ANALYSIS OF GENE EXPRESSIONS ASSOCIATED WITH INCREASED ALLELOPATHY IN RICE (Oryza sativa L.) UNDER LOW NITROGEN TREATMENT

M. E. EL-DENARY¹ AND E. A. ELSHAMEY²

1. Department of Genetics, Faculty of Agriculture, Tanta University, Egypt
2. Rice Research & Training Center, Agricultural Research Center, Egypt

Allelopathy of plants is strongly coupled with external biotic and abiotic stress factors. Abiotic stress factors such as heavy metal, weak solar radiation, higher temperature, and nutrient starvation increase allelopathic activity due to increased production of allelochemicals (Einhellig, 1999; Wu et al., 1999; Belz, 2007; Kato-Noguchi, 2009). Many plants are able to induce defense mechanism against biotic stress factors such as pathogen and insect attacks (Pena-Cortes et al., 2005; Okada et al., 2007). Allelopathy of rice has been shown to be induced by jasmonic and salicylic acids, which are important signaling molecules regulating inducible defense genes against the pathogen infection (Bi et al., 2007; Kato-Noguchi, 2009). The production of sorgoleone, sorghum allelochemical was also induced by velvetleaf root extracts (Dayan, 2006), although it is unclear how active components in the extracts reach sorghum plants in natural ecosystems. Phenolic acids are often mentioned as putative allelochemicals, and are the most commonly investigated compounds among potential allele-chemicals since they have been found in a wide range of plants (Dalton, 1999). However, phenolic acids are unlikely to be responsible for rice allelopathy because their concentrations in rice root exudates never reach phytotoxic levels (Olofsdotter et al., 2002; Seal et al., 2004). Another potential allelochemical isolated from root exudates of the rice cultivar Koshihikari is momilactone B (Kato-Noguchi et al., 2002). Momilactone B inhibits the growth of typical rice weeds like barnyardgrass and Echinochloa colonum at concentrations greater than 1 μmol/L. Rice plants secrete momilactone B from the roots into the rhizosphere over their entire life cycle (Kato-Noguchi et al., 2008). The secretion of momilactone B was also confirmed for other rice cultivars (Kong et al., 2004). These observations suggest that rice allelopathy may be primarily dependant on the secretion of momilactone B (Kato-Noguchi et al., 2008 and 2010).

It is therefore suggested that allelopathic potential of rice is dependent mainly on the species and the content of allelochemicals produced in phenylpropanoid metabolism pathway, especially under stressful conditions (Kim and Shin, 2008). Under decrease N availability, phenolic content and PAL activity in plants may be increased due to decrease demand of proteins required for growth. In

Egypt. J. Genet. Cytol., 43: 113-131, January, 2014
this process, ammonium ions released by PAL can be assimilated to increase N cycling. Different genes were up-regulated in Rikuto Norin22 when exposed to low of N. For instance, the genes that encode for putative protein kinase interactor 1, metallothionein-like protein type 1, catalase 2 (CAT-2), ribosomal proteins, and S1/P1 nuclease were detected simultaneously in Rikuto Norin22. These genes are involved in signal transduction, stress resistance and detoxification, protein synthesis, and programmed cell death-associated endonuclease (Song et al., 2008).

Since plant response on the molecular level is the initial but vital step in the process of plant defense. Several techniques are now available to examine differential gene expressions, such as suppression subtractive hybridization (SSH) (Chang et al., 2010).

The objective of this study was to investigate the involvement of chemical-mediated rice/barnyardgrass interaction in rice allelopathy. The effects of barnyardgrass, nutrient-limited conditions and root exudates of barnyardgrass on allelopathic activity and momilactone B concentration in rice seedlings were examined.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

Rice genotypes; Rikuto Norin22 (allelopathic) and Sakha103 (non-allelopathic) and barnyardgrass seeds were obtained from the Rice Research and Training Center (RRTC), Sakha, Kafr El-Sheikh, Egypt. This study was carried out at the Experimental Farm of (RRTC) and University of Arkansas, Department of Agriculture, Stuttgart, USA. The cultivations were carried out in a clay soil type along summer season (May of 2011 and 2012) under conditions of no rainfall and humidity 70-80 %. Seeds of accession Rikuto Norin22 were surface sterilized in 70% (v/v) aqueous ethanol and grown on a sheet of moist filter paper at 25°C with a 12h photoperiod for 10 days in a growth chamber as described by (Kato-Noguchi et al., 2002). Seeds of barnyardgrass were germinated and grown for 10 days as described above.

Uniform rice and barnyardgrass seedlings (fifty of each) were then transferred onto a holed plate of polystyrene foam that was floated on the medium of 1 L Hoagland nutrient solution was developed by (Hoagland and Arnon, 1933) in plastic container (15 cm×10 cm×10 cm height); (Kim et al., 2005; Song et al., 2008). Only roots of both plants (rice and barnyardgrass) were immersed in the medium through the hole of the plate and all manipulations were carried out under sterile conditions. The pH value of the medium was maintained at 6.0 throughout the experiments by using 0.5 mol/L NaOH or H2SO4 (Kim et al., 2005; Song et al., 2008). After 10 days, incubation at 25°C with a 12 h photoperiod, rice seedlings were harvested for bioassay and momilactone B determination.
**Nutrient stress treatment**

Four concentrations of nitrogen (in Hoagland nutrient solution), 1 N (normal strength), 0.3 N (0.3-fold strength), 0.1 N (0.1-fold strength) and 0.03 N (0.03-fold strength) were prepared for the medium of rice incubation. After germination, uniform 50 rice seedlings were transferred onto a holed plate of polystyrene foam that was floated on 1 L of these medium, and grown for 10 days as described above.

**Determination of momilactone B in rice seedlings**

Rice seedlings (10 g fresh weight) were homogenized in 100 mL 50% (v/v) cold aqueous methanol and the homogenate was filtered through filter paper (No. 2; DBNRR. Arkansas, USA). The residue was homogenized again with 100 mL of methanol and filtered and the two filtrates were combined. An aliquot of the extract was evaporated to dryness, dissolved in 0.2 mL of methanol and added to a sheet of filter paper (No. 2) in a 3-cm Petri dish. Methanol was evaporated in a draft chamber. Then, the filter paper in the Petri dishes was moistened with 0.8 mL of a 0.05% (v/v) aqueous solution of Tween 20. The final assay concentration for clean up was one rice plant equivalent extract per mL. The two filtrates were combined and evaporated to yield an aqueous residue. Then, the aqueous residue was loaded onto a column of synthetic polystyrene adsorbent and purified, and momilactone B was quantified as described by (Kato-Noguchi *et al.*, 2002).

**Barnyardgrass bioassay**

After germination in the darkness at 25°C for 120 h, 10 uniform barnyardgrass seedlings were placed into the Petri dishes. The length of their shoots and roots was measured after 48 h of incubation in the darkness at 25°C. Inhibitory activity (%) was determined by the formula: [(control plant length - plant length treated with rice extract)/control plant length] x 100. Control bioassays did not contain rice extracts according to (Navarez and Olofsdotter, 1996).

**Molecular analysis**

It was done to estimate the differential gene expression in rice seedlings in the presence of barnyardgrass under low nitrogen nutrient.

**Preparation of plant materials**

Rice accessions Rikuto Norin22 and Sakha103 introduced from Egypt were employed as the donor plants and barnyardgrass was used as the receiver plant. 100 seeds of the two rice accessions and barnyardgrass were germinated on seedling trays. When the seedlings reached the 3rd-leaf (rice) and 2nd-leaf (barnyardgrass) stages, respectively, fifty uniform seedlings of each were selected, transplanted into a Styrofoam plate (holes spaced at 5 x 6 cm²), and the seedlings stabilized with a cotton plug were inserted into each. The Styrofoam plate was floated on a pot (45 x 35 x 15 cm²) filled with 10 L Hoagland nutrient solution (normal nutrient condition) (Fajer *et al.*, 1992;
Blez and Hurle, 2004; Kim et al., 2005; Xiong et al., 2005). Rikuto Norin22 and Sakha103 seedlings were grown under the same condition. After seven days of recovery in the Hoagland nutrient solution, 30 rice seedlings and 10 weed seedlings were transplanted into a Styrofoam plate to form a hydroponic rice-weed mixture with the barnyardgrass seedlings in the center surrounded by the rice seedlings. This setting can induce rice allelopathy (Blez and Hurle, 2004; Kim et al., 2005; Xiong et al., 2005).

Two nitrogen (N) levels; 0.03 N (the lower N treatment) and 1.0 N (normal N Hoagland solution) were used and pH values of the treatment solutions were all maintained at 6.0 throughout the experiment by using 1 M NaOH or 0.5 M H₂SO₄ (Kim et al., 2005; Shen and Lin, 2007). Roots of the two rice accessions were sampled after seven days of N treatment; roots were frozen in liquid nitrogen, and stored at -80°C until analysis.

**Construction of subtractive library**

Suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) was employed to construct a subtractive library. Total RNA extracted from the roots with Trizol kit (Ca, USA), and genomic DNA was removed with DNase1. Tester (sample at 0.03 N) and driver (sample at 1 N) RNA samples were prepared by pooling equal amounts of RNA from Rikuto Norin22 roots in 0.03 N and 1 N Hoagland solutions. Synthesis of cDNA was according to the method of SMART PCR cDNA synthesis. LD-PCR products were purified and digested with 15 units RsaI at 37°C for 4 h. SSH was carried out by using the PCR-selected cDNA subtraction Kit (Ca, USA) based on the manufacturer’s instructions. After two hybridizations, a nested PCR was performed to selectively amplify the differentially expressed fragments.

Products of PCR were purified by E.Z.N.A. Cycle-pure Kit (Ca, USA), ligated into pMD18-T vector (Ca, USA), and then transformed into E. coli DH5α cells by CaCl₂. The bacteria were plated onto ampicillin-containing Luria-Bertani (LB) agar plates, which were overlaid with X-Gal and isopropyl-beta-D-thiogalactopyranoside (IPTG). After overnight incubation at 37°C, white colonies (putative positive clones) were picked and cultured in 1.5 ml microfuge tubes with 500 µl LB liquid medium (AMP+).
**Screening and Identification of clones**

Every clone was amplified by PCR (primed with primer 1 and primer 2R in the clontech PCR-select cDNA subtraction Kit). PCR products were blotted onto Hybond-N+ nylon membranes (Ca, USA), which were dried for 2 h at 80°C under vacuum. Tester cDNA and driver cDNA were used as forward and reverse probes, respectively; both were hybridized with the selected clones, clones that showed strong hybridization signed with the forward SSH probe, but not with the reverse probe were positive clones. Reverse northern blots were performed according to the manufactures instructions of DIG-HIGH prime DNA Labeling and Detections Starter Kit® (Ca, USA). The hybridization membranes were scanned by ArtixScan 1010 (Ar, USA).

**Sequence analysis**

Thirty-five clones were selected and sequenced at USDA Biological Engineering Technology and Service, Ca, USA. The identification of these genes was searched by NCBI (The National Center for Biotechnology Information, USA, www.ncbi.nlm.nih.gov) using Blastn and Blastx.

**Real-time fluorescent quantitative polymerase chain reaction (qRT-PCR)**

First strand cDNA was synthesized from the same DNA-free-total RNA extracts used for SSH with ExScript RT reagent Kit (Ca, USA). After dilution (1:10, v/v), cDNA samples were amplified with gene-specific primers (5-CTCGCCGTTCCACTCCTTG-3 and 5-GCTCGGCTCGTTATTCCT-3 for PAL; 5-TGCTGTATCATGGGAAACTAAA-3 and 5-AGTACAGATAGCCAAGGAGGTT-3 for Cytochrome P450). RT-PCR was performed according to the method of SYBR Green I with the SYBR Premix EX Taq (Ca, USA). Actin was used as the references gene detected with 5-TGTAAGCAACTGGGATGA-3 and 5-CCTTCGTAGATTGGGACT-3 primers. The amplification procedures were: an initial denaturation at 95°C for 10 sec followed by 41 cycles of 95°C for 5 sec and 60°C for 20 sec. The specificity of the amplification was verified by the melting curve at the end of the PCR cycle. Fluorescence was read at every temperature increment of 0.2°C with a hold time of 2 sec. The relative quantification (ratio) of a target gene was calculated with the following formula: ratio=2−ΔΔCT (Pfaffl, 2001) and was analyzed using software SPSS11.5.

**RESULTS AND DISCUSSION**

**Effects of allelopathic activity on barnyardgrass and momilactone B concentration in rice**

Rice seedlings were incubated with (mix-incubation) or without (mono-incubation) barnyardgrass seedlings for 10 days, and allelopathic activity of rice extracts were determined by barnyardgrass bioassay. The extracts of mono-incubated rice inhibited the growth of barnyardgrass roots and shoots by 15 and 12%, respectively, and the extracts of mixed-incubated
rice inhibited the growth of barnyardgrass roots and shoots by 79 and 75%, respectively. Thus, the mixed-incubation induced 5.3-6.3-fold increase in allelopathic activity in the rice extracts against barnyardgrass roots and shoots, respectively, as shown in (Table 1).

The concentration of momilactone B in mono-incubated rice seedlings was 2.7 nmol/seedling and that in mixed-incubated rice seedlings was 18.6 nmol/seedling. Thus, the concentration was 6.9-fold greater in mixed-incubated rice seedlings than in mono-incubated rice seedlings (Fig. 1).

This result suggests that the production of momilactone B in rice seedlings may be increased by the presence of barnyardgrass seedlings. Momilactone B inhibited the growth of roots and shoots of barnyardgrass at concentrations greater than 1 µmol/L. The effectiveness of momilactone B on the growth inhibition of rice seedlings themselves was less than 1% of that on the growth inhibition of barnyardgrass (Kato-Noguchi et al., 2008). It was reported that momilactone B among rice allelochemicals may play a critical role in rice allelopathy (Kato-Noguchi et al., 2002 and 2010). Therefore, the increased concentration of momilactone B in rice seedlings under mixed-incubation with barnyardgrass may be associate with increased allelopathic activity of rice seedling.

Rice seedlings were incubated without barnyardgrass seedlings in the media containing different nutrient concentrations for 10 days, and allelopathic activity of the rice extracts was determined by barnyardgrass bioassay. The allelopathic activity of rice extracts was increased significantly at nutrient concentrations lower than 0.1 N. The extracts of rice seedlings incubated in 1 N (normal strength) medium inhibited the growth of barnyardgrass roots and shoots by 16 and 14%, respectively, whereas the extracts of rice seedlings incubated in 0.03 N medium inhibited the growth of barnyardgrass roots and shoots by 44 and 46%, respectively as shown in Table (2). Thus, allelopathic activities of rice seedlings incubated with 0.1 N medium against barnyardgrass root and shoot growth were 2.6 and 3.1 fold greater than those of rice seedlings incubated with 1 N medium, respectively. These results indicate that the growth conditions under nutrient starvation may increase allelopathic activity of rice seedlings. It was also reported that phosphate or nitrogen limited growth conditions increased allelopathic activity of rice (Shen and Lin, 2007; Song et al., 2008). When rice and barnyardgrass are grown together, there may be greater competition for nutrients between rice and barnyardgrass. This competition may cause nutrient deficiency stress and thus increase the allelopathic activity of rice seedlings.

In this regards, concentrations of momilactone B in rice seedlings were increased significantly at nutrient concentrations lower than 0.1 N (Fig. 2). The momilactone B concentration in the rice
seedlings incubated in 0.1 N medium was 2.6-fold greater than that of rice seedlings incubated in 1 N (normal nutrient) medium, which suggests that the growth conditions under low nutrient concentration may increase the momilactone B production in the rice seedlings. The elevated rice allelopathic activity by low nutrient conditions (Table 2) may be due to the accumulation of momilactone B in rice seedlings (Fig. 2). Momilactone B had strong allelopathic activity against barnyardgrass (Kato-Noguchi et al., 2008 and 2010).

However, inhibition activities of rice seedlings incubated in 0.01 N medium against barnyardgrass root and shoot growth, respectively, were still 1.9 and 1.7 fold less than those of rice seedlings incubated with barnyardgrass (Table 1). The momilactone B concentration in rice seedlings incubated with 0.01 N medium was 2.7-fold less than in rice seedlings incubated with barnyardgrass (Fig. 1). Therefore, there may be some other mechanisms by which allelopathic activity and momilactone B concentration are increased in rice seedlings by mixed-incubation of rice with barnyardgrass, other than potential nutrient competition between two species. Therefore, plants are able to elevate the defense mechanisms against several biotic stress conditions by detection of various compounds. Interestingly, momilactone B in rice was also increased by jasmonic acid treatment (Kato-Noguchi, 2009).

The chemical cross talk between host and symbiotic plants is an essential process for the development of physical connections in symbiosis and parasitism (Callaway, 2002; Palmer et al., 2004; Bais et al., 2006). Barnyardgrass is one of the most common and noxious weeds in rice paddy fields (Xuan et al., 2006). Although barnyardgrass is adapted rice production system due to its similarity in growth habit, the reason why barnyardgrass so often invades into the rice paddy fields is unknown. There might be some special interactions between both plant species.

In summary, the present study suggests that allelopathic activity and momilactone B concentration in rice seedlings increased in the presence of barnyardgrass seedlings. These increases were probably caused by nutrient competition between the two species. As momilactone B possesses strong phytotoxic and allelopathic activities (Kato-Noguchi et al., 2002, 2008 and 2010), the elevated production and secretion of momilactone B of rice may provide a competitive advantage for root establishment through local suppression of pathogens and inhibition of the growth of competing plant species including barnyardgrass.

Molecular analysis of differential gene expression in rice seedlings under low nitrogen level

To test the induced gene expression in allelopathic rice, the forward subtracted cDNA library was constructed by SSH to detect the molecular response of Rikuto Norin22 to low N (0.03 N) treatment (Fig. 3). A total of 994 clones were obtained
from the forward subtractive library; 188 of them were selected for reverse northern blots after PCR (Fig. 4A and B). Gene expressions in 35 Rikuto Norin22 clones were positive for up regulation. In allelopathic rice cv. Rikuto Norin22 exposed to low nitrogen (N) stress and barnyardgrass competition.

These clones were sequenced, and the cDNA sequences were searched against standard databases (www.ncbi.nlm.nih.gov). According to nucleic acid homologies and encoded protein sequences, 32 out of the 35 clones were successfully assigned. The putative functions of the assigned expressed sequence tags (ESTs) are given in (Table 3). Clones 312, 403 and 891 encode for the same putative triosephosphate isomerase, and clone 323 showed the highest homology to putative glycine hydroxymethyltransferase from rice (Oryza sativa), all of which are involved in plant primary metabolism. Clones 278, 547 and 663 had homology to phenylalanine ammonia-lyase (PAL), putative o-methyltransferase from (Oryza sativa) and cytochrome P450 from Plutella xylostella, respectively.

These enzymes were associated with de novo synthesis of phenolic-based allelochemicals. Clones 34, 84, 257, 642, 935, 489 and 272 showed the highest homologies to putative SCARECROW gene regulator-like, no apical meristem (NAM) protein, subtilisin-chymotrypsin inhibitor, DNA binding protein S1FA family protein, S1/P1 nuclease, Bol A-like protein, and putative CLB1 protein (calcium-dependent lipid binding), respectively from (Oryza sativa), which participate in plant growth and cell cycle regulation.

Clones 803, 943, and 243 had the greatest homologies to putative protein kinase-interactor 1, catalase 2 (CAT-2), and myosin-like protein, respectively. In contrast, clones 201, 120, 715, and 917 had homologies to the same metallothionein-like protein type 1, and an expressed protein from (Oryza sativa) that plays a critical role in stress resistance and signal transduction. Clones 188, 341, 640, 177 and 657 encode for the same putative 60S ribosomal protein L22-2 and 40S ribosomal protein S13, respectively, but clones 486, 399, 349, 622 and 743 showed the highest homologies to putative 40S ribosomal protein S9, putative 60S ribosomal protein L5, putative chaperonin 10, putative apoptosis-related protein, and HSP20-like chaperone, respectively, from (Oryza sativa). Those enzymes function in protein synthesis and degradation. The four assigned sequences encoded for by clones 298, 308, 645 and 700 have unknown function (Table 3).

Differential expression of two genes by qRT-PCR gene expression of both PAL and P450, which are involved in de novo allelochemical synthesis, was increased in Rikuto Norin22 and Sakha103 when exposed to low nutrient conditions with limited N availability. The relative expression levels of the two genes in Rikuto Norin22, however, were higher than those in Sakha103. After seven days of the low N treatment, the relative tran-
script abundance of PAL in root of Rikuto Norin22 increased by 11.38 times, while in Sakha103 increased by only 1.15 times. The expression of P450 gene for the two rice accessions was increased by 10 and 1.1 times under the same low N condition for Rikuto Norin22 and Sakha103 respectively as shown in Figs. (5 and 6). This finding was reconfirmed by SSH analysis (Table 3).

Figure (6), show expression of PAL, Cytochrome P450 and Actin in rice plants, PAL and Cytochrome P450 are present in Rikuto Norin22 variety and absent in Sakha 103, but Actin gene was presented in both varieties as references gene. The change in allelopathic potential seems to result from differential gene expression mediated by different N concentrations, since N deprivation and other stress factors can induce gene expression associated with secondary metabolism in many crops (Sheveleva et al., 1997; Scheible et al., 2004). Up-regulation of the putative genes that encode for PAL, O-methyltransferase, triosephosphate-isomerase, and cytochrome P450, which are involved in de novo synthesis of phenolic allelochemicals and detoxification of toxic substances, was detected in Rikuto Norin22 by SSH at low N. PAL is the first key enzyme in phenylpropanoid metabolism that can be regulated by various biotic and a biotic factors to different extents depending on different plant species. P450 is directly involved in the formation of p-coumaric acid (Anterola et al., 2002), an intermediate for methylated polyphenolic synthesis, and detoxification of plant autotoxic substances.

Triosephosphate isomerase is vital in the glycolytic pathway, and provides substrate for phenylpropanoid metabolism. It is suggested that allelopathic potential can be enhanced under N-limited conditions, which may result in the activation of genes that encode for PAL and P450. In turn, this may lead to increased phenolic allelochemicals that can suppress the growth of accompanying weeds under limited N conditions in hydroponic solution. Phenolic allelochemicals are derived mainly from L-phenylalanine via t-cinnamate (Scheible et al., 2004).

Stimulation of phenylpropanoid metabolism can be triggered by changes in N levels and mediated by the induction of enzymes in the early steps of phenylpropanoid biosynthesis (Fritz et al., 2006). Under low N availability, phenolic content and PAL activity in plants may increase because of decreased demand for proteins required for growth. In this process, ammonium ions released by PAL can be assimilated to increase N cycling, for example, via the GS/GOGAT system. The resulting N-free carbon skeletons of t-cinnamate can be shunted into phenylpropanoid metabolism (Razal et al., 1996).

This hypothesis seems to explain why the allelopathic potential increases in response to N stress by phenolic compounds that have low turn over rates, but
not by terpenoids that have higher turnover rates. In addition, the present study shows that molecular changes in plant growth and the cell cycle are also involved in the enhancement of rice allelopathic potential under limited N conditions. For example, the putative SCARECROW (SCR) gene regulator-like is involved in cell division (Wysocka-Diller et al., 2000). Subtilisin-chymotrypsin inhibitor in rice is one of bifunctional α-amylase/subtilisin inhibitors, which functions in the regulation of seed development and root growth of rice seedlings. The genes that encode for putative CLB1 protein and Bol A-like protein appear to be involved in the modulation of the cell cycle (Yamagata et al., 1998).

The up-regulation of these genes detected by SSH in the present study suggests that root growth of allelopathic rice was facilitated in low N conditions, which, in turn, led to the increased root/shoot ratio as auto adaptation to the limiting nutrient conditions. The present study also indicates that other genes were up-regulated in Rikuto Norin22 when exposed to low N. For instance, the genes that encode for putative protein kinase interactor 1, metallothionein-like protein type 1, catalase 2 (CAT-2), ribosomal proteins, and S_j/P_1 nuclease were detected simultaneously in Rikuto Norin22 (Table 3). These genes are involved in signal transduction, stress resistance and detoxification, protein synthesis, and programmed cell death-associated endonuclease (Bauwe and Kolukisaoglu, 2003). Therefore, the transcriptional activation of various biosynthetic pathway genes can be an important step for regulating the accumulation of phenylpropanoids during a plant’s response to nutrient deficiency.

Extensive molecular work is still needed to understand the network of signal transduction and the associated control for increasing chemical defence and growth adaptation under the limiting nutrient conditions in rice/weed mixtures (Dixon et al., 2002).

In summary, rice allelopathy has been confirmed as an inducible genetic trait (Bi et al., 2007) that is associated with molecular regulation of secondary metabolic pathways. It may be feasible to trigger allelopathic potential through alteration of agricultural practice or manipulation of environmental conditions. Also, from above data in yield and allelopathic characters we found the accession Giza182 like accession Rikuto Norin22 as strong allelopathy, and from obvious studies we found the accessions Giza182 as restorer line (El-Mowafi et al., 2005). So, we recommend by using accession Giza182 as parental line to produce hybrid rice has highly yield and strong allelopathy like hybrid (IR69625A x Giza182) data is not shown (El Denary et al., 2012).

Finally, it is in great need to elucidate the molecular genetics and enzymology of associated biosynthetic pathways, and to establish a series of specific management strategies that can be applied to
crop production to avoid down-regulation of the relevant genes associated with allelopathic activity (Belz, 2007; Macias et al., 2007).

SUMMARY

Our previous studies showed that allelopathic activity and momilactone B concentration in rice seedlings were increased (6.9 times) in the presence of barnyardgrass seedlings. In this study we attempt to demonstrate the response of rice to the grass and Up-regulation of the putative genes that encode phenylalanine ammonia-lyase (PAL), O-methyltransferase, triosephosphateisomerase, and cytochrome P450, which are involved in phenolic allelochemicals synthesis. Suppression subtractive hybridization (SSH) technique was used in this study to investigate the up-regulate expression of genes in Rikuto Norin22 exposed to low N levels and co-cultured with/without barnyardgrass in hydroponics. PAL is the first key enzyme in phenylpropanoid metabolism that can be regulated by various biotic and abiotic factors to different extents depending on different plant species and P450 is directly involved in the formation of p-coumaric acid. Rice genotypes, Giza182 like accession Rikuto Norin22 has strong allelopathic activity. In addition, our studies indicate that the accessions Giza182 is restorer line (data is not shown). So, we recommend using accession Giza182 as parental line in producing hybrid rice that has high yield and strong allelopathy like the hybrid (IR69625A x Giza182).

REFERENCES

Anterola, A. M., J. H. Jeon, L. B. Davin and N. G. Lewis (2002). Transcriptional control of monolignol biosynthesis in Pinustaeda: factors affecting monolignol ratios and carbon allocationin phenylpropanoid metabolism. J. Biol. Chem., 277: 272-280.

Bais, H. P., T. L. Weir, L. G. Perry, S. Gilroy and J. M. Vivanco (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. Annual Review Plant Biol., 57: 233-266.

Bauwe, H. and U. Kolukisaoglu (2003). Genetic manipulation of glycine-decarboxylation. J. Exp. Bot., 54: 523-535.

Belz, R. G. (2007). Allelopathy in crop/weed interactions update. Pest. Man. Sci., 63: 308-26.

Belz, R. G. and K. Hurle (2004). A novel laboratory screening bioassay for crop seedling allelopathy. J. Chem. Ecol., 30: 175-198.

Bi, H. H., R. Z. Zeng, L. M. Su, M. An and S. H. Luo (2007). Rice allelopathy induced by methyl jasmonate and methyl salicylate. J. Chem. Ecol., 33: 1089-103.

Chang-Xun Fang, Hai-Bin He, Qing-Shui Wang, Long Qiu, Hai-Bin Wang, Yue-E Zhuang, Jun Xiong and
Wen-Xiong Lin (2010). Genomic analysis of allelopathic response to low nitrogen and barnyardgrass competition in rice (Oryza sativa L.) Plant Growth Regul., 61: 277-286.

Callaway, R. M. (2002). The detection of neighbors by plants. Trends Plant Sci., 17: 104-105.

Dalton, B. R. (1999). The occurrence and behavior of plant phenolic acids in soil environments Principals and practices in plant ecology: allelochemical interactions. Boca Raton, FL: CRC Press, p. 57-74.

Dayan, F. E. (2006). Factors modulating the levels of the allelochemical sorgolene in Sorghum bicolor. Planta, 224: 339-346.

Diatchenko, L., Y. C. Lau, A. P. Campbell, A. Chenchik and F. Moqadam (1996). Suppression subtractive hybridization: A method for generating 694 J. Chem. Ecol., 34: 688-695.

Einhellig, F. A. (1999). An integrated view of allelochemicals amid multiple stresses. In: Inderjit, Dakshini KMM, Foy CL, editors. Principals and practices in plant ecology: allelochemical interactions. Boca Raton, FL: CRC Press., p. 479-94. Inderjit. Plant phenolics in allelopathy. Bot. Rev., 62: 186-202.

Dixon, R. A., L. Achnine, P. Kota, C. J. Liu, M. S. Reddy and L. J. Wang (2002). The phenylpropanoid pathway and plant defense genomics perspective. Mol. Plant Pathol., 3: 371-390.

El Denary, M. E., S. A. Dora, M. I. Abo Yousef and E. A. El-Shamey (2012). Genetic behavior for momilactone B in some lines and their hybrids of rice. J. Agric. Chem. & Biotechn., Mansoura Univ., 3: 295-309.

El-Mowafy, H. F., A. O. Bastawisi, M. I. Abo Yousef and F. U. Zanan (2005). Exploitation of rice heterosis under Egyptian conditions. 10\th Nat. Conf. Agron., 7-10 Oct. Suez Canal Univ. Fac. of Env. Agric. Sci, El-Arish.

Fajer, E. D., M. D. Bowers and F. A. Bazzaz (1992). The effect of nutrients and enriched CO\textsubscript{2} environments on production of carbon-based allelochemicals in plantago: A test of the carbon/nutrient balance. Am. Nat., 140: 702-723.

Fritz, C., N. Palacios-Rojas, R. Feil and M. Stitt (2006). Regulation of secondary metabolism by the carbon-nitrogen status in tobacco: nitrate inhibits large sectors of phenylpropanoid metabolism. Plant J., 46: 533-548.

Hoagland, D. R. and D. I. Arnon (1933). The water culture method for...
growing plants without soil. Univ. of California. Berkeley, Agr. Expt. Sta. Circ., 347: 1-39.

Kato-Noguchi, H., T. Ino, N. Sata and S. Yamamura (2002). Isolation and identification of a potent allelopathic substance in rice root exudates. Physiol. Plant, 115: 401-405.

Kato-Noguchi, H., K. Ota and T. Ino (2008). Release of momilactone A and B from rice plants into the rhizosphere and its bioactivities. Allelopathy J., 22: 321-328.

Kato-Noguchi, H. (2009). Stress-induced allelopathic activity and momilactone B in rice. J. Plant Physiol., 59: 153-8.

Kato-Noguchi, H., M. Hasegawa, T. Ino, K. Ota and H. Kujime (2010). Contribution of momilactone A and B to rice allelopathy. J. Plant Physiol., 167: 787-91.

Kim, S. Y., A. V. Madrid, S. T. Park, S. J. Yang and M. Olofsdotter (2005). Evaluation of rice allelopathy in hydroponics. Weed Res., 45: 74-79.

Kim, K. U. and D. H. Shin (2008). Progress and prospect of rice allelopathy research. In: Zeng RS, Mallik AU, Luo SM (eds) Allelopathy in sustainable agriculture and forestry. Springer, New York, p. 189-233.

Kong, C. H., W. Liang, X. Xu, F. Hu, P. Wang and Y. Jiang (2004). Release and activity of allelo-chemicals from allelopathic rice seedlings. J. Agric. Food Chem., 52: 2861-2865.

Macias, F. A., J. M. Molinillo, R. M. Varela and J. G. Galindo (2007). Allelopathy - a natural alternative for weed control. Pest Manag. Sci., 63: 327-348.

Navarez, D. C. and M. Olofsdotter (1996). Relay seeding technique for screening allelopathic rice (Oryza sativa L.). International Weed Control Congress, Copenhagen, Denmark, 25-28 June, 1285-1290.

Okada, A., T. Shimizu, K. Okada, T. Kuzuyama, J. Koga and N. Shibuya (2007). Elicitor induced activation of the methylerythritol phosphate pathway toward phytoalexins biosynthesis in rice. Plant Mol. Biol., 65: 177-87.

Olofsdotter, M., M. Rebulanan, A. Madrid and W. Dali (2002). Why phenolic acids are unlikely primary allelochemicals in rice. J. Chem. Ecol., 28: 229-242.

Palmer, A. G., R. Gao, J. Maresh, W. K. Eribil and D. G. Lynn (2004). Chemical biology of multihost/pathogen interaction: chemical perception and metabolic complementation. Annual Rev. Phytopathol., 42: 439-64.
Pena-Cortes, H., P. Barrios, F. Dorta, V. Polanco, C. Sunchez and E. Sunchez (2005). Involvement of jasmonic acid and derivatives in plant response to pathogen and insects and in fruit ripening. J. Plant Grow. Regul., 23: 246-60.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nuc. Acids Res., 29: 2002-2007.

Razal, R. A., S. Ellis, S. Singh, N. G. Lewist and G. H. Towers (1996). Nitrogen recycling in phenylpropanoid metabolism. Photochemistry, 41: 31-35.

Seal, A. N., T. Haig and J. E. Pratley (2004). Evaluation of putative allelochemicals in rice roots exudates for their role in the suppression of arrowhead root growth. J. Chem. Ecol., 30: 63-78.

Scheible, W. R., R. Morcuende, T. Czechowski, C. Fritz, D. Osuna, N. Palcios and D. Schindelkach (2004). Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory in structure of Arabidopsis in response to nitrogen. Plant Physiol., 136: 483-499.

Shen, L. and W. Lin (2007). Effects of phosphorus levels on allelopathic potential of rice cocultured with barnyardgrass. Allelopathy J., 19: 393-402.

Sheveleva, E., W. Chmara, H. J. Bohnert and R. G. Jensen (1997). Increased salt and drought tolerance by D-ononitol production in transgenic Nicotiana tabacum L. Plant Physiol., 115: 1211-1219.

Song, B., J. Xiong, C. Fang, L. Qiu, R. Lin and Y. Liang (2008). Allelopathic enhancement and differential gene expression in rice under low nitrogen treatment. J. Chem. Ecol., 34: 688-695.

Wu, H., J. Pratley, D. Lemerle and T. Haig (1999). Crop cultivars with allelopathic capability. Weed Res., 39: 171-180.

Wysocka-Diller, J. W., Y. Helariutta, H. Fukaki, J. E. Malamy and P. N. Benfey (2000). Molecular analysis of SCARECROW function reveals a radial patterning mechanism common to root and shoot. Development, 127: 595-603.

Xiong, J., W. X. Lin, J. J. Zhou, M. H. Wu, X. X. Chen and H. Q. He (2005). Studies on biointerference between barnyardgrass and rice accessions at different nitrogen regimes. Proceedings Fourth World Congress on Allelopathy Charles Sturt University, Wagga Wagga, NSW, Australia, 501-504.
Xuan, T. D., I. M. Chung, T. D. Khanh and S. Tawata (2006). Identification of phytotoxic substances from early growth of barnyardgrass (Echinochloa crus-galli) root exudates. J. Chem. Ecol., 32: 895-906.

Yamagata, H., K. Kunimastu, H. Kamusuka, T. Kuramota and T. Iwaski (1998). Rice characterization, localization and changes in developmental and germinating seeds. Biosci. Biotech. Biochem., 62: 978-985.

Table (1): Effect of Rikuto Norin22 rice extracts (mono and mixed) on Shoot length and Root length of barnyardgrass.

| No. | Shoot length (cm) | Root Length (cm) |
|-----|------------------|------------------|
|     | Cont. | Mono/Inhib. % | Mixed/Inhib.% | Cont. | Mono/Inhib.% | Mixed/Inhib.% |
| 1st time | 14 | 12.3 | 12.1% | 3.5 | 75.0% | 11.6 | 9.9 | 14.6% | 2.5 | 78.4% |
| 2nd time | 13 | 12.4 | 4.60% | 3.5 | 75.0% | 11.7 | 9.8 | 15.5% | 2.5 | 78.6% |
| 3rd time | 14 | 12.3 | 12.1% | 3.4 | 75.7% | 11.6 | 9.9 | 14.6% | 2.4 | 79.3% |

Table (2): Shoot length and Root length of barnyardgrass affected by Rikuto Norin22 under different concentration from nutrition solution.

| No. | Shoot length (cm) | Root Length (cm) |
|-----|------------------|------------------|
|     | Cont. | 1.0 N | 0.3 N | 0.1 N | 0.03 N | Inhib % | Cont. | 1.0 N | 0.3 N | 0.1 N | 0.03N | Inhib % |
| 1st time | 14 | 11.7 | 10.9 | 9.9 | 7.9 | 43.5 | 11.6 | 9.8 | 8.8 | 8.5 | 6.2 | 46.5 |
| 2nd time | 13 | 11.8 | 11.0 | 9.9 | 7.8 | 40.0 | 11.7 | 9.7 | 8.7 | 8.5 | 6.2 | 47.0 |
| 3rd time | 14 | 11.7 | 10.9 | 10.0 | 7.8 | 44.2 | 11.6 | 9.7 | 8.8 | 8.4 | 6.3 | 45.6 |
Table (3): Proposed identities of genes up-regulated in allelopathic rice Rikuto Norin22.

| Clone | Length (bp) | Accession Number | Best Homologue in Database                                      | Score | ID%/E Value | Source               |
|-------|-------------|------------------|---------------------------------------------------------------|-------|--------------|----------------------|
|       |             |                  | **Primary metabolism**                                        |       |              |                      |
| 312   | 619         | ABR26842         | Putative triosephosphate isomerase                           | 576   | 99/0         | O. sativa            |
| 323   | 386         | NM-00195         | Putative glycine hydroxymethyltransferase                    | 341   | 99/0         | O. sativa            |
| 403   | 705         |                  | Putative triosephosphate isomerase                           | 580   | 99/0         | O. sativa            |
| 891   | 593         |                  | Putative triosephosphate isomerase                           | 535   | 100/0        | O. sativa            |
|       |             |                  | **Phenolic synthesis**                                       |       |              |                      |
| 278   | 498         | ABR2632          | Phenylalanine ammonia-lyase                                   | 460   | 99/0         | O. sativa            |
| 547   | 441         | NM-0047          | Putative o-methyltransferase                                  | 404   | 99/0         | O. sativa            |
| 663   | 542         | BAE5526          | Cytochrome P450                                               | 80    | 72/1.2       | P. xylostella        |
|       |             |                  | **Plant growth/cell cycle regulation**                       |       |              |                      |
| 34    | 504         | AAM0982          | Putative SCARECROW gene regulat-like                         | 113   | 99/2e-55     | O. sativa            |
| 84    | 391         | NM-00151         | No apical meristem (NAM) protein                             | 231   | 100/7e-126   | Zea mays             |
| 257   | 544         | ABA98076         | Subtilisin-chymotrypsin inhibitor                             | 192   | 97/2e-102    | O. sativa            |
| 642   | 572         | NM-00139         | DNA binding protein SIFA family protein                      | 359   | 98/0         | O. sativa            |
| 935   | 551         | NM-00156         | S1/P1 Nuclease                                               | 120   | 86/1e-59     | O. sativa            |
| 489   | 552         | BAD81948         | Bo1A-like protein                                            | 285   | 100/6e-158   | O. sativa            |
| 272   | 290         | NM-00169         | Putative CLB1 protein (calc-depen lipid)                      | 220   | 98/2e-119    | O. sativa            |
|       |             |                  | **Stress resistance/Signal transduction**                    |       |              |                      |
| 803   | 384         | NM-001271        | Putative protein kinase interactor 1                         | 240   | 99/3e-131    | O. sativa            |
| 943   | 623         | A55096           | Catalase 2 (CAT-2)                                           | 182   | 97/3e-12     | O. sativa            |
| 243   | 621         | NM-001062        | Myosin-like protein                                          | 328   | 100/0        | O. sativa            |
| 201   | 232         | AAB70646         | Metallothionein-like protein type 1                          | 150   | 98/8e-78     | O. sativa            |
| 120   | 230         | AAB70646         | Metallothionein-like protein type 1                          | 157   | 99/6e-82     | O. sativa            |
| 715   | 618         | NM-001095        | Metallothionein-like protein type 1                          | 287   | 95/7e-147    | O. sativa            |
| 917   | 522         | NM-001657        | Expressed protein                                            | 465   | 98/0         | O. sativa            |
Table (3): Cont’

| Protein synthesis/degradation | Accession | Description | Value | Similarity | Species |
|-------------------------------|-----------|-------------|-------|------------|---------|
| 177  | ABR25479  | Putative 40S ribosomal protein S13 | 458   | 99/0       | O. sativa |
| 188  | NM-001036 | 60S ribosomal protein L22-2, putative | 327   | 99/0       | O. sativa |
| 341  | NM-001056 | 60S ribosomal protein L22-2, putative | 404   | 100/0      | O. sativa |
| 486  | ABA94682  | 40S ribosomal protein S9, putative | 404   | 98/0       | O. sativa |
| 399  | A2WXX9    | Putative 60S ribosomal protein L5 | 265   | 94/3e-146  | O. sativa |
| 349  | BAD36474  | Putative chaperonin 10 | 254   | 99/2e-139  | O. sativa |
| 640  | NM-001656 | 60S ribosomal protein L22-2, putative | 276   | 98/9e-153  | O. sativa |
| 657  | ABR25489  | Putative 40S ribosomal protein S13 | 154   | 100/4e-81  | O. sativa |
| 622  | NM-001762 | Putative apoptosis-related protein | 532   | 98/0       | O. sativa |
| 743  | NM-001956 | HSP20-like chaperone | 341   | 99/0       | O. sativa |

| Function unknown | Accession | Description | Value | Similarity | Species |
|------------------|-----------|-------------|-------|------------|---------|
| 308              | NM-0047   | Function unknown | 252   | 100/2e-138 | O. sativa |
| 645              | 391       | No significant similarity found |      |            |         |
| 700              | 395       | No significant similarity found |      |            |         |
| 298              | 329       | No significant similarity found |      |            |         |

*** O. sativa = Oryza sativa  Z. mays = Zea mays  P. xylostella = Plutella xylostella
Fig. (1): Momilactone B concentration in rice.

Fig. (2): Effect of nutrient concentration in medium on momilactone B concentrations in rice.

Fig. (3): The results of SSH. M, DNA marker DL 3.0 Kb, S1, first PCR product of subtracted tester cDNA, US1, first PCR product of unsubtracted tester cDNA, S2, second PCR product of subtracted tester cDNA and US2, second PCR product of unsubtracted tester cDNA.

Fig. (4): Reverse northern blots showing differential screening of partly subtracted library clones in allelopathic rice cv. Rikuto Norin 22 exposed to nitrogen (N) stress and barnyardgrass competition. Forward (A) and reverse (B) hybridizations using a tester cDNA probe of Rikuto Norin22 under low (A) or normal (B) nitrogen nutrition. Arrows indicate positive clones.
Fig. (5): SYBR-Green RT-PCR Analysis of Phenolic-based allelochemicals Pathway Gene Expression in Rikuto Norin22 and Sakha103. Total RNA was reverse transcribed, and aliquots were amplified using primer pairs specific for PAL and Cytochrome P450 and the internal reference Actin. RNA levels for each gene were expressed relative to the amount of Actin RNA, as described in Methods.

Fig. (6): Expression of PAL, Cytochrome P450 and Actin in rice plants by DNA marker DL 3.0 Kb {Rikuto norin22 (lane 2, 3, 5 and 6) and Sakha103 (lane 1 and 4)} under low nitrogen level. The assays were performed using cDNA.
