Original Article

Identification and comparative analysis of microRNAs in barnyardgrass (*Echinochloa crus-galli*) in response to rice allelopathy

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ABSTRACT

Rice allelopathy is a hot topic in the field of allelopathy, and behaviour of donor allelopathic rice has been well documented. However, few studies address the effects on receiver barnyardgrass (BYG). We found that expression of miRNAs relevant to plant hormone signal transduction, nucleotide excision repair and the peroxisome proliferator-activated receptor and p53 signalling pathways was enhanced in BYG co-cultured with the allelopathic rice cultivar PI312777, the expression levels of these miRNAs in BYG plants were positively correlated with allelopathic potential of the co-cultured rice varieties. Treatment of BYG plants with rice-produced phenolic acids also increased miRNA expression in BYG, while treatment with rice-produced terpenoids had no obvious effect on miRNA expression. In the hydroponic system, the largest number of *Myxococcus sp.* was found in the growth medium containing rice with the highest allelopathic potential. The addition of phenolic acids in the hydroponic medium also increased the number of *Myxococcus sp.* More interestingly, inoculation with *Myxococcus xanthus* significantly increased miRNA expression in the treated BYG. Jointed treatments of ferulic acid and *M. xanthus* led to strongest growth inhibition of BYG. The results suggest that there exist involvement of *Myxococcus sp.* and mediation of miRNA expression in rice allelopathy against BYG.

Key-words: allelochemicals; miRNA; *Myxococcus sp.*; phenolic compounds.

Abbreviations: AP, apurinic/apyrimidinic; ARF1, auxin response factor 1; ARF15, auxin response factor 15; BYG, barnyardgrass; C4H, cinnamate 4-hydroxylase; CA, cinnamic acid; FA, ferulic acid; GIF2, GRF interacting factor 2; HA, p-hydroxybenzoic acid; IAA, indole-3-acetic acid; OE, overexpression; PAL, phenylalanine ammonia-lyase; PPAR, peroxisome proliferator-activated receptor; qPCR, quantitative PCR; RAD23, DNA repair protein RAD23; RNAi, RNA interference; RT, reverse-transcription; sRNA, small RNA.

INTRODUCTION

Allelopathy is a biological phenomenon that refers to the beneficial or harmful effects of one organism on another by influencing its growth, survival and reproduction through the release of chemicals into the environment (Rice 1984), and is characteristic of certain plants, algae and microorganisms. As one of the most important food crops, rice (*Oryza sativa* L.) provides over 21% of the calorific needs of the world’s population (Fitzgerald et al. 2009). However, weeds cause major losses in rice yields and also reduce the quality of the crop. Although barnyardgrass (BYG) is one of the most problematic weeds in transplanted and direct-seeded rice worldwide, some rice germplasms are allelopathic to BYG. Allelopathy in rice was first observed in the 1980s in seed production plots that were naturally infested with duck salad (*Heteranthera limosa*) at the Arkansas Rice Research Institute, USA (Dilday et al. 1989). The phenomenon has also been reported in many Asian and African countries (Fujii 1992; Olofsdotter et al. 1995; Hassan et al. 1998; Kim & Shin 1998; Weston & Duke 2003). Song et al. (2008) and He et al. (2012b) show that allelopathic effect from resource competition in this system, and the underlying mechanism has been explored in recent years (He et al. 2012b; Kato-Noguchi & Ino 2012; Xu et al. 2012; Fang et al. 2013; Gealy et al. 2013a,b; Kato-Noguchi & Peters 2013).

Isolation and identification of responsible allelochemicals are crucial for understanding rice allelopathy. However, there is disagreement among researchers about rice allelochemicals. Early studies have identified an array of phenolic acids in the exudates of allelopathic rice cultivars (Seal et al. 2004; He et al. 2012a), and some phenolic acids were phytotoxic to weeds (Rimando et al. 2001). However, Olofsdotter et al. (2002) claimed that phenolic acids released from living allelopathic rice roots were unlikely to reach phytotoxic levels. Later momilactone B was regarded as a...
main allelochemical in rice because of its very high phytotoxicity (Kato-Noguchi & Ino 2012; Xu et al. 2012; Kato-Noguchi & Peters 2013). However, the evaluation of the phytotoxicity of these putative allelochemicals was conducted in the laboratory, and was not representative of actual environmental conditions. Plant allelochemicals released from roots can be transformed by surrounding microorganisms, and the degraded products may play a key role in allelopathic growth inhibition (Bertin et al. 2003; Inderjit 2005). Phenolic acids, flavonoids and momilactones have all been found to be degraded and transformed in the soil of paddy fields (Blum 2011; Kato-Noguchi & Peters 2013; Weston & Mathesius 2013). Our recent study found that the allelopathic rice variety PI312777 recruited more microbial populations than less allelopathic rice did, and the main genera of microbes in rice rhizosphere belongs to myxobacteria (Xiong et al. 2012). Myxococcus sp. strongly inhibits seed germination and seedling growth of BYG in the agar plate test (Supporting Information Fig. S1).

Despite these arguments, it is accepted that allelopathy incontrovertibly triggers the expression and regulation of genes in both the donor and receiver plants. Earlier studies have documented that allelopathy is a quantitative trait (Dilday et al. 1998; Jensen et al. 2001), which is mediated by both genetic effects and environment factors. Rice allelopathy is an inducible trait and can be induced by both biotic and abiotic stress conditions, such as higher accompanying weed densities and nutrient deficiency (Song et al. 2008; He et al. 2012a; Kato-Noguchi & Ino 2012). Previous studies have demonstrated that the expression of genes relevant to phenylpropanoid metabolism was enhanced when allelopathic rice was exposed to stressful conditions. Bi et al. (2007) found that the transription of the phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) genes in allelopathic rice leaves was significantly increased after their exposure to methyl jasmonate and methyl salicylate. Song et al. (2008) and Fang et al. (2009) also showed that the level of PAL expression was significantly increased in the allelopathic rice cultivar PI312777 compared with the non-allelopathic rice cultivar Lemont when the nitrogen supply was restricted or when the leaves were sprayed with exogenous salicylic acid, which resulted in an enhanced inhibitory effect on target weeds. PAL plays a key regulatory role in controlling the biosynthesis of all phenylpropanoid products (Weisshaar & Jenkins 1998). Other genes involved in the biosynthesis of phenolic compounds, including C4H, ferulic acid (FA) 5-hydroxylase and caffeic acid O-methyltransferases, display higher induction in the allelopathic rice cultivar PI312777 than that in the non-allelopathic rice cultivar Lemont under different rice/weed ratio conditions, which was attributed to the higher phenolic acid content in PI312777 plants (He et al. 2012a). All these studies focused on the response of allelopathic rice to weeds. However, few study addressed response of the target weed in the mixed system.

Recently, a class of transcriptional and post-transcriptional regulators of gene expression was discovered, known as microRNAs (miRNAs), that essentially belong to the small non-coding RNA with 21–24 nucleotides (Llave et al. 2002; Lim et al. 2003). This class of non-coding RNAs plays a regulatory role in plant development (Reinhart et al. 2002) and responses to environmental signals (Sunkar & Zhu 2004; Sunkar et al. 2012; Shaik & Ramakrishna 2014).

In this study, we analysed the miRNA expression profile in BYG plants and co-cultured allelopathic rice, and compared the expression levels of miRNAs in BYG under various treatment conditions. The change in the expression levels of target genes and their relevant metabolic pathways was determined to confirm the biological function of miRNAs further. Potential involvement of *Myxococcus sp.* in rice allelopathy against BYG and mediation of miRNA expression in BYG were also evaluated.

### MATERIALS AND METHODS

#### Plant materials

Rice (*O. sativa* L.) varieties PI312777 (high allelopathic potential) and Lemont (low allelopathic potential) were selected as the donor plants (Dilday et al. 1994b) and BYG (*Echinoclao crus-galli* L.) collected from a paddy field was used as the receiver plant. In addition, two transgenic lines of PI312777 – the PAL-RNA interference (PAL-RNAi) line and PAL-overexpression (PAL-OE) line – were also used, because the RNAi line has lower allelopathic potential, while the OE line has a higher allelopathic potential than PI312777. The generation of these transgenic rice lines was followed by the method as described in our previous study (Fang et al. 2013).

#### Isolation of RNA for miRNA sequencing

Allelopathic rice PI312777 and BYG seeds were surface-sterilized with sodium hypochlorite for 30 min, placed in Petri dishes in a temperature-controlled growth chamber and then sown in separate seedling plates. At the three-leaf stage, uniform seedlings from each plant species were selected and transferred into a Styrofoam plate (with 24 holes distributed evenly). The seedlings were affixed to the plate by inserting a cotton plug into each hole. The Styrofoam plate was allowed to float in a pot filled with 5 L of rice culture solution, which was a minor optimization of the composition of Yoshida et al. (1976), the contents of which are shown in Supporting Information Table S2. The culture solution was changed every week. The outside of each pot was painted black to prevent the growth of algae. The pH value of the solution was maintained at 5.5 throughout the experiment, which was conducted in a greenhouse in which the temperature ranged from 25 °C (night minimum) to 32 °C (day maximum) and the humidity ranged from 40% (day minimum) to 60% (night maximum). After 7 d, 12 rice seedlings and 12 BYG seedlings were co-cultured in the same pot for 7 d. The root tip tissue of both the rice and BYG plants were sampled and then immediately frozen in liquid nitrogen and stored at −80 °C for isolation of the total RNA. The total RNA was isolated using the TRIzol method [Invitrogen Trading (Shanghai) Co. Ltd.] according to the manufacturer’s instructions and the...
trace genomic DNA was digested using DNase I [TaKaRa Biotechnology (Dalian) Co., Ltd.].

**Rice/BYG co-cultured for different periods**

Two allelopathic rice varieties – PI312777 and Lemont – and receiver BYG were used in this experiment. Both the rice and weed seeds were treated and grown under the same conditions as those described earlier. When the rice and BYG seedlings had reached the four- or three-leaf stage, respectively, 12 rice seedlings and 12 BYG seedlings were transplanted from the plate and placed in a 5 L pot. Both the rice and BYG root tips were sampled after co-culture for 3, 5, 7 and 9 d. 24 uniform rice or BYG seedlings in independent 5-L pots were used as the control, and all settings were replicated four times. The root samples were frozen immediately in liquid nitrogen and stored at −80 °C for the isolation of miRNAs.

**Different co-culture densities in the rice/weed system**

In this experiment, the PI312777, the PAL-RNAi and PAL-OE transgenic lines, and the Lemont varieties were used as the donor plant and BYG was used as the receiver. After carrying out the aforementioned procedures to prepare the pretreated plant seedlings, 12 seedlings of either PI312777 or Lemont were planted with four, six or 12 BYG seedlings, represented as rice : BYG = 3:1, 2:1 and 1:1. The for PAL-RNAi and PAL-OE transgenic lines, 12 rice seedlings and 12 BYG seedlings were cultured in the same pot. Twenty-four uniform rice or BYG seedlings in independent 5-L pots were used as the control; all of the settings were replicated nine times. After 7 d, BYG root samples from the various treatments and the control were tested for the detection of indole-3-acetic acid (IAA) and apurinic/apyrimidinic (AP) sites. The remaining BYG seedlings were also harvested and packed in triplicate. These samples were all oven-dried at 120 °C for 30 min, and at 80 °C for 48 h to measure dry plant weight and evaluate the rice allelopathic potential under different rice:BYG co-culture conditions.

**Treatment of BYG with different phenolic acids and terpenoids**

Three phenolic acids [p-hydroxybenzoic acid (HA), cinnamic acid (CA) and ferulic acid (FA)] and four terpenoids [(-) carvone (C\textsubscript{10}H\textsubscript{14}O), (+) carvone (C\textsubscript{10}H\textsubscript{16}O), (-) menthone (C\textsubscript{10}H\textsubscript{18}O) and (+) cedrol (C\textsubscript{15}H\textsubscript{26}O)] were used as the test compounds. Only BYG was used as a receiver. The BYG seedlings were prepared as described, and were exposed at the four-leaf stage to different concentrations of phenolic acids (HA, 0.4, 0.5 and 0.6 mM; CA, 0.12, 0.20 and 0.28 mM; and FA, 0.02, 0.10 and 0.18 mM) or terpenoids [ (+) cedrol, 1, 7 and 13 μM; (-) menthone, 5.55 and 105 μM; (-) carvone, 30, 130 and 230 μM; and (+) carvone, 3, 23 and 43 μM]. The concentrations were set following the bioassay results on BYG in our previous study. Each treatment and a control of BYG cultured in normal nutrient solution were repeated four times. After placing the BYG in a solution containing allelochemicals for 7 d, the root tips from the BYG in each treatment group were sampled, immediately frozen in liquid nitrogen and stored at −80 °C for the isolation of miRNAs.

**Treatment of BYG with Myxococcus xanthus**

The bacterium M. xanthus was cultured in CTT medium (1% Casitone, 10 mM of Tris-hydrochloride [pH 7.6], 1 mM of potassium phosphate, 8 mM of magnesium sulphate) (Hodgkin & Kaiser 1977) for 48 h. Forty millilitres of culture medium that contained 0.3 g fresh weight of M. xanthus was diluted six times with sterile nutrient solution and used to treat the BYG; The control comprised sterile nutrient solution with CTT at a ratio of 6:1. The BYG seedlings were treated in the various solutions for 7 d, after which the root tips were sampled for the isolation of miRNAs.

**Small RNA (sRNA) sequencing and identification of miRNAs**

DNA-free total RNA from PI312777 and BYG were used to construct sRNA libraries, using a TruSeq small RNA Sample Preparation Kit (Illumina, Inc. U. S. A.). The sRNA libraries were sequenced in the Illumina HiSeq™ 2000 system, followed by sequenced analysis, the details of which are shown in Supporting Information Table S2. Finally, Gene Ontology (GO) enrichment goatools (https://github.com/tanghaibao/goatools) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment was carried out to annotate and classify the candidate miRNAs.

**Isolation of miRNAs from BYG root and reverse-transcription (RT)**

BYG root miRNAs were extracted and isolated using a miRNA pure Mini Kit (Beijing ComWin Biotech Co., Ltd.) following the manual. A total of 1 μg of miRNAs was reverse-transcribed following the stem-loop method to design RT primers; 13 candidate miRNAs were chosen from the BYG miRNA library and U6 was used as the reference gene. Details of RT are shown in Supporting Information Table S2.

**Quantitative PCR (qPCR) analysis of miRNA expression**

Stem-loop qPCR was performed with a realplex® Real-Time PCR System (Eppendorf) using the SuperReal PreMix (SYBR Green) [Tiangen Biotech (Beijing) Co., Ltd.] and miRNA-specific forward and general reverse primers (Supporting Information Table S1). The qPCR protocol details are given in Supporting Information Table S2. The relative expression level of the miRNAs was calculated using the 2−ΔΔCt method (Livak & Schmittgen 2001), and the data were normalized on the basis of U6 Ct values.
Detection of target gene expression

The total RNA of the BYG samples was extracted using TRIzol [Invitrogen Trading (Shanghai) Co., Ltd.], and the genomic DNA was digested using DNase I [TaKaRa Biotechnology (Dalian) Co., Ltd.]. A 1-μL aliquot of total RNA from each sample was reverse-transcribed into cDNA using a TIANscript RT Kit [Tiangen Biotech (Beijing) Co., Ltd.]. Three target genes involved in plant hormone signal transduction and one gene relating to nucleotide excision repair were used to compare the relative expression levels in BYG that received various treatments. The primers for the four target genes are listed in Supporting Information Table S1. Semi-quantitative RT-PCR was performed to detect the target gene expression in BYG that received various treatments. The β-actin gene was used as the reference gene. Details of the semi-quantitative RT-PCR are shown in Supporting Information Table S2. The PCR products were detected in 1.2% (w/v) agarose gel.

DNA extraction from the hydroponic solution

To evaluate the relationship between the quantity of microbes and the rice allelopathic potential, replicates of each treated plant were taken and the hydroponic solutions were uniformly mixed; 100 mL of the mixture were then filtered three times using filter paper to remove the sediment, the filtrate was then filtered again through a millipore filter (0.22 μm nominal pores), on which the microorganisms from the hydroponic solution were collected and used to extract genomic DNA following the freeze-melt method with sodium dodecyl sulfate lysis. Details of the procedure are shown in Supporting Information Table S2. These crude nucleic acid solutions were purified using the EZNA™ Gel Extraction Kit (Omega Bio-Tek Inc. U.S.A.) and re-dissolved in 30 μL of elution buffer. DNA damage was evaluated according to the AP site using the DNA Damage Quantification Kit-AP Site Counting [Dojindo Molecular Technologies, Inc. China].

Effect of M. xanthus and FA on BYG growth

A 0.3 g fresh weight sample of M. xanthus with 10 mL of 0.1 mM FA was added to 100 g of sterile soil in a glass culture vessel; cultures of sterile soil with M. xanthus alone or with the addition of 0.1 mM FA were also carried out and sterile soil mixed with 10 mL of sterile double-distilled water was used as a control. After 3 d, 30 surface-sterilized BYG seeds were sown into the soil in the superclean bench, and the vessels were then kept in an incubator at 23 °C for 10 h (dark conditions) or 28 °C for 14 h (light conditions, 20,000 lux). The root length of the BYG from each vessel was measured, and the allelopathic inhibition of each treatment on BYG was calculated. The soil was sampled to extract DNA using an EZNA™ Soil DNA Kit (Omega Bio-Tek Inc. U.S.A.) and the number of M. xanthus was determined by qPCR following the protocol described earlier.

RESULTS

sRNA expression profiles in allelopathic rice and BYG

According to the sRNA sequence results, more sRNA was expressed in BYG than in allelopathic rice PI312777 (Fig. 1a). In the rice/BYG co-culture system, sRNA expression in the target weed was obviously affected by co-cultured PI312777: more than 62% of the total sRNA was expressed in BYG roots, which represented a twofold increase in comparison with the rice (Table 1). The unique sRNA was then annotated by using Rfam (10.1; http://rfam.sanger.ac.uk/). The results showed that 0.48% of the sRNA in BYG and 2.44% of rice sRNA was regarded as positive miRNA (Fig. 1b).

Web Gene Ontology Annotation Plot (WEGO) annotation and KEGG pathway enrichment analysis of the identified miRNAs

The positive miRNAs from PI312777 and BYG were both used to determine the rice genome from the Genbank database (http://www.ncbi.nih.gov/Genbank/) using Blastn. The known miRNAs were then investigated for target genes. According to WEGO analysis, miRNAs can generally be divided into three categories: the cellular component, molecular function and biological process (Fig. 2). A further KEGG enrichment analysis showed that in this PI312777/BYG co-cultured system, the presence of PI312777 dramatically influenced several vital pathways in BYG,
including plant hormone signal transduction, nucleotide excision repair and the peroxisome proliferator-activated receptor (PPAR) and p53 signalling pathways, while in PI312777 plants only tropane, piperidine and pyridine alkaloid biosynthesis were affected (Table 2). The negative effect of allelopathic rice on BYG was then further investigated in the following studies.

miRNAs expression in BYG co-cultured with the allelopathic rice variety PI312777 and the non-allelopathic rice variety Lemont

For plant hormone signal transduction, the relevant miRNAs, including miRchromosome_5_50931 (miRchr5_50931), miR172b, miR393 and miR393b, were mostly up-regulated in the BYG from the PI312777/BYG system compared with those in the monoculture BYG. In contrast, these miRNAs were mostly down-regulated in the BYG from the Lemont/BYG system, indicating that the high rice allelopathic potential resulted in increased expression levels of the target miRNAs in BYG.

In addition, the abundance of miR437 and miR1871 in the PPAR signalling pathway, of miR535, miR396a, miR396b and miR396c in the p53 signalling pathway and of miR529b in both pathways was also more strongly affected by the allelopathic potential of PI312777. The relative expression levels of these miRNAs at different times after the initiation of co-culture were all higher in BYG in the PI312777/BYG than in the Lemont/BYG system. The expression levels of the miRNAs relevant to nucleotide excision repair, including miR444b.1 and miR444c.1, were higher in PI312777/BYG than those in Lemont/BYG, especially on day 7, which confirmed the sRNA sequencing results (Fig. 3a,b).

IAA concentration and DNA AP sites in BYG

The IAA content in the BYG roots was reduced when the plant was co-cultured with rice, especially with the allelopathic rice variety PI312777. The lowest IAA content in the BYG roots was found in the system of rice/weed (1:1). Variations in the allelopathic potential among rice varieties resulted in changes of the IAA concentrations in the co-cultured BYG, with increasing levels in plants co-cultured with the PAL-RNAi lines and decreasing levels in the PAL-OE co-culture system. These results further confirmed the changes in the expression of relevant miRNAs and the target genes (Fig. 4).

The BYG co-cultured with PAL-OE lines contained the largest number of AP sites, followed by the BYG co-cultured with PI312777 at a ratio of 1:1, then at a ratio of 1:2, the BYG co-cultured with PAL-RNAi and the BYG co-cultured with PI312777 at a ratio of 1:3. The smallest number of AP sites was found in the BYG co-cultured with Lemont and the control BYG (Fig. 4).

BYG growth in rice : BYG co-culture systems at different rice : BYG ratios

A further evaluation of rice allelopathic inhibition in BYG showed that the inhibitory rates of rice on BYG dry weight increased as the number of BYG plants increased. However, the inhibitory rates of PI312777 on BYG were much higher than those of Lemont at the same rice : BYG ratios. The PAL-OE line of PI312777 showed the highest inhibitory rate on BYG, while the allelopathic potential of PAL-RNAi rice on BYG was significantly lower than that of PI312777 under the same conditions (Fig. 5).

Table 1. Comparison of small RNA (sRNA) expression in allelopathic rice PI312777 and barnyardgrass

<table>
<thead>
<tr>
<th>Type</th>
<th>Unique sRNA</th>
<th>Percentage of unique (%)</th>
<th>Total sRNA</th>
<th>Percentage of total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barnyardgrass specific</td>
<td>2 997 165</td>
<td>62.44</td>
<td>4 972 406</td>
<td>31.56</td>
</tr>
<tr>
<td>Allelopathic rice PI312777 specific</td>
<td>1 504 768</td>
<td>31.35</td>
<td>1 806 104</td>
<td>11.46</td>
</tr>
<tr>
<td>Barnyardgrass and allelopathic rice PI312777 specific</td>
<td>297 760</td>
<td>6.02</td>
<td>8 975 645</td>
<td>56.97</td>
</tr>
</tbody>
</table>

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Myxococcus sp. population in the hydroponic system

To determine the size of the Myxococcus sp. populations in the hydroponic system, standard curves related Ct values to the numbers of Myxococcus sp. Each standard represented between $10^5$ and $10^8$ copies of *Escherichia coli*. The calibration curve for the bacterial copies was linear ($R^2 = 0.9938$) and spread over four orders of magnitude (Supporting Information Fig. S2). The results showed that the allelopathic rice co-culture system contained larger numbers of Myxococcus sp. than that of the non-allelopathic rice, and increased with weed density in the PI312777 co-culture system. However, no significant difference was found in the Lemont co-culture system with various weed densities. In addition, the OE of *PAL* in PI312777 also resulted in an increased *Myxococcus sp.* population compared with the control PI312777 plant, while the *PAL*-RNAi transgenic line of PI312777 contained a smaller *Myxococcus sp.* population than PI312777. The numbers were increased in the rice/BYG co-culture system; the *PAL*-OE/BYG mixture contained the largest number of *Myxococcus sp.*, followed by the PI312777 co-culture, and the smallest number was found in the *PAL*-RNAi co-culture (Fig. 6).

miRNA expression in *M. xanthus*-treated BYG

To investigate possible involvement of *Myxococcus sp.* in allelopathic effect of rice on BYG, the expression level of...
miRNAs was determined in BYG treated with *M. xanthus*. The results showed that the expression level of miRNAs was increased in the *M. xanthus*-treated BYG, which indicated the allelopathic potential of *M. xanthus* on BYG (Fig. 7a).

A further comparison showed that the level of expression of four target genes – *auxin response factor 1* (ARF1), *auxin response factor 15* (ARF15), *GRF interacting factor 2* (GIF2) and *DNA repair protein RAD23* (RAD23) – was obviously down-regulated in the treated BYG, and a correlation between miRNA and target gene expression was found (Fig. 7b).

**miRNA expression in BYG treated with different allelochemicals**

Our previous studies showed that the selected phenolic acids and terpenoids at the tested concentrations used in the hydroponic culture system have similar inhibitory effects on BYG in bioassays under laboratory conditions. The results showed that the phenolic acids greatly increased the level of expression of the miRNAs involved in plant hormone signal transduction, especially *miR172b* and *miR393*, which were more than 19-fold higher after each treatment. The expression level of *miR444b.1* and *miR444c.1* also greatly increased in all of the phenolic acid-treated BYG, indicating that treatment with phenolic acids reduced the DNA repair ability of BYG, and was in turn harmful to BYG growth development. In addition, the expression level of the miRNAs related to the PP AR and p53 signalling pathways also increased. All of these results demonstrated that phenolic acids significantly influenced plant growth and development through the expression of relevant miRNAs (Fig. 8a).

In contrast, both *miRchr5_50931* and *miR393* were down-regulated in all of the BYG treated with terpenoids compared with the normal control plants, and the expression of *miR172b* and *miR393b* was enhanced after most of the...
terpenoid treatments, but the increase was distinctly smaller than that observed with phenolic acid treatment. Moreover, the change in the level of expression of miR444b.1 and miR444c.1 also differed from that after phenolic acid treatment; these two miRNAs were either up-regulated or down-regulated after different terpenoid treatments (Fig. 8b). However, the change in expression level was no more than threefolds, which was significantly smaller than that after phenolic acid treatment.

**Myxococcus sp. populations in BYG treated with allelochemicals in the hydroponic system**

The population of *Myxococcus sp.* was also determined by qPCR, which showed that their number was higher in the phenolic acid-treated BYG than in the control plants, except for those treated with 0.6 mM of HA, 0.12 mM of CA and 0.18 mM of FA. The largest *Myxococcus sp.* population was found after treatment with 0.02 mM FA (about 800 cells per mL of solution); the other five treatments that enhanced the *Myxococcus sp.* population resulted in more than 330 cells per mL of solution compared with the control (124 cells per mL of solution) (Fig. 9a). In contrast to treatment with phenolic acids, of the 12 different treatments with terpenoids, five reduced the population and resulted in fewer than 100 cells/mL of solution. No significant difference was found with three other treatments (230/μM of carveol, 55 μM of menthone and 3 μM of carvone), although the other four treatments (5 μM of menthone,
1 μM of cedrol, 13 μM of cedrol and 30 μM of carveol) resulted in an increased Myxococcus sp. population, but the numbers were all less than 270 cells per mL of solution (Fig. 9b). This result demonstrated that phenolic acids and terpenoids had different effects on the multiplication of Myxococcus sp.

Allelopathic inhibition of *M. xanthus* and FA on BYG

Compared with the control, *M. xanthus* with FA showed the strongest allelopathic inhibition in BYG, with a 64.82% inhibition rate on BYG root length; treatment of BYG with...
M. xanthus or FA alone resulted in 8.26 and 34.64% inhibition rates, respectively (Fig. 10a,c). Moreover, the soil to which M. xanthus and FA were added had the largest numbers of M. xanthus, with 3025 cells per g of soil, and significantly larger numbers than that to which M. xanthus (640 cells per g of soil) or FA (278 cells per g of soil) was added alone; the control soil only contained 44 cells per g of soil after 7 d (Fig. 10b). These results indicated the allelopathic potential of M. xanthus with FA.

**DISCUSSION**

Allelopathy now receives more attention from agroecologists (Fitter 2003; Gealy *et al*. 2013b). An environment-friendly weed control based on the use of chemical weapons from a donor plant against the target weed, without the introduction of herbicides or pesticides, is appropriate for sustainable agriculture. About 3–4% of the rice accessions throughout the world have shown allelopathic potential towards weeds (Dilday *et al*. 1994a), and specific varieties have been used to investigate the underlying mechanism. Previous studies mostly focused on exploring the response and reaction of the donor plants. However, a few studies have centred on the target weed, which is an equally important field of research. According to our results, rice allelopathy significantly inhibited plant hormone signal transduction in BYG; the level of four miRNAs – miRchr5_50931, miR172b, miR393 and miR393b – was increased in the BYG co-cultured with PI312777. Among these, miR172b and miRchr5_50931 are negative regulators of auxin response factors (ARF1 and ARF15), which are transcription factors that bind with auxin response elements and regulate the expression of auxin response genes (Guilfoyle & Hagen 2007), which can be induced by auxin, also known as IAA, and play a major role in the developmental and physiological responses of plants. Allelopathic
inhibition by PI312777 resulted in increased expression of miR172b and miRchr5_50931 in the co-cultured BYG, and the two miRNAs in BYG were clearly a response to the rice allelopathic effect. Extension of the duration of the rice/weed co-culture resulted in enhanced miRNAs expression, which in turn led to the down-regulation of ARF expression, thereby preventing the growth development of the BYG. The IAA content was also lower in the co-cultured than in the mono-cultured BYG, and the lower level of expression of AFRs and auxin response genes has been attributed to a reduction in IAA. The allelopathic potential of PI312777 was enhanced by the OE of PAL in the rice, and BYG co-cultured with this transgenic rice showed higher levels of expression of miRNAs than that co-cultured with the wild type, while the PAL-RNAi line of PI312777, a less allelopathic transgenic rice, had less influence on the expression level of these miRNAs. These results showed that rice allelopathy particularly suppressed the IAA signal in the target BYG. In contrast to PI312777, the co-culture of BYG with the non-allelopathic rice accession Lemont had relatively little effect, due to its weak allelopathic potential.

Another negative effect of rice allelopathy is DNA damage, represented by AP sites in DNA locations that neither have a purine nor a pyrimidine base. Among the various treatments, more AP sites were detected in the PI312777/BYG co-culture system, especially under conditions of high BYG density; the findings suggested that the allelopathic effect on BYG was due to damage to the genomic DNA, which prevented regular cell division. The expression of two miRNAs – miR444b.1 and miR444c.1 – was increased in the BYG co-cultured with PI312777 for various periods and was enhanced BYG density. These two miRNAs degraded a gene that encodes the DNA repair protein, RAD23, which is relevant to DNA damage repair. The decreased expression of RAD23 in the co-cultured BYG, especially that co-cultured with PI312777, resulted in a decreased capacity to repair the DNA damage from phytotoxicity. In addition, the degree of DNA damage was greater in the PAL-OE/BYG system, in correlation with the increased expression of miRNAs and the down-regulation of RAD23, but was lower in the BYG co-cultured with the less allelopathic PAL-RNAi transgenic PI312777 rice. Several signal pathways, including the PPAR and p53 signalling pathways, were also influenced in the co-cultured BYG. PPARs are a group of nuclear receptor proteins that function as transcription factors that regulate the expression of genes (Michalk et al. 2006). In mammals, PPARs play an essential role in the regulation of cellular differentiation, development and metabolism (Rosen & Spiegelman 2001). An increase in miRNAs relevant to the PPAR signalling pathway implied that rice allelopathic inhibition of BYG growth development is correlated with signal transduction, and strong suppression of the pathway was found in the most allelopathic rice co-culture system. For the other signalling pathway, the p53 transcription factor regulates multiple biological functions, including DNA damage and oxidative stress (Finkel & Holbrook 2000; Maclaine & Hupp 2009). This gene is continuously degraded in the cell under normal conditions. When external or cellular stress causes DNA damage, p53 degradation is inhibited, and p53 protein accumulates in the nucleus. In our study, increased expression of the miRNAs involved in the p53 signalling pathway in the BYG co-cultured with allelopathic rice indicated a weakness in its DNA repair capacity, which resulted in more serious DNA damage than that found in the control plant, based on the determination of the DNA AP sites.

Although some conflict remains over the main allelochemicals in rice, the investigation of the gene response of the BYG treated with exogenous putative allelochemicals has supplied useful evidence. Our studies showed that the expression of these miRNAs was greatly increased in BYG treated with different phenolic acids. However, the treatment of BYG with terpenoids produced no significant change in or down-regulation of the expression of the miRNAs involved in plant growth development and DNA repair. These findings revealed the phytotoxicity of phenolic acids on the target weed.

Microorganisms, an important link between allelochemicals and target plants, have received increasing attention (Bertin et al. 2003; Blum 2003; Inderjit 2005; Dayan et al. 2010; Khan et al. 2013; Zuo et al. 2014), and the biotransformation of allelochemicals by particular environmental microorganisms has been reported to be necessary and to contribute to an increase in allelopathy (Wang et al. 2013). In this study, a specific microbe, the Myxococcus species, showed a close relationship with rice allelopathic inhibition. Our previous studies found that the rhizospheric soil of PI312777 contained a larger Myxococcus population than that of Lemont (Xiong et al. 2012). In this study, the population of Myxococcus species was significantly higher in the PI312777/BYG system than in the Lemont/BYG system, to which an increase in BYG density contributed. However, no significant difference in BYG density was seen in the Lemont system. These results indicated the vital function of...
Myxococcus during the rice allelopathic inhibitory process. The Myxococcus species belongs to the myxobacteria, a group of proteobacteria that reside mainly in the soil (Reichenbach 2001; Velicer & Vos 2009) and produce a large number of natural products (Diez et al. 2012). The allelopathic effect may occur directly through the release of natural products by the particular allelopathic bacterium, and growth-inhibiting allelochemicals produced by plant growth-promoting bacteria have been isolated and characterized previously (Duke et al. 2000; Barazani & Friedman 2001). Our studies found that the addition of M. xanthus to the BYG mono-culture system resulted in enhanced levels of expression of the miRNAs that are relevant for growth inhibition. The increase in the expression of these miRNAs marked inhibiting their target genes. These interesting outcomes finally led to the allelopathic capacity of M. xanthus to suppress BYG, which is an important link between the donor plant allelochemicals and the receiver plants, and these specific bacteria acted as transforming factors of plant allelochemical exudates.

Allelochemicals, especially phenolic acids, significantly promoted the proliferation of Myxococcus sp. in the

Figure 10. Effects of application of ferulic acid (FA) and inoculation of Myxococcus xanthus on seedling growth of barnyardgrass and final population number of M. xanthus in soil. Pre-sterilized soil was used; M. xanthus and FA were added to the soil separately or together for 3 d; pre-sterilized soil with same volume of sterilized water was used as the control. Thereafter, germinated BYG seeds were sown in the soil, the root length of the BYG was measured and the inhibitory rates (IR) were calculated as follows: $IR = (1 - \text{treatment/control}) \times 100\%$. IR > 0 and IR < 0 indicate inhibitory and stimulatory effects, respectively. Soils from various treatments were then sampled to extract genomic DNA to determine the final population numbers of M. xanthus using the quantitative PCR. For each rice : BYG ratio, superscript letters indicate statistical groups that are significantly different ($P < 0.01$). (a) The root length and the relevant IR of BYG; (b) the M. xanthus population numbers in different soils; (c) the phenomenon of BYG under different soil conditions.
hydroponic system. Among the phenolic acids tested, low concentrations of FA and HA had a remarkable inductive effect, which further demonstrated their importance in the process of rice allelopathy. In contrast, the terpenoids tested showed little positive effect on Myxococcus sp., the population numbers of which were much lower when terpenoids were added to the hydroponic system rather than phenolic acids. A comparison of the growth-promoting effect of different allelochemicals on a characteristic bacterium represents the cross-talk interaction between predominant allelochemicals and microorganisms, thereby clearly distinguishing between the primary and secondary actions of different allelochemicals.

In conclusion, phenolics and other compounds released from allelopathic rice plants can recruit myxobacteria Myxococcus sp., and then, these allelochemicals and myxobacteria jointly increase expression of relevant miRNAs of the co-cultured BYG, which lead to inhibition of plant hormone signal transduction and decreases of DNA repair capacity in BYG, consequently inhibited BYG growth (Fig. 11).

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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. Allelopathic inhibition of Myxococcus sp. on BYG.

Figure S2. The standard curve of IAA concentration (I), the number of AP sites (II) and rice allelopathy.

Table S1. The primers used in this study.

Table S2. The details of methods.