsACS2* reduced the

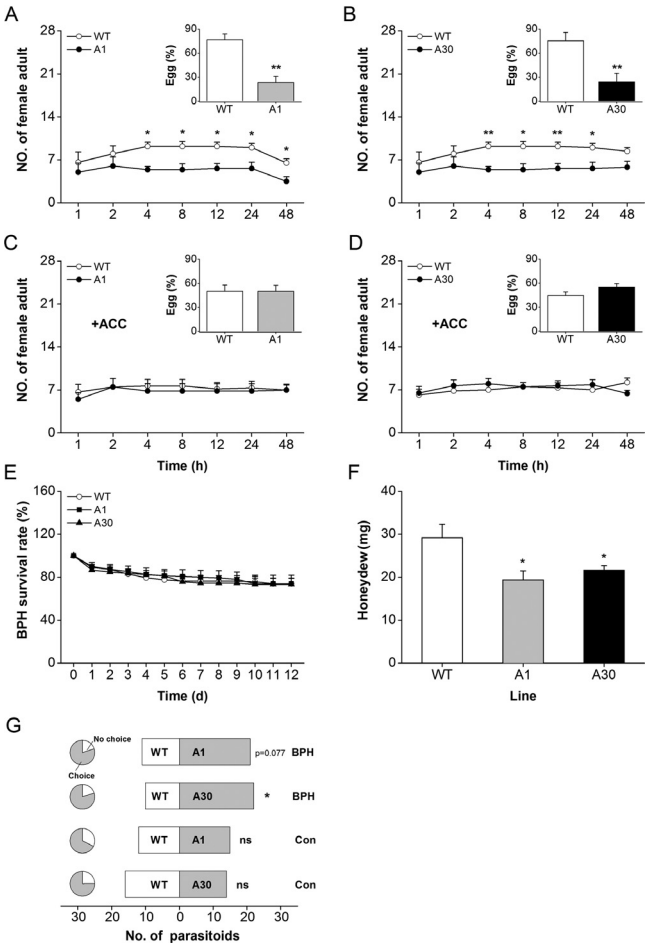

Figure 6 Ethylene Decreases Resistance against the Rice Brown Planthopper (BPH).

(A, B) Mean number of BPH female adults (+SE, *n* = 6) settling on wild-type (WT) versus as-*acs* plants in paired choice assays.

(C, D) Mean number of BPH female adults (+SE, *n* = 6) on WT and as-*acs* plants that were individually treated with ACC. Inserts: Mean percentage (+SE, *n* = 6) of BPH eggs per plant on pairs of plants as stated above.

(E) Mean survival rate (+SE, *n* = 10) of BPH nymphs on as-*acs* lines or WT plants 1–12 d after the start of feeding.

(F) Mean amount of honeydew per day (+SE, *n* = 18) secreted by BPH adult females feeding on as-*acs* lines or WT plants.

(G) Number of *Anagrus nilaparvatae* parasitoids attracted to non-infested WT plants (Con) versus non-infested plants of ac-*acs* lines (A1 and A30); BPH-infested WT plants (BPH) versus BPH-infested WT plants of as-*acs* lines (A1 and A30). Asterisks indicate significant differences between as-*acs* and WT plants (* $P < 0.05$; ** $P < 0.01$, (A, B, F): Student's *t*-tests, (G): χ^2 test).

levels of herbivore-induced ethylene, which in turn modulated the production of direct and indirect defense compounds and the resistance of rice to SSB and BPH (Figures 2–6). All changes in as-*acs* lines could be restored to WT levels by supplying the ACS product ACC (Figures 3, 5, and 6). These data show that OsACS2 is required for the herbivore-induced production of ethylene, and that the ethylene signaling pathway plays an important role in resistance of rice against herbivores.

Rice has six ACS genes. Previous studies revealed that these OsACS genes respond to environmental stresses. For instance, the transcripts of *OsACS1* and *OsACS5* were induced by submergence (Zarembinski and Theologis, 1997; Van der Straeten et al., 2001), and *OsACS5* was also induced by application of ethylene and GA, but inhibited by ABA (Van der Straeten et al., 2001). *OsACS3*, *OsACS4*, and *OsACS6* were found to be constitutively expressed, whereas *OsACS1* and *OsACS2* were up-regulated when infected by *M. oryzae* (Iwai et al., 2006). Here, we found that *OsACS2* mRNA levels also increased after mechanical wounding and herbivore infestation, but not after application of JA, SA, and ethephon, an ethylene releaser (Figure 1). Taken together, these results suggest that the different *OsACSs* may have specific response patterns to environmental signals, which may reflect their different roles in regulating ethylene-mediated plant processes. In this context, *OsACS2* may enable plants to respond to biotic stress by up-regulating ethylene production. Support for this hypothesis comes from other plant systems. For example, the transcript levels of tobacco *NtACS2* and *NtACS3*, belonging to type 2 of ACSs (Supplemental Figure 2), increase when plants are infected by tobacco mosaic virus (TMV) (Kim et al., 2003). Similarly, *NaACS3a* in *Nicotiana attenuata* is induced by treatment with mechanical wounding plus *Manduca sexta* oral secretions (OS) (von Dahl et al., 2007) and *LeACS2* transcripts in *Lycopersicon esculentum* increase following *Macrosiphum euphorbiae* infestation (Mantelin et al., 2009). This suggests that type 2ACSs are reactive to biotic stresses. Herbivore attack generally results in the release of ethylene in various plant species, including *N. attenuata* and *A. thaliana* (De Vos et al., 2005; von Dahl et al., 2007). In rice, SSB and BPH infestation strongly induced the release of ethylene in previous studies (Lu et al., 2006, 2011; Qi et al., 2011; Zhou et al., 2011). Here we demonstrate that antisense expression of *OsACS2* significantly suppressed the release of SSB- and BPH- induced ethylene: a 60%–70% reduction in transcript levels of *OsACS2* in as-*acs* lines resulted in a 40%–60% decrease in ethylene levels (Figure 2). The findings indicate that OsACS2 mediates the biosynthesis of herbivore-induced ethylene in rice.

In general, the ethylene signaling pathway plays an important, but variable, role in regulating plant resistance to herbivores. For example, in *A. thaliana*, the ethylene pathway negatively regulates plant resistance to the chewing generalist *S. littoralis* and *Spodoptera exigua*, whereas it has no influence on resistance to the chewing specialists *Pieris rapae* and *Plutella xylostella* as well as the phloem-feeding aphids *Myzus persicae* and *Brevicoryne brassicae* (Stotz et al., 2000; Mewis et al., 2005; Bodenhausen and

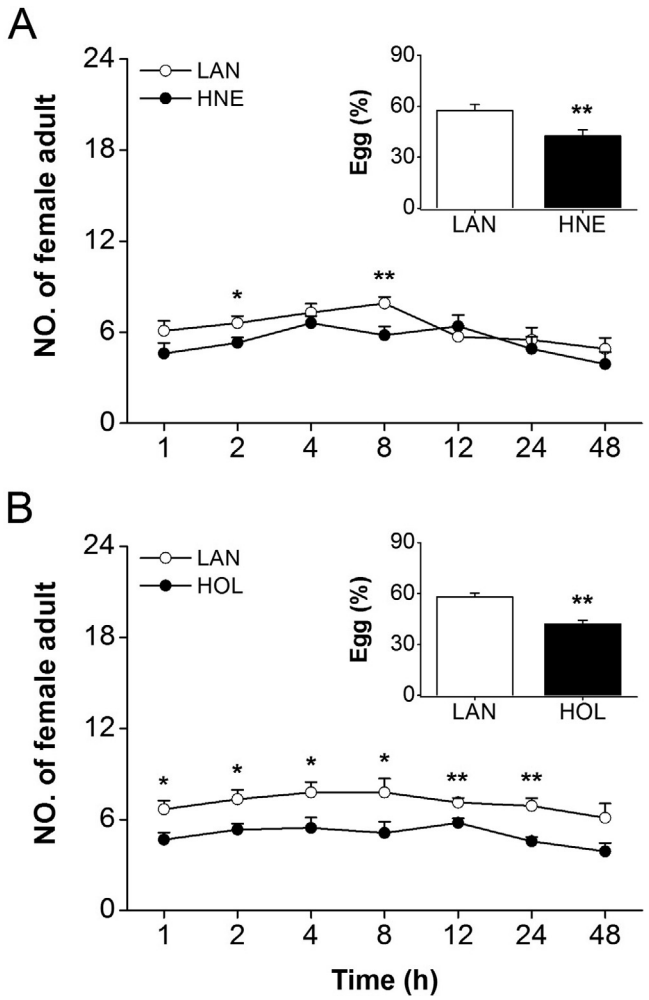

Figure 7 Two Volatile Chemicals, 2-Heptanone and 2-Heptanol, Are Repellent to the Brown Planthopper (BPH).

(A) Mean number of BPH female adults (+SE, n = 8) settling on wild-type (WT) plants treated with lanolin alone (LAN) versus WT plants treated with 250 nmol of 2-heptanone in 10 μl of lanolin (HNE) in paired choice assays.

(B) Mean number of BPH female adults (+SE, n = 8) settling on WT plants treated with lanolin alone (LAN) versus WT plants treated with 250 nmol of 2-heptanol in 10 μl of lanolin (HOL) in paired choice assays. Inserts: Mean percentage (+SE, n = 10) of BPH eggs per plant on pairs of plants as stated above. Asterisks indicate significant differences in as-*acs* lines compared to WT plants (* $P < 0.05$; ** $P < 0.01$, Student's t-tests).

Reymond, 2007). In tomato, plant resistance to the aphids *M. persicae* (Boughton et al., 2006) and *M. euphorbiae* (Mantelin et al., 2009) are negatively modulated by ethylene signaling. In maize, pharmacological studies revealed that ethylene positively mediates plant resistance to the chewing generalist *S. frugiperda* (Harfouche et al., 2006). Here we found that ethylene signaling positively regulates rice resistance to a chewing specialist SSB, but negatively modulates resistance to the phloem-feeding specialist BPH (Figures 5 and 6).

As stated above, ethylene pathway regulates many plant defense compounds, including for example hydroxyproline-rich glycoproteins that are involved in cell wall strengthening (Toppan et al., 1982; Roby et al., 1985). Therefore, ethylene signaling may mediate plant herbivore resistance via the reinforcement of structural or chemical barriers (Adie et al., 2007). Here we found that antisense expression of *OsACS2* decreased elicited ethylene, but not JA and SA levels (Supplemental Figure 7), which in turn reduced TrypPI activity and the release of SSB-induced volatiles and decreased resistance against SSB (Figures 3–5 and Supplemental Table 1). Exogenous application of ACC to as-*acs* lines restored TrypPI activity, volatile release, and rice resistance to SSB (Figures 3B and 5B, and Supplemental Table 3). Our previous studies revealed that SSB infestation synchronously elicits multiple signal molecules, including JA, SA, ethylene, and H_2O_2 (Zhou et al., 2009; Lu et al., 2011; Qi et al., 2011; Zhou et al., 2011), and that JA-mediated signaling plays an important role in the production of herbivore-induced TrypPIs (Zhou et al., 2009; Lu et al., 2011; Qi et al., 2011) and the emission of volatiles in rice (Lou et al., 2005b). Taken together, these data suggest that the production of SSB-induced TrypPIs and volatiles in rice is synergistically regulated by both the JA and ethylene signaling pathways, as reported in other plants (Xu et al., 1994; O'Donnell et al., 1996; Schmelz et al., 2003; Zhang et al., 2013). Moreover, the results also indicate that ethylene signaling positively regulates rice resistance to SSB, possibly via the activation of TrypPIs (Zhou et al., 2009; Lu et al., 2011). Further research should investigate whether ethylene signaling can also influence rice resistance to SSB by mediating other defense compounds or cell wall strengthening.

A particularly noteworthy finding of our study is that ethylene deficiency has herbivore-specific effects on rice defense responses and resistance. Contrary to SSB, ethylene deficiency enhanced the release of BPH-induced volatiles and increased direct and indirect resistance to the plant hopper (Figures 4 and 6). BPH infestation is known to elicit multiple signaling pathways, including JA, SA, ethylene, and H_2O_2 signaling (Zhou et al., 2009; Lu et al., 2011; Qi et al., 2011; Zhou et al., 2011). Compared with SSB infestation, BPH infestation generally weakly induces JA pathway but strongly elicits SA and H_2O_2 (Zhou et al., 2009). Ethylene may function as an inhibitor of these BPH-dominant pathways—a mechanism which could explain the contrasting effects of ethylene deficiency on the two herbivores. Other elements of the rice defense signaling cascade, including JA (Zhou et al., 2009) and OsERF3 (Lu et al., 2011), have been shown to have similar divergent effects.

It has been reported that green leafy volatiles (Qi et al., 2011) and herbivore-induced volatiles (Xiao et al., 2012) emitted from rice can influence feeding and oviposition preference of BPH. Moreover, a recent study demonstrates that ethylene is important in mediating interactions between chewing and phloem-feeding insects and higher trophic levels in *A. thaliana* (Zhang et al., 2013). We found that antisense expression of *OsACS2* increased the amount of BPH-induced volatiles (Supplemental Table 2) and some of these volatile chemicals, including 2-heptanone, 2-heptanol (Figure 7), and linalool (Xiao et al., 2012), had a repellent role to BPH. However, ethylene itself had no effect on the host location of BPH (Supplemental Figure 9). Thus, the effect of ethylene signaling pathway on BPH feeding and oviposition preference is likely due to the increased emission of repellent rice volatiles, such as 2-heptanone, 2-heptanol, and linalool.

From an agroecological perspective, our results suggests that altered ethylene production may lead to resistance trade-offs: ethylene-deficient plants in the field are likely to be more susceptible to chewing herbivores like SSB, but at the same time may attract fewer BPH adults, which, depending on the composition of the herbivore community, may be beneficial or costly to the plant. Clearly, the role of ethylene in plant–herbivore community interactions deserves further attention.

In summary, we demonstrate that OsACS2 regulates the biosynthesis of herbivore-induced ethylene production, defense induction, and resistance in rice. We propose that ethylene may be an important node that mediates trade-offs between resistance against insects with different feeding modes.

METHODS

Plant Growth

The rice genotype Xiushui 11 (WT) and two as-*acs* transgenic lines (see below) were used in this study. Plants were grown as described by Lu et al. (2011). Forty-five-day-old plants that individually planted in 500-ml hydroponic plastic pots were used for the experiments.

Insects

Colonies of SSB, LF, and BPH were originally obtained from rice fields in Hangzhou, China, and reared on Xianyou 63 (a rice variety that is susceptible to SSB, LF, and BPH) rice seedlings as described in Zhou et al. (2009).

Isolation and Characterization of *OsACS2* cDNA

The primers ACS-F1 (5′-AGTCCGACACCAAATCAATG-3′) and ACS-R1 (5′-CTCCGAATCTCCGATACCC-3′) were designed based on the sequence of rice *OsACS2* (TIGR ID: Os04g48850). Using RT–PCR, we obtained the full-length cDNA of *OsACS2* from total RNA isolated from WT plants infested by SSB for 24 h. The amplified fragments were cloned into the pMD19-T vector (TaKaRa) and sequenced. DNA analysis was carried out using BLAST searches (http://blast.ncbi.nlm.nih.gov/). Amino acid sequences were deduced using DNAMAN (www.lynnon.com/). Phylogenetic tree analysis was performed by Clustalx (www.clustal.org/).

Generation of Transgenic Plants

A 421-bp sequence of *OsACS2* was PCR-amplified using the primers 5′-GCTCTAGAGGTGAAGCTGAACGTGTC-3′ and 5′-GGGGTACCGTACCTGCATCGGAAAGA-3′, and was digested by *Kpn*I and *Xba*I, and cloned into pCAMBIA1301, yielding an antisense transformation vector pCAMBIA–asACS2 (Supplemental Figure 3). The vector was inserted into the Xiushui 11 to generate T_0 transgenic plants using *A. tumefaciens*-mediated transformation (Zhou et al., 2009). The procedure of transforming rice, screening the homozygous T_2 plants, and identifying the number of insertions are described in Zhou et al. (2009). Two homozygous T_2 lines (A1 and A30), each with a single insertion (Supplemental Figure 4), were used for experiments.

Plant Treatments

To wound plants mechanically, plant stems (about 2-cm length of the lower part) were individually pierced 200 times using a needle. Non-manipulated plants were used as controls (Con). For SSB treatment, plant stems were infested using a third-instar larva of SSB that had been starved for 2 h. Controls (Con) were not manipulated. For LF treatment, plant leaves at node 4 (the youngest fully expanded leaf was defined as leaf node 1) were infested by a third-instar larva of LF that had been starved for 2 h. Non-manipulated plants were used as controls (Con). For BPH treatment, plant stems were infested with 12 gravid female adults of BPH that were confined in a glass cage (diameter 4 cm, height 8 cm, with 48 small holes, diameter 0.8 mm). Plants with an empty cage were used as controls (Non-infested). For JA or SA treatment, plants were individually sprayed with 2 ml of JA solution (100 μg ml^{-1}) or SA (70 μg ml^{-1}) in 50 mM sodium phosphate buffer. Controls (BUF) were sprayed with 2 ml of buffer. For ethephon treatment, one plant was covered with a sealed glass cylinder (diameter 4 cm, height 50 cm), and then a 50-ml glass beaker with 20 ml of 1 mmol L^{-1} ethephon (Sigma-Aldrich Company, USA) in distilled water (pH = 7.2) was placed into the cylinder. Controls (Con) were similarly treated with 20 ml of distilled water. For ACC treatment, ACC was applied in the nutrient solution to a final concentration of 100 μM (Young et al., 2004). An equal volume of water was added into the nutrient

solution of controls (Con). For 2-heptanone or 2-heptanal treatment, rice stems were individually treated with 2-heptanone (250 nmol) or 2-heptanal (250 nmol) in 10 μl of lanolin paste. Control (lanolin) plants were treated with 10 μl of pure lanolin. For replicate numbers, see individual experiments below.

Quantitative Real-Time PCR

For qRT–PCR analysis, five independent biological samples were used. Total RNA was isolated using the SV Total RNA Isolation System (Promega), following the manufacturer's instructions. One mg total RNA sample was reverse-transcripted using the PrimeScript™ RT–PCR Kit (TaKaRa). The qRT–PCR assay was performed on the CFX96™ Real-Time System (Bio-Rad) using Premix Ex Taq™ Kit (TaKaRa). A rice actin gene *OsACT* (TIGR ID: Os03g50885) was used as an internal standard to normalize cDNA concentrations. The primers and probe sequences used for mRNA detection of target genes by qRT–PCR are provided in Supplemental Table 4.

JA and SA Analysis

Plants were randomly assigned to SSB and control treatments. Two *as-acs* lines (A1 and A30) and one WT line were used. The stems were harvested at 0, 1.5, and 3 h after SSB infestation, and JA and SA levels were analyzed by gas chromatography–mass spectrometry (GC–MS) using internal standards as described in Lou and Baldwin (2003). Each treatment at each time interval was replicated five times.

Ethylene Analysis

For ethylene measurements, two independent experiments were performed. In the first experiment, plants of the two *as-acs* lines (A1 and A30) and one WT line were randomly assigned to SSB and control treatments. Three plants were covered with a sealed glass cylinder (diameter 4 cm, height 50 cm), and ethylene production was determined at 12, 24, and 48 h after the start of the experiment using the same method as described by Lu et al. (2006). In the second experiment, the two *as-acs* lines (A1 and A30) and one WT line were randomly assigned to BPH and control treatments. Ethylene accumulation was measured at 8 and 24 h after BPH infestation. Each treatment at each time interval was replicated five times.

TrypPI Activity Analysis

Plants from each line (A1, A30, and WT lines) were randomly assigned to SSB and control treatments. Plant stems (0.2–0.3 g per sample) were harvested at 0 and 3 d after the start of the experiment. The TrypPI activity was measured using a radial diffusion assay as described in Van Dam et al. (2001). Each treatment at each time interval was replicated five times.

Volatile Collection and Isolation

The collection, isolation, and identification of rice volatile used the same method as described in Lou et al. (2005b). Volatiles emitted from individual plants of each line (A1, A30, and WT lines) that were infested with SSB, BPH for 24 h, or left uninfested were collected. Quantities for each compound were expressed as percentage of peak areas relative to the internal standard (IS, diethyl sebacate) per 8 h of trapping for one plant. Collections were replicated five to eight times for each treatment.

Olfactometer Bioassays

Behavioral responses of *A. nilaparvatae* females to rice volatiles were performed in a Y-tube olfactometer using the same method as described in Lou et al. (2005a). The response of the parasitoid exposed to the following pairs of odor sources was determined: (1) a BPH-infested plant of each *as-acs* line (A1 or A30) versus a BPH-infested WT plant (infestation for 24 h); (2) a control plant of each *as-acs* line versus a control WT plant. In total, five pairs of plants and 40 female parasitoids were used for each odor source combination.

The effect of ethylene on BPH behavior was investigated in an H-shaped olfactometer using the same method as described in Lou and Cheng (2003). Odor sources were caged in two glass arms (12 cm diameter × 30 cm height) of the H-shaped olfactometer, and the two arms were connected by a small glass tube (6 cm diameter × 15 cm long). Two ends of the small glass tube were covered with nylon mesh, and its middle part was provided with a small hole (1 cm diameter) to release BPH individuals. The behavioral response of BPH to two pairs of odor sources was determined: (1) a 10-ml cotton-covered glass bottle containing 20 ppm ethylene (ethylene treatment; 1 h later, the ethylene concentration in the arm is 0.1 ppm, which is equivalent to that emitted from a plant that was infested by 12 gravid female BPH adults for 8 h) versus a 10-ml cotton-covered glass bottle with pure air (control); (2) a 10-ml cotton-covered glass bottle containing 20 ppm ethylene plus one WT plant versus a 10-ml cotton-covered glass bottle with pure air plus one WT plant. In all bioassays, 15 gravid female BPH adults that were starved for 2 h were introduced into the small glass tube of the H-shaped olfactometer. One hour later, the number of BPH in each half of the small glass tube was recorded. Each experiment was replicated eight times.

Herbivore Resistance Experiments

For SSB performance, freshly hatched SSB larvae were allowed to feed on plants of each line (A1, A30, and WT

lines; three larvae per plant). Twenty replicate plants from each line were used. Larval mass was measured 12 d after the start of the experiment. To determine the difference in plant tolerance to SSB infestation, A1, A30, and WT lines were individually infested with a SSB third-instar larva. Every day, the damage levels of plants were checked and photographs were taken.

To investigate the colonization and oviposition behavior of BPH female adults, pots with two plants (a transgenic plant versus a WT plant or a WT plant treated with lanolin versus a WT plant treated with 2-heptanone or 2-heptanal) were confined with glass cylinders (diameter 4 cm, height 8 cm, with 48 small holes, diameter 0.8 mm). Each cylinder received 12 gravid BPH females. The number of BPH individuals on each plant was counted at 1, 2, 4, 8, 12, 24, and 48 h after the initial release. After the last count, BPH eggs on each plant were counted under a microscope. The experiment was replicated six times. The survival rates of BPH nymphs on WT and as-*acs* plants were also determined. Plant stems were individually covered with a glass cylinder, into which 15 BPH neonates were released. Every day, the numbers of surviving BPH on each plant was recorded until 12 d after the release of the herbivores. The experiment was replicated 10 times.

To assess the effect of as-*acs* lines on BPH feeding, a newly emerging female adult of BPH was placed into a small parafilm bag (6 × 5 cm), which was then fixed on the stems of individual plants of each line (A1, A30, and WT lines). Each plant was infested with two females. The amount of honeydew excreted by one female adult per plant was weighed 24 h after the start of the experiment. The experiment was replicated 18 times.

Data Analysis

The differences in herbivore performance, expression levels of genes, and phytohormones on different treatments, lines, or treatment times were determined by Student's *t*-tests. Differences in TrypPIs and volatiles were carried out by one-way analysis of variance (ANOVA). TrypPI and volatile data were log- or arcsin-transformed before analysis to meet requirements of normality. If the ANOVA was significant ($P < 0.05$), Duncan's multiple range tests were used to detect significant differences between groups. Parasitoid responses were assessed using χ^2 tests. All tests were carried out with Statistica 6 (Statistica, SAS Institute Inc., Cary, NC, USA).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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