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Barnyard grass stress up regulates the biosynthesis of phenolic compounds in allelopathic rice

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ABSTRACT

Allelopathic rice cultivar PI312777 (PI) and non-allelopathic rice cultivar Lemont (Le) were mixed with barnyard grass (*Echinochloa crus-galli* L., BYG) at various ratios (rice:weed ratios of 4:1, 2:1, and 1:1) in hydroponic cultures. The expression of four genes, i.e. phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), ferulic acid 5-hydroxylase (F5H), and caffeic acid *O*-methyltransferases (COMT), which are involved in the biosynthesis of the phenolic compounds in rice, were evaluated by a quantitative real-time polymerase chain reaction (*qRT-PCR*). The contents of phenolic compounds in leaves, roots, and culture solutions of the two rice cultivars were determined using high performance liquid chromatography (HPLC). The results showed that all of the four genes were up-regulated in leaves and roots of the allelopathic rice PI at all rice:weed ratios. However, three of the four genes, C4H, F5H, and COMT, were down-regulated in the leaves and roots of the non-allelopathic rice Le. The degree to which PAL was up-regulated in leaves and roots was much higher in PI than in Le. The contents of phenolic compounds in PI leaves, roots, and culture solutions were higher than that in Le leaves, roots, and culture solutions. The higher expression of the genes involved in the phenylpropanoid metabolism and the higher contents of phenolic compounds in PI are consistent with the higher inhibitory rates of PI on BYG. These results indicate that the PAL gene in PI is more sensitive to BYG stress than in Le, and barnyard grass up regulates the biosynthesis of phenolic compound in allelopathic rice.

Introduction

The phenomenon of plant allelopathy was noticed around 300 BC. As early as 1 AD the first observations of weed and crop allelopathy were recorded by Theophrastus and Pliny II (Weston, 2005). The study of plant allelopathy was formalized in the 1980s, when Dilday discovered that a few rice cultivars had a special ability to inhibit the growth of paddy weeds, specifically red stem (*Ammannia coccinea* Rottb.) and duck salad (*Heteranthera limosa* (Sw.) Willd.) (Dilday et al., 1994). Allelopathy, properly applied, can reduce the need for chemical herbicides in rice cultivation, reducing the risk of environmental contamination, human health problems, and the development of herbicide-resistant weeds (Olofsdotter, 1998). The use of rice allelopathy in integrated weed management is one of the most interesting new avenues to sustainable agriculture.

Allelopathic characteristics in rice are quantitatively inherited and allelopathic potential of rice is affected by environmental interactions (Courtois and Olofsdotter, 1998; Dilday et al., 2000; Jensen et al., 2001). Under low-nitrogen conditions, the allelopathic potential of allelopathic rice cultivar PI312777 (PI) became enhanced. Nine genes related to phenylpropanoid metabolism, including phenylalanine ammonia-lyase (PAL), became up regulated and the content of phenolic compounds in rice was increased (Xiong et al., 2010). The same results were observed under low-potassium and low-phosphorus conditions (Wang et al., 2008, 2010). When rice leaves were irradiated with UV light, the activities of diterpene cyclase, PAL and cinnamate-4-hydroxylase (C4H) were induced and PAL activity in Kouketsumochi (an allelopathic rice cultivar) was elevated to over two times higher than that in AUS 196 (a non-allelopathic rice cultivar). The *p*-coumaric acid content in allelopathic rice was found to be about 3–5 times higher than in any non-allelopathic rice cultivars (Shin et al., 2000). There are

Abbreviations: BA2H, benzoic acid 2-hydroxylase; BYG, barnyard grass (*Echinochloa crus-galli* L.); C4H, cinnamate-4-hydroxylase; COMT, caffeic acid *O*-methyltransferases; F5H, ferulic acid 5-hydroxylase; HPLC, high performance liquid chromatography; IR, inhibitory rate; Le, non-allelopathic rice cultivar Lemont; PAL, phenylalanine ammonia-lyase; PI, allelopathic rice cultivar PI312777; *qRT-PCR*, quantitative real-time polymerase chain reaction.

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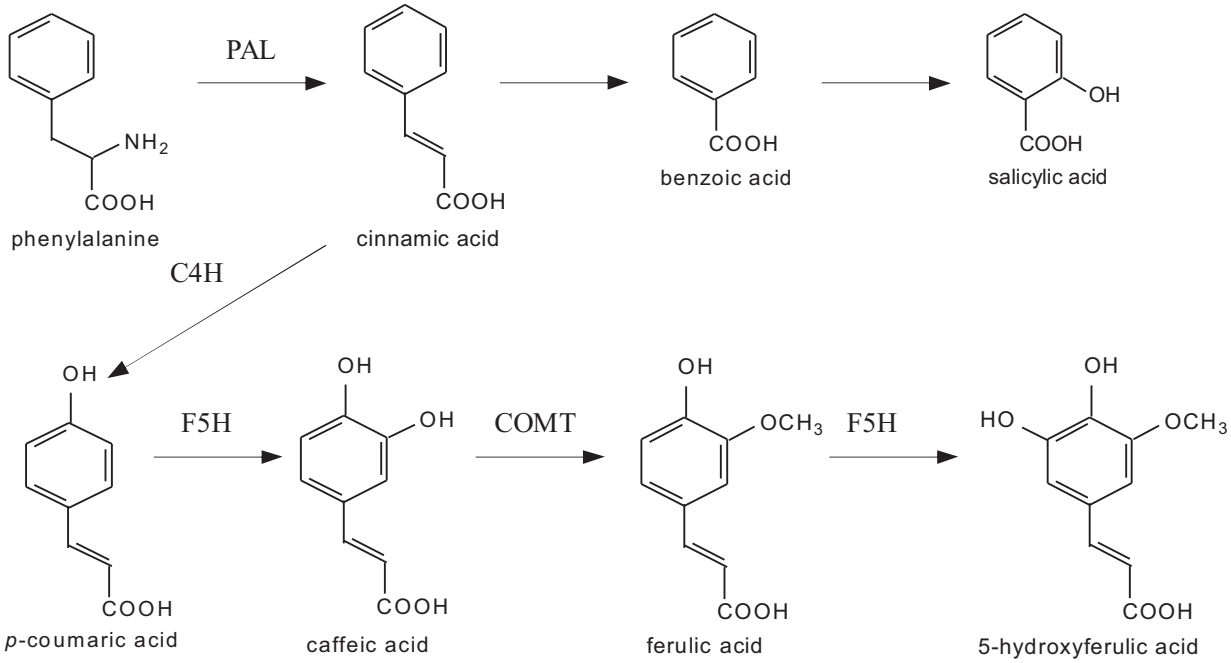

Fig. 1. Pathway of phenylpropanoid metabolism (Dixon and Paiva, 1995; Silverman et al., 1995; Buchanan et al., 2000; Sawada et al., 2006). PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; F5H, ferulic acid 5-hydroxylase; COMT, caffeic acid *O*-methyltransferases; BA2H, benzoic acid 2-hydroxylase.

relatively few articles on the allelopathy of rice under weed stress. Kim et al. (2000) studied the relationship between rice allelopathy and barnyard grass (*Echinochloa crus-galli* L., BYG) stress. The experiment involved four combinations of rice (Kouketsumochi) and BYG (1 + 4, 2 + 3, 3 + 2, and 4 + 1). The highest inhibition, 82.3%, was obtained at a 1 + 4 combination of rice and BYG and the lowest at a 4 + 1 combination. This suggested that Kouketsumochi rice exerts stronger allelopathic effects when grown under more competitive conditions. Kato-Noguchi (2011) reported that the allelopathic activity of rice seedlings was 5.3–6.3 times higher when rice and BYG seedlings were grown together. The concentration of momilactone B (a putative allelochemical derivatives from terpenoid metabolism) in rice seedlings grown with BYG seedlings was 6.9 times higher than the concentration observed in rice seedlings grown in monoculture. Our previous research showed that the allelopathic rice PI and non-allelopathic rice non-allelopathic rice cultivar Lemont (Le) had distinct morphological, physiological, and photosynthetic responses to BYG in rice:weed mixed culture systems (He et al., 2010). Analysis by suppression subtractive hybridization technique showed that under low nitrogen and BYG stress, the expression of the genes associated with allelochemical synthesis and detoxification were up-regulated in PI (Song et al., 2008; Fang et al., 2010).

Phenolic compounds have attracted a great deal of attention from rice allelopathy researchers (Chou and Lin, 1976; Mattice et al., 1998; Rimando et al., 2001; Chung et al., 2001, 2002; Seal et al., 2004). Plant phenolic compounds are derived from cinnamic acid, which is formed from phenylalanine by PAL. This key enzyme in the biosynthesis of phenolic compounds catalyzes the transition from primary (shikimate pathway) to secondary (phenylpropanoid pathway) metabolism (Dixon and Paiva, 1995; Silverman et al., 1995; Buchanan et al., 2000; Sawada et al., 2006). Cinnamic acid is a branch point in the phenylpropanoid pathway. One branch is to benzoic acid derivatives (molecules with a C6–C1 skeleton), such as benzoic acid and salicylic acid. Another branch leads to cinnamic acid derivatives (molecules with a C6–C3 skeleton), for example to *p*-coumaric acid by C4H, to caffeic acid by ferulic acid 5-hydroxylase (F5H), to ferulic acid by caffeic acid *O*-methyltransferases (COMT), and to 5-hydroxyferulic acid by F5H (Fig. 1). Rice allelopathic potential was enhanced under abiotic stress, accompanied by an increase of phenolic compounds and up regulation of related biosynthesis genes (Shin et al., 2000; Wang et al., 2008, 2010; Xiong et al., 2010). No report describes the response of phenylpropanoid genes in allelopathic rice to biotic stress such as weeds. In this study, we evaluate the performance of four related genes, PAL, C4H, F5H, and COMT in the biosynthesis of phenolic compounds, and the levels of certain phenolic compounds in allelopathic rice and non-allelopathic rice grown hydroponically under BYG stress at various rice:weed ratios.

Materials and methods

Rice/Echinochloa crus-galli L. (BYG) mixed culture system

In this rice/weed mixed culture system, allelopathic rice PI and non-allelopathic rice Le (Dilday et al., 2000) were mixed with BYG at various ratios in a hydroponic culture system (Fig. 2). The rice:weed ratios in single pots were 20 rice seedlings:5 BYG seedlings (4:1), 20 rice seedlings:10 BYG seedlings (2:1), and 20 rice seedlings:20 BYG seedlings (1:1). The controls were 20 seedlings of each rice cultivar or 20 BYG seedlings, grown in monoculture. The experiments were conducted in a greenhouse at the agro ecological experimental station of Fujian Agriculture and Forestry University, Fuzhou, China. The temperature ranged from 25 °C to 35 °C.

Germinated seeds of PI, Le and BYG were sown in sand. Forty uniform rice seedlings (3-leaf stage) and BYG seedlings (2-leaf stage) were transplanted into Styrofoam floats with 40 perforated holes (5 cm × 8 cm). The seedlings were stabilized with cotton plugs inserted into each hole. The Styrofoam plates with seedlings were floated in a plastic pot (45 cm × 35 cm × 15 cm) containing 10-L Hoagland solution (Hoagland and Arnon, 1950). Seven days after recovery, rice and BYG seedlings were transplanted at different rice:weed ratios (Fig. 2) into pots with fresh Hoagland solution. The treatments were conducted in triplicates. Additional distilled water was added to each pot daily to maintain a 10-L solution volume. Seven days after treatment, all plant seedlings were harvested. The

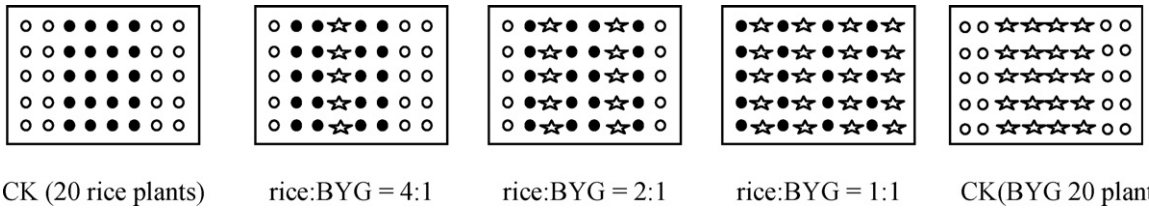

Fig. 2. Scheme of rice/BYG mix cultured system at different ratios of rice:BYG. (●) Rice seedlings; (☆) BYG seedlings; (○) blank (empty holes). BYG, barnyard grass; PI, allelopathic rice cultivar PI312777; Le, non-allelopathic rice cultivar Lemont; CK, control.

BYG seedlings were oven-dried at 120 °C for 30 min and at 80 °C for 48 h so that plant dry weight could be measured. The rice seedlings were frozen at −80 °C for subsequent analysis. The culture solutions were collected and filtered before the determination of the phenolic compound content.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

The *qRT-PCR* was performed as described previously (Wang et al., 2010). Gene-specific primers are shown in Table 1s (Supplementary). The actin gene was used as an internal control. Total RNA was extracted from 0.5-g samples (leaves and roots) using TRIzol reagent (Invitrogen) (Supplementary, Fig 1s). RNA integrity was electrophoretically verified by OD_{260}/OD_{280} nm absorption ratio > 1.90. The *qRT-PCR* was conducted with an ExScriptTMRT Reagent Kit and SYBR Premix Ex TaqTM Kit (TaKaRa) using a CFD3120 MiniOpticon instrument (BIO-RAD) in accordance with the manufacturer's instructions. PCR program is as follows: amplification conditions were an initial denaturation at 95 °C for 10 s, followed by 41 cycles of 95 °C for 5 s, and 60 °C for 20 s. The specificity of amplification was verified by the melting curve at the end of PCR cycle. Fluorescence was read at temperature increments of 0.2 °C with a hold time of 2 s (Supplementary, Fig 2s). The amplification products have been verified by sequencing, and comparison of the sequences with the NCBI database (Supplementary, Table 2s). To calculate PCR efficiencies for each gene, reactions with 10-folds serial dilutions of templates were used and R^2 values were computed (Pfaffl, 2001). The relative quantification (ratio) of each target gene was calculated with the following formula: ratio = $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Three biological replicates were analyzed for each pot, and six replicate PCR reactions were performed for each biological sample. The results were analyzed using Opticon Monitor 3 software.

Extraction of phenolic compounds

The phenolic compounds of rice tissue and culture solutions were extracted as described previously with minor modifications (Wang et al., 2010). One gram rice tissue samples (roots and leaves) were dipped in 5 mL 80% methanol (high performance liquid chromatography (HPLC)-grade, adjusted to pH 2.6 with 2 M HCl), shaken at 35 °C and 225 rpm for 12 h. The extracts were centrifuged and filtered. The filtrates were dried in a vacuum oven at 35 °C to remove solvent. The dried residues were re-dissolved in 2 mL pure HPLC-grade methanol, and filtered through a 0.45 μm nylon filer for HPLC analysis.

From each experiment, 1 L of culture solution was collected and adjusted to pH 2.6 with 2 M HCl. Samples were evaporated until they were almost dry using a rotary evaporator at 50 °C. The residues were re-dissolved in 2 mL pure HPLC grade methanol and filtered through a 0.45 μm nylon filer for HPLC analysis.

HPLC analysis of phenolic compounds

Ten phenolic compounds, namely catechins, protocatechuic acid, *m*-dihydroxybenzene, caffeic acid, *p*-hydroxybenzoic acid, syringic acid, vanillic acid, salicylic acid, *trans*-ferulic acid, and cinnamic acid were selected as authentic standards and purchased from the National Institute for the Control of Pharmaceutical and Biological Products, China. Each phenolic compound was dissolved in HPLC-grade methanol.

A Waters model 1525 instrument with a dual λ UV detector (Waters model 2487) was used for HPLC. Separation of the 10 authentic standards was performed on a 300 × 3.9-mm uBondapak C18 column. The mobile phase consisted of mixture of solvent A (water:acetic acid, 95:5, v/v) and solvent B (HPLC-grade methanol). Gradient elution was performed as follows: 0–2 min, 50% B; 2–3 min, 60% B; 3–6 min, 70% B; 6–20 min, 60% B. The flow rate was 1.0 mL min^{-1}. The detection wavelength was 254 nm. The injection volume was 1 μL. Putative phenolic compounds were identified by comparison of their retention times to those of authentic standards (Supplementary, Table 3s) and their contents were quantified based on comparison of their peak areas to authentic standards.

Data analysis

All experimental data were subjected to a one-way analysis of variance (ANOVA) followed by least significant difference (LSD) analysis at the 5% level of probability. Statistical analysis was performed using the DPS data processing system (Tang and Feng, 2007).

Results

BYG growth in rice/BYG mixed culture system at different rice/BYG ratios

In this paper, the effect of two different allelopathic rice cultivars with different allelopathic behavior on BYG growth were explored by rice/BYG mixed culture at various ratios. The results showed that the inhibitory rates of rice on plant dry weight of BYG increased as the number of BYG plants increased. However, the inhibitory rates of PI on BYG were much higher than those of Le at the same rice:BYG ratios (Table 1).

Gene's expression of phenolic metabolism in rice at different rice/BYG ratios

The expression levels of four phenylpropanoid genes, PAL, C4H, F5H, and COMT were analyzed by *qRT-PCR*. The results showed that all four genes were up-regulated in PI leaves and roots (Fig. 3). The degree of up-regulation increased as the number of BYG plants increased, except for F5H in PI roots. However, three of the four genes, except for PAL, were down-regulated in Le leaves and roots and the degree of down-regulation increased as the number of BYG

Table 1
The inhibitory rates (IR) of two rice cultivars on plant dry weight of barnyard grass (BYG). PI, allelopathic rice cultivar PI312777; Le, non-allelopathic rice cultivar Lemont; IR = (1 − treatment/control) × 100%. IR > 0 and IR < 0 indicate inhibitory and stimulatory effects, respectively. Each experiment was performed in triplicate. All experimental data are presented as mean ± SD. For each cultivar, superscript letters indicate statistical groups that are significantly different ($p < 0.05$).

Rice/BYG ratio	IR (%)	
	PI	Le
4:1 (20 rice seedlings:5 BYG seedlings)	35.53 ± 2.63[c]	13.16 ± 3.95[b]
2:1 (20 rice seedlings:10 BYG seedlings)	43.42 ± 1.32[b]	21.71 ± 2.63[a]
1:1 (20 rice seedlings:20 BYG seedlings)	46.71 ± 1.97[a]	24.34 ± 1.97[a]

plants increased. PAL was up regulated to a greater extent in PI than in Le. Specifically, the up regulation of PAL expression under BYG stress was 3.7 (at the rice:BYG ratio of 4:1), 2.2 (at the rice:BYG ratio of 2:1), and 2.2 (at the rice:BYG ratio of 1:1) times higher in PI leaves than in Le leaves and 6.7 (at the rice:BYG ratio of 4:1), 3.0 (at the rice:BYG ratio of 2:1), and 2.3 (at the rice: BYG ratio of 1:1) times higher in PI roots than in Le roots (Fig. 3).

Phenolic content of rice leaves, roots, and culture solutions at different rice/BYG ratios

The concentrations of each of the 10 phenolic compounds in mixture and their sensitivity were pretested by HPLC. The results showed that the mixture of 10 phenolic compounds could be analyzed simultaneously by their retention time and values of their peak area (Supplementary, Table 3s).

The contents of the 10 phenolic compounds were higher in PI samples than in Le samples, especially in the presence of BYG stress (Table 2). Taking a rice:BYG ratio of 1:1 as an example, the total contents of the 10 phenolic compounds were about 6 times higher in PI leaves, roots and culture solution than that in Le leaves, roots and culture solution. Furthermore, the total contents of phenolic compounds in PI were significantly increased as the number of BYG plants increased. The total contents of the 10 phenolic compounds in PI leaves, roots and culture solutions at a rice:BYG ratio of 1:1 were about 7, 6 and 4 times as much as when PI was grown in monoculture, respectively. The highest total contents were 110 $\mu g\, g^{-1}$ fresh weight in PI leaves, 101 $\mu g\, g^{-1}$ fresh weight in PI roots, and 7×10^{-7} M in PI culture solutions at a rice:BYG ratio of 1:1. The levels of individual phenolic compounds were very different in PI. The three most abundant phenolic compounds in PI were salicylic acid, *trans*-ferulic acid, and *p*-hydroxybenzoic acid in rice leaves, roots, and culture solutions at all rice:weed ratios.

Discussion

In this study, we observed that the PAL gene was up regulated to a greater extent in allelopathic rice PI than in non-allelopathic rice Le at all rice:BYG ratios. In addition, the expression of downstream genes involved in phenylpropanoid biosynthesis, C4H, F5H, and COMT, were found to be quite distinct in the two test rice cultivars (Fig. 3). PAL is the first key enzyme in the general phenylpropanoid pathway, which catalyzes the deamination of phenylalanine to cinnamic acid, the first intermediate of the phenylpropanoid pathway. Cinnamic acid is further converted to molecules with C6–C3 and C6–C1 skeletons by subsequent enzymatic steps (Fig. 1). PAL can be induced by various stress conditions (Dixon and Paiva, 1995; Silverman et al., 1995; Buchanan et al., 2000; Sawada et al., 2006). Our results suggest the PAL gene in PI is more sensitive to BYG stress than in Le, and the subsequent genes (C4H, F5H and COMT) up regulate in PI. This resulted in the synthesis of more phenolic compounds in PI than in Le under BYG stress (Table 2). Our results are similar to those from our previous studies in which two rice cultivars were exposed to nutrient stresses such as low nitrogen or low phosphorus or low potassium (Wang et al., 2008, 2010; Xiong et al., 2010). Kim et al. (2000) found that PAL activity in Kouketsumochi (an allelopathic rice cultivar) was about 17% higher than in Dongjinbyeo (a non-allelopathic rice cultivar), and the Kouketsumochi rice produced about 5 times as much cinnamic acid as the Dongjinbyeo rice. Shin et al. (2000) reported that when rice leaves were irradiated with UV light, PAL activity in Kouketsumochi was increased to over two times higher than that in AUS 196 (a non-allelopathic rice cultivar). The activity of C4H in Kouketsumochi was induced and showed peak activity at 24-h after UV irradiation, though there was no change in AUS 196. Furthermore, the content of *p*-coumaric acid was about 3–5 times higher in allelopathic rice

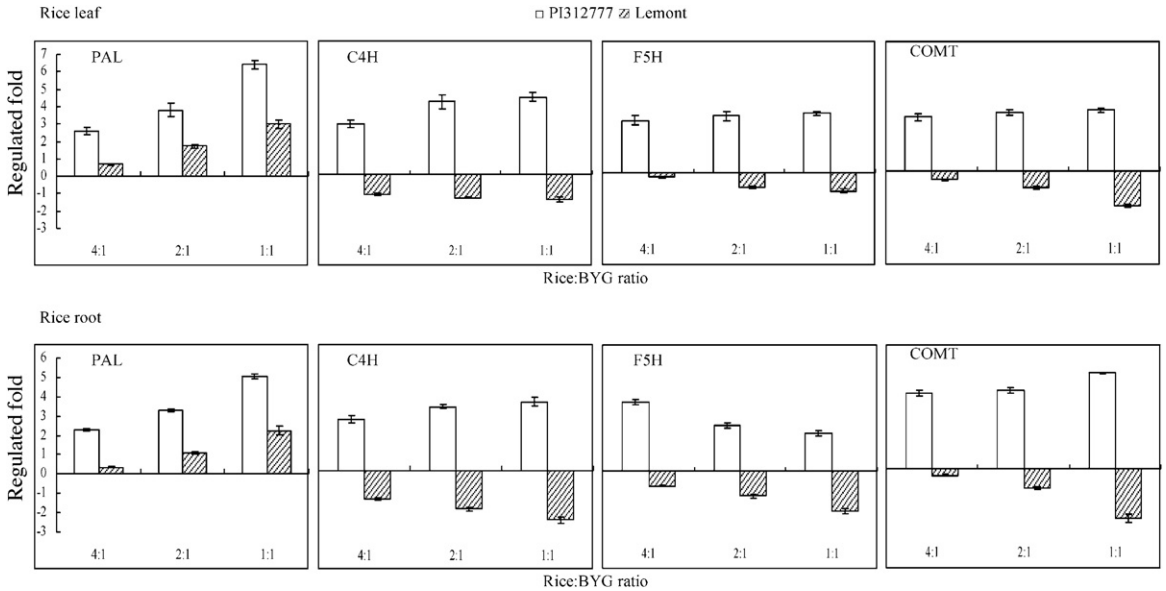

Fig. 3. Expression of the four genes in phenylpropanoid metabolism of two rice cultivars under BYG stress. PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; F5H, ferulic acid 5-hydroxylase; COMT, caffeic acid *O*-methyltransferases; BYG, barnyard grass; PI, allelopathic rice cultivar PI312777; Le, non-allelopathic rice cultivar Lemont.

Table 2
Content of phenolic compounds in rice leaves, roots and culture solutions when grown in different rice:barnyard grass ratios. PI, allelopathic rice cultivar PI312777; Le, non-allelopathic rice cultivar Lemont; CK, control (rice cultivar grown in monoculture); ND, not detected. Each experiment was performed in triplicate. All experimental data are presented as mean ± SD.

Phenolic compound	PI				Le			
	CK	4:1	2:1	1:1	CK	4:1	2:1	1:1
Rice leaf ($\mu g\,g^{-1}$ fresh weight)								
Catechin	1.06 ± 0.03	1.16 ± 0.02	1.17 ± 0.05	1.16 ± 0.02	0.97 ± 0.03	1.00 ± 0.02	1.04 ± 0.04	1.04 ± 0.03
Protocatechuic acid	0.29 ± 0.01	0.50 ± 0.02	0.69 ± 0.02	0.76 ± 0.02	0.20 ± 0.01	0.30 ± 0.02	0.37 ± 0.02	0.39 ± 0.02
m-Dihydroxybenzene	0.90 ± 0.03	1.22 ± 0.02	1.90 ± 0.07	2.12 ± 0.08	0.77 ± 0.01	0.88 ± 0.02	0.90 ± 0.02	0.93 ± 0.02
Caffeic acid	0.37 ± 0.02	1.06 ± 0.02	2.21 ± 0.03	2.80 ± 0.04	0.26 ± 0.01	0.39 ± 0.02	0.36 ± 0.02	0.36 ± 0.02
p-Hydroxybenzoic acid	1.73 ± 0.04	3.59 ± 0.07	4.91 ± 0.05	5.49 ± 0.07	1.08 ± 0.03	1.40 ± 0.03	1.61 ± 0.02	1.60 ± 0.03
Syringic acid	ND	ND	1.18 ± 0.03	1.58 ± 0.02	ND	ND	0.50 ± 0.02	0.57 ± 0.01
Vanillic acid	ND	ND	ND	4.63 ± 0.10	ND	ND	ND	2.07 ± 0.04
Salicylic acid	6.60 ± 0.47	7.55 ± 0.66	42.71 ± 0.58	62.13 ± 1.32	4.41 ± 0.13	4.46 ± 0.11	4.59 ± 0.14	5.11 ± 0.21
trans-Ferulic acid	3.79 ± 0.11	8.56 ± 0.18	16.51 ± 0.21	23.92 ± 0.38	2.93 ± 0.26	6.05 ± 0.22	5.69 ± 0.26	4.74 ± 0.28
Cinnamic acid	1.78 ± 0.03	2.79 ± 0.05	4.40 ± 0.09	5.27 ± 0.17	1.34 ± 0.07	2.64 ± 0.11	2.35 ± 0.11	2.23 ± 0.09
Subtotal	16.52	26.43	75.68	109.86	11.96	17.12	17.41	19.04
Rice root ($\mu g\,g^{-1}$ fresh weight)								
Catechin	1.35 ± 0.22	1.45 ± 0.20	1.49 ± 0.12	1.58 ± 0.11	1.19 ± 0.14	1.22 ± 0.10	1.30 ± 0.15	1.46 ± 0.16
Protocatechuic acid	0.50 ± 0.01	0.58 ± 0.02	0.68 ± 0.02	0.69 ± 0.02	0.32 ± 0.02	0.32 ± 0.02	0.31 ± 0.01	0.32 ± 0.02
m-Dihydroxybenzene	1.25 ± 0.03	1.82 ± 0.12	1.87 ± 0.08	2.03 ± 0.09	0.93 ± 0.08	1.03 ± 0.07	1.05 ± 0.07	1.18 ± 0.08
Caffeic acid	0.36 ± 0.02	1.07 ± 0.04	2.16 ± 0.03	2.75 ± 0.05	0.26 ± 0.01	0.34 ± 0.01	0.33 ± 0.02	0.35 ± 0.02
p-Hydroxybenzoic acid	1.68 ± 0.05	2.76 ± 0.07	0.44 ± 0.02	4.85 ± 0.03	1.17 ± 0.03	1.47 ± 0.02	1.85 ± 0.02	1.89 ± 0.02
Syringic acid	ND	ND	1.21 ± 0.01	1.47 ± 0.01	ND	ND	0.50 ± 0.01	0.61 ± 0.01
Vanillic acid	ND	ND	0.00	4.06 ± 0.02	ND	ND	ND	2.01 ± 0.02
Salicylic acid	5.48 ± 0.47	11.26 ± 1.10	41.15 ± 0.53	59.74 ± 0.46	4.67 ± 0.05	4.83 ± 0.08	4.53 ± 0.06	4.77 ± 0.07
trans-Ferulic acid	5.37 ± 0.41	8.02 ± 0.36	15.92 ± 0.17	19.33 ± 0.16	2.72 ± 0.11	6.11 ± 0.21	4.35 ± 0.14	4.32 ± 0.13
Cinnamic acid	1.51 ± 0.10	3.16 ± 0.27	4.01 ± 0.17	4.19 ± 0.14	1.49 ± 0.07	1.97 ± 0.06	1.88 ± 0.08	1.55 ± 0.06
Subtotal	17.5	30.12	68.93	100.69	12.75	17.29	16.1	18.46
Rice culture solution ($\times 10^{-9}$ M)								
Catechin	ND	ND	ND	ND	ND	ND	ND	ND
Protocatechuic acid	ND	1.94 ± 0.06	4.62 ± 0.08	5.26 ± 0.09	ND	ND	1.82 ± 0.14	1.93 ± 0.11
m-Dihydroxybenzene	9.46 ± 0.14	18.05 ± 1.11	22.25 ± 0.60	23.13 ± 0.86	9.33 ± 0.34	10.07 ± 0.44	10.60 ± 0.55	10.58 ± 0.50
Caffeic acid	2.43 ± 0.16	8.52 ± 0.14	13.52 ± 0.14	17.94 ± 0.09	1.96 ± 0.08	2.41 ± 0.07	2.51 ± 0.07	2.05 ± 0.08
p-Hydroxybenzoic acid	23.26 ± 0.47	29.92 ± 0.55	40.77 ± 0.28	58.83 ± 0.32	9.85 ± 0.22	9.92 ± 0.25	11.05 ± 0.28	13.67 ± 0.30
Syringic acid	5.34 ± 0.12	6.89 ± 0.12	8.36 ± 0.16	8.94 ± 0.13	1.48 ± 0.14	2.64 ± 0.12	2.41 ± 0.11	3.54 ± 0.09
Vanillic acid	ND	ND	30.43 ± 0.63	43.17 ± 0.32	ND	ND	ND	11.20 ± 0.32
Salicylic acid	79.44 ± 5.67	119.54 ± 5.63	204.05 ± 7.24	372.61 ± 5.20	33.87 ± 0.38	37.40 ± 0.52	40.01 ± 0.42	49.23 ± 0.55
trans-Ferulic acid	36.74 ± 1.12	93.39 ± 1.03	97.73 ± 1.75	129.23 ± 2.16	12.21 ± 1.04	14.00 ± 0.90	13.50 ± 1.10	13.76 ± 0.81
Cinnamic acid	18.33 ± 0.30	27.57 ± 1.18	32.78 ± 1.83	36.37 ± 1.18	7.19 ± 0.22	6.81 ± 0.20	7.15 ± 0.25	7.86 ± 0.30
Subtotal	175	305.82	454.51	695.48	75.89	83.25	89.05	113.82

Tang gan, Kouketsumochi and Taichung Native 1 than any other non-allelopathic rice (Shin et al., 2000). All this evidence indicates differential response to environmental stresses exists among allelopathic rice cultivars and non-allelopathic rice cultivars. The genes involved in phenylpropanoid biosynthesis in allelopathic rice cultivars could be up regulated by abiotic or biotic stresses to increase the biosynthesis of phenolic compounds.

Although the role of phenolic compounds in rice allelopathy has been studied quite extensively (Chou and Lin, 1976; Mattice et al., 1998; Rimando et al., 2001; Chung et al., 2001, 2002; Seal et al., 2004), it remains controversial. Firstly, phenolic compounds in plants exist either in the free form or the bound form. The free phenolic acids are usually conjugated to other organic molecules such as glucose, carbohydrates or organic acids, etc. Only has the free phenolic compounds released to the environment have an effect on neighboring plants. Therefore, compounds in rice root exudates are more biologically significance than compounds in rice tissues. However, Olofsdotter et al. (2002) suggested that the concentration of phenolic acids released by rice cultivars was too low to the detriment of weed species. Secondly, it is difficult to get a consistent picture from publications on the allelopathic effects of phenolic acids when applied in mixtures because some papers suggested synergistic effects (Einhellig and Rasmussen, 1979; Einhellig et al., 1982; Lehman et al., 1994; Chung et al., 2002; Jia et al., 2006). Others suggested antagonistic effects (Duke et al., 1983; Inderjit and Bhowmik, 2002). It is commonly accepted that allelopathy is the result of the combined effects of several compounds, rather than one specific compound solely (Einhellig, 1995; Blum, 1996; Reigosa et al., 1999; Kruse et al., 2000; Inderjit and Duke, 2003). A number of putative allelochemicals in rice have been reported, such as long-chain fatty acid esters, benzaldehydes, terpenoids, momilactone, steroids, blumenol A and grasshopper ketone, as well as phenolic acids (Chou and Lin, 1976; Mattice et al., 1998; Kim and Kim, 2000; Rimando et al., 2001; Chung et al., 2002; Seal et al., 2004; He et al., 2006; Macias et al., 2006; Kato-Noguchi, 2011; Kato-Notuchi et al., 2012; Xu et al., 2012). It is important that although some chemical compounds in mixture such as amino acids, carbohydrates and other organic compounds may not act directly as allelopathic agents, they can modify the activity of allelochemicals. A mixture can show allelopathic activity even when the concentrations of individual compounds are significantly below their active thresholds (Blum et al., 1993, 1999; Blum, 1996, 2004; Pue et al., 1995). Our results in this paper showed the inhibitory rates of rice/BYG mixed culture solutions on BYG were increased as the number of BYG plants increased, and the levels of phenolic compounds are elevated in allelopathic rice in the presence of BYG (Tables 1 and 2). We suggest it is the result of the combined effects of compounds in culture solution.

This is the first report on the expression of specific genes involved in the phenylpropanoid metabolism and the contents of

phenolic compounds in rice:BYG mixed cultures at various rice:BYG ratios. Understanding the response mechanism of rice allelopathy under abiotic and biotic stress can help us to find a suitable way to increase rice allelopathy, for example up regulate genes related to phenylpropanoid metabolism. It has been reported that allelopathic potential of rice could be induced or enhanced by exogenous salicylic acid, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzonic acid, methyl jasmonate and methyl salicylate (Bi et al., 2007; Fang et al., 2009; Xu et al., 2010). Improvement of rice allelopathy by integrated regulation technology may be an effective way to suppress competitive weeds in the field, reduce the need for synthetic herbicides and thus foster sustainable agriculture.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2012.06.018.

References

Bi HH, Zeng RS, Su LM, An M, Luo SM. Rice allelopathy induced by methyl jasmonate and methyl salicylate. J Chem Ecol 2007;33:1089–103.
Blum U. Allelopathic interaction involving phenolic acids. J Nematol 1996;28:259–67.
Blum U. Fate of phenolic allelochemicals in soil—the role of soil and rhizosphere microorganisms. In: Macias FA, Galindo JCG, Molinillo JMG, Gutler HG, editors. Allelopathy: chemistry and mode of action of allelochemicals. CRC Press; 2004. p. 57–76.
Blum U, Gerig TM, Worsham AD, King LD. Modification of allelopathic effects of *p*-coumaric acid on morning glory seedling biomass by glucose, methionine, and nitrate. J Chem Ecol 1993;19:2791–811.
Blum U, Shafer SR, Lehmen ME. Evidence for inhibitory interactions involving phenolic acid in field soils: concepts vs. experimental model. Crit Rev Plant Sci 1999;18:673–93.
Buchanan BB, Gruissem W, Jones RL. Biochemistry & molecular biology of plants. American Society of Plant Physiologists; 2000.
Chou CH, Lin HJ. Autointoxication mechanisms of *Oriza sativa*. I. Phytotoxic effects of decomposing rice residues in soil. J Chem Ecol 1976;2:353–67.
Chung IM, Ahn JK, Yun SJ. Identification of allelopathic compounds from rice (*Oryza sativa* L.) straw and their biological activity. Can J Plant Sci 2001;81:815–9.
Chung IM, Kim KH, Ahn JK, Chun SC, Kim CS, Kim JT, et al. Screening of allelochemicals on and identification of potentially allelopathic compounds from rice (*Oryza sativa*) variety hull extracts. Crop Prot 2002;21:913–20.
Courtois B, Olofsdotter M. Incorporating the allelopathy trait in upland rice breeding programs. In: Olofsdotter M, editor. Allelopathy in rice. Proceedings of the workshop on allelopatny in rice. Manila, Philippines: International Rice Research Institute; 1998. p. 57–67.
Dilday RH, Lin J, Yan W. Identification of allelopathy in the USDA-ARS rice germplasm collection. Aust J Exp Agric 1994;34:907–10.
Dilday RH, Mattice JD, Moldenhauer KA. An overview of rice allelopathy in the USA. In: Kim KU, Shin DH, editors. Rice allelopathy. Proceedings of the workshop in rice allelopathy. Taegu, Korea: Kyungpook National University; 2000. p. 15–26.
Dixon RA, Paiva NL. Stress-induced phenylpropanoid metabolism. Plant Cell 1995;7:1085–97.
Duke SO, Williams RD, Markhart AH. Interaction of moisture stress and three phenolic compounds on lettuce seed germination. Ann Bot 1983;52:923–6.
Einhellig FA. Mechanisms of action of allelochemicals in allelopathy. In: Inderjit, Dakshini KMM, Einhellig FA, editors. Allelopathy: organisms, processes, and applications. ACS Symposium Series 582. Washington, DC: American Chemical Society; 1995. p. 96–116.
Einhellig FA, Rasmussen JA. Effects of three phenolic acids on chlorophyll content and growth of soybean and grain sorghum seedlings. J Chem Ecol 1979;5:815–23.
Einhellig FA, Schon MK, Rasmussen JA. Synergistic effects of four cinnamic acid compounds on grain sorghum. J Plant Growth Regul 1982;1:251–8.
Fang CX, Xiong J, Qiu L, Wang HB, Song BQ, He HB, et al. Analysis of gene expressions associated with increased allelopathy in rice (*Oryza sativa* L.) induced by exogenous salicylic acid. Plant Growth Regul 2009;57:163–72.
Fang CX, He HB, Wang QS, Qiu L, Wang HB, Zhuang YE, et al. Genomic analysis of allelopathic response to low nitrogen and barnyardgrass competition in rice (*Oryza sativa* L.). Plant Growth Regul 2010;61:277–86.
He HB, Lin WX, Wang HB, Fang CX, Gan QF, Wu WX, et al. Analysis of metabolites in root exudates from allelopathic and non allelopathic rice seedlings. Allelopathy J 2006;18:247–54.
He HB, Wang HB, Wu HW, Guo XK, Liu CH, Chen RS, et al. Effects of allelopathic rice on barnyardgrass stress: I. Morphological and physiological responses. Allelopathy J 2010;26:185–96.
Hoagland DR, Arnon DI. The water culture method of growing plants without soil. Berkeley, CA: California Agriculture Experiment Station Circular; 1950.
Inderjit, Bhowmik PC. Allelochemicals phytotoxicity in explaining weed invasiveness and their function as herbicide analogues. In: Inderjit, Mallik AU, editors. Chemical ecology of plant: allelopathy in aquatic and terrestrial ecosystems. Basal: Birkhauser; 2002. p. 187–97.
Inderjit, Duke SO. Ecophysiological aspects of allelopathy. Planta 2003;217:529–39.
Jensen BL, Courtois B, Shen L, Li ZK, Olofsdotter M, Mauleon RP. Locating genes controlling allelopathic effects against barnyardgrass in upland rice. Agron J 2001;93:21–6.
Jia C, Kudsk P, Mathiassen SK. Joint action of benzoxazinone derivatives and phenolic acids. J Agric Food Chem 2006;54:1049–57.
Kato-Noguchi H. Barnyardgrass-induced rice allelopathy momilactone B. J Plant Physiol 2011;168:1016–20.
Kato-Notuchi H, Tamura K, Sasaki H, Suenaga K. Identification of two phytotoxins, blumenol A and grasshopper ketone, in the allelopathic Japanese rice variety Awaakamai. J Plant Physiol 2012;169:682–5.
Kim KW, Kim KU. Searching for rice allelochemicals. In: Kim KU, Shin DH, editors. Rice allelopathy. Proceedings of the workshop in allelopathy in rice. Taegu, Korea: Kyungpook National University; 2000. p. 83–95.
Kim KU, Shin DH, Lee IJ, Kim HY. Rice allelopathy in Korea. In: Kim KU, Shin DH, editors. Rice allelopathy. Proceedings of the workshop in rice allelopathy. Taegu, Korea: Kyungpook National University; 2000. p. 57–82.
Kruse M, Strandberg M, Strandberg B. Ecological effects of allelopathic plants—a review. Sikeborg, Denmark: National Environmental Research Institute. NERI Technical Report 370, No. 315; 2000.
Lehman ME, Blum U, Gerig TM. Simultaneous effects of ferulic and p-coumaric acids on cucumber leaf expansion in split-root experiments. J Chem Ecol 1994;20:1773–82.
Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $-2^{-\Delta\Delta Ct}$ method. Methods 2001;25:402–8.
Macias FA, Chinchilla N, Varela RM, Molinllo JMG. Bioactive steroids from *Oryza sativa* L. Steroids 2006;71:603–8.
Mattice J, Lavy T, Skulman B, Dilday RH. Searching for allelochemicals in rice that control ducksalad. In: Olofsdotter M, editor. Allelopathy in rice. Proceeding of the workshop on allelopathy in rice. Manila, Philippines: International Rice Research Institute; 1998. p. 81–98.
Olofsdotter M. Allelopathy in rice. In: Olofsdotter M, editor. Allelopathy in rice. Proceedings of the workshop on allelopatny in rice. Manila, Philippines: International Rice Research Institute; 1998. p. 1–5.
Olofsdotter M, Rebulanan M, Madrid A, Dali W, Navarez D, Olk DC. Why phenolic acids are unlikely primary allelochemicals in rice. J Chem Ecol 2002;28:229–42.
Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:2002–7.
Pue KJ, Blum U, Gerig TM, Shafer SR. Mechanisms by which noninhibitory concentrations of glucose increase inhibitory activity of *p*-coumaric acid on morning glory seedling biomass accumulation. J Chem Ecol 1995;21:833–47.
Reigosa MJ, Adela SM, Luis G. Ecophysiological approach in allelopathy. Crit Rev Plant Sci 1999;18:577–608.
Rimando AM, Olofsdotter M, Dayan FE, Duke SO. Searching for rice allelochemicals: an example of bioassay—guided isolation. Agron J 2001;93:16–20.
Sawada H, Shim IS, Usui K. Induction of benzoic acid 2-hydroxylase and salicylic acid biosynthesis—modulation by salt stress in rice seedlings. Plant Sci 2006;171:263–70.
Seal AN, Pratley JE, Haig T, An M. Identification and quantitation of compounds in a series of allelopathic and non-allelopathic rice root exudates. J Chem Ecol 2004;30:1647–62.
Shin DH, Kim KU, Sohn DS, Kang SG, Kim HY, Lee IJ, et al. Regulation of gene expression related to allelopathy. In: Kim KU, Shin DH, editors. Rice allelopathy. Proceedings of the workshop in rice allelopathy. Taegu, Korea: Kyungpook National University; 2000. p. 109–24.
Silverman P, Seskar M, Kanter D, Schweizer P, Metraux JP, Raskin I. Salicylic acid in rice: biosynthesis, conjugation, and possible role. Plant Physiol 1995;108:633–9.
Song BQ, Xiong J, Fang CX, Qiu L, Lin RY, Liang YY, et al. Allelopathic enhancement and differential gene expression in rice under low nitrogen treatment. J Chem Ecol 2008;34:688–95.
Tang QY, Feng MG. DPS data processing system: experimental design, statistical analysis, and data mining. Beijing: Science Press; 2007.
Wang HB, He HB, Xiong J, Qiu L, Fang CX, Zeng CM, et al. Effects of potassium stress on allelopathic potential of rice (*Oryza sativa* L.). Acta Ecol Sin 2008;28:6219–27.
Wang HB, He HB, Ye CY, Lu JC, Chen RS, Liu CH, et al. Molecular physiological mechanism of increased weed suppression ability of allelopathic rice mediated by low phosphorus stress. Allelopathy J 2010;25:239–48.

Weston LA. History and current trends in the use of allelopathy for weed management. In: Harpern JDI, An M, Wu H, Kent JH, editors. Proceedings of the 4th world congress on alleloapthy. Wagga Wagga, NSW, Australia: Charles Sturt University; 2005. p. 15–21.

Xiong J, Wang HB, Qiu L, Wu HW, Chen RS, He HB, et al. *q*RT-PCR analysis of key enzymatic genes related to phenolic acid metabolism in rice accessions (*Oryza Sativa* L.) exposed to low nitrogen treatment. Allelopathy J 2010;25:345–56.

Xu GF, Zhang FD, Li TL, Wu D, Zhang YH. Induced effects of exogenous phenolic acids on allelopathy of a wild rice accession (*Oryza longistaminata*, S37). Rice Sci 2010;17:134–40.

Xu M, Galhano R, Wiemann P, Bueno E, Tiernan M, Wu W, et al. Genetic evidence for natural product-mediated plant–plant allelopathy in rice (*Oryza sativa*). New Phytol 2012;193:570–5.