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Differentially Expressed Genes of Potentially Allelopathic Rice in Response against Barnyardgrass

Ahmad Junaedi[1,2], Woo-Suk Jung[1], Ill-Min Chung[1], Kwang-Ho Kim[1*]

[1] *Department of Applied Life Science, Konkuk University, Seoul 143-701, Korea*
[2] *Dept. of Agronomy and Horticulture, Bogor Agricultural University, Bogor 16680, Indonesia*

Abstract

Differentially expressed genes (DEG) were identified in a rice variety, Sathi, an *indica* type showing high allelopathic potential against barnyardgrass (*Echinochloa crus-galli* (L.) Beauv. var. *frumentaceae*). Rice plants were grown with and without barnyardgrass and total RNA was extracted from rice leaves at 45 days after seeding. DEG full-screening was performed by GeneFishing™ method. The differentially expressed bands were re-amplified and sequenced, then analyzed by Basic Local Alignment Search Tool (BLAST) searching for homology sequence identification. Gel electrophoresis showed nine possible genes associated with allelopathic potential in Sathi, six genes (namely DEG-1, 4, 5, 7, 8, and 9) showed higher expression, and three genes (DEG-2, 3 and 6) showed lower expression as compared to the control. cDNA sequence analysis showed that DEG-7 and DEG-9 had the same sequence. From RT PCR results, DEG-6 and DEG-7 were considered as true DEG, whereas DEG-1, 2, 3, 4, 5, and 8 were considered as putative DEG. Results from blast-n and blast-x search suggested that DEG-1 is homologous to a gene for S-adenosylmethionine synthetase, DEG-2 is homologous to a chloroplast gene for ribulose 1,5-bisphosphate carboxylase large subunit, DEG-8 is homologous to oxysterol-binding protein with an 85.7% sequence similarity, DEG-5 is homologous to histone 2B protein with a 47.9% sequence similarity, DEG-6 is homologous to nicotineamine aminotransferase with a 33.1% sequence similarity, DEG-3 has 98.8% similarity with nucleotides sequence that has 33.1% similarity with oxygen evolving complex protein in photosystem II, DEG-7 is homologous to nucleotides sequence that may relate with putative serin/threonine protein kinase and putative transposable element, and DEG-4 has 98.8% similarity with nucleotides sequence for an unknown protein.

Key words: allelopathy, barnyardgrass, BLAST searching, differentially expressed genes, rice

Introduction

The release of rice varieties with high allelopathic potential is an urgent need for a more sustainable weed management system for rice production that will reduce the use of herbicides and the burden of hand weeding (Chung et al. 2002; Jensen et al. 2001). Courtis and Olofsdotter (1998) stated that an understanding of the structure and genetic control of the allelopathic trait is needed to incorporate this trait in rice breeding programs. Molecular studies are the prospective approach for better exploring and understanding allelopathy (Bhowmik and Inderjit 2003; Mallik 2005).

Ebana et al. (2001) reported seven quantitative trait loci (QTLs) from an F_2 population derived from the cross between an *indica*-type line PI312777 (high allelopathic rice) and a *japonica* cultivar Rexmont (less allelopathic rice). Jensen et al. (2001) reported four QTLs using a population derived from a cross between cultivar IAC165 (*japonica* upland variety) and cultivar CO39 (*indica* irrigated variety). Lee at al. (2005) reported nine QTLs in F_2 and F_3 segregates of the cross between Sathi and Nongan. Lin et al. (2005) studied inter-simple sequence repeat (ISSR) molecular marker to estimate genetic diversity in 57 rice accessions. Shin et al. (2000) found that expression of enzyme phenylalanine ammonia-lyase (PAL) and cinnamic acid 4-hydroxylase (CA4H) were induced by UV light, and momilactone-A was accumulated. Treatment of UV-challenged rice leaves gave rise to the inhibition of barnyardgrass growth. Dayan et al. (2005) studied lipid recorsinols as markers to evaluate the allelopathic potential of rice varieties. Whereas Duke et al. (2005) reported transcriptional profiling experiments using microarrays to examine the effects of various herbicides and natural phytotoxins on the *Arabidopsis* transcriptome.

Differentially expressed gene(s) (DEG) refers to a gene

* **To whom correspondence should be addressed**
Kwang-Ho Kim
E-mail: khkim@konkuk.ac.kr
Tel: +82-2-450-3732

(some genes) that is differentially expressed at the mRNA level in two or more samples. GeneFishing™ DEG technique allows for the detection of differentially expressed genes in two or more samples by the random PCR method using Seegene's ACP™ (Annealing Control Primer) technology (Hwang et al. 2003; Kim et al. 2004; Pohjanvirta 2004). Some applications of this method have been reported (Cui et al. 2005; Hwang et al. 2004).

This experiment was aimed at observing the differentially expressed genes in a high allelopathic potential Sathi variety of rice, in the presence and absence of barnyardgrass grown with the rice plant.

Materials and Methods

Plant materials

Rice plants (Sathi variety) were grown in growth chambers using soil media in wagner pots (20 cm/25 cm of diameter/height), one plant per pot. Growth chamber was settled for 13 h day (28 °C, 70% RH, 14000 lux light intensity) and 11 h night (23 °C, 65% RH). Rice plants were grown in submerged conditions. At 28 days, five plants of 3-days germinated barnyardgrass (*Echinochloa crus-galli* (L.) Beauv. var. *frumentaceae*) were planted around a rice hill. Rice plants grown without barnyardgrass were used as the control.

RNA isolation

Rice leaves were collected from 45-day-old plants for total RNA extraction. Total RNA was extracted with the TRIzol® Reagent (Invitrogen™ life technologies) according to the manual instructions. Quality of RNA was confirmed by gel electrophoresis and the concentration was calculated after measured in 260/280 nm of spectrophotometer.

First-strand cDNA synthesis

Total RNAs were used for the synthesis of first-strand cDNAs by reverse transcriptase. Reverse transcription was performed for 1.5 h at 42 °C in a final reaction volume of 20 μl containing 3 μg of the purified total RNA, 4 μl of 5X reaction buffer (Promega, Madison, WI, USA), 5 μl of dNTPs (each 2 mM), 2 μl of 10 μM dT-ACP1 (5'-CTGTGAATGCTGCGACT ACGATIIIIIT(18)-3'), 0.5 μl of RNasin® RNase Inhibitor (40 U/μl; Promega), and 1 μl of Moloney murine leukemia virus reverse transcriptase (200 U/μl; Promega). First-strand cDNAs were diluted by the addition of 80 μl of ultra-purified water for the GeneFishing™ PCR, and stored at -20 °C until use.

ACP-based GeneFishing™ PCR and agarose gel detection

Differentially expressed genes were screened by ACP-based PCR method (Kim et al. 2004) using the GeneFishing™ DEG kits (Seegene, Seoul, South Korea). Second-strand cDNA synthesis was conducted at 50 °C during one cycle of first-stage PCR in a final reaction volume of 20 μl containing 3-5 μg of diluted first-strand cDNA (about 50 ng cDNA), 1 μl of dT-ACP2 (10 μM), 1 μl of 10 μM arbitrary ACP, and 10 μl of 2X Master Mix (Seegene). The PCR protocol for second-strand synthesis was one cycle at 94 °C for 1 min, followed by 50 °C for 3 min, and 72 °C for 1 min. After second-strand DNA synthesis was completed, the second-stage PCR amplification was done for 40 cycles following protocol: 94 °C for 40 s, followed by 65 °C for 40 s, 72 °C for 40 s, followed by a 5 min final extension at 72 °C. The amplified PCR products were separated in 2% agarose gel stained with ethidium bromide.

Direct sequencing

The differentially expressed bands were re-amplified and extracted from the gel using the GENCLEAN® II Kit (Q-BIO gene, Carlsbad, CA, USA), and directly sequenced with ABI PRISM® 3100-*Avant* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Primers were then constructed referring to these sequence results.

Confirmation by RT-PCR

The differential expression of DEG was confirmed by RT-PCR using each gene specific primer pair. The first-strand cDNA was normalized by the human beta-actin gene. The normalized cDNA was used as a template. The PCR reaction was conducted in a final reaction volume of 20 μl containing 2-4 μg (about 50 ng) of diluted first-strand cDNA, 1 μl of primer 5 (10 μM), 1 μl of primer 3 (10 μM), and 10 μl of 2X Master Mix (Seegene, Seoul, South Korea). The PCR amplification protocol was an initial 3 min denaturation at 94 °C, followed by 20~25 cycles of 94 °C for 40 s, 60 °C for 40 s, 72 °C for 40 s, followed by a 5 min final extension at 72 °C. The amplified PCR products were separated in 2% agarose gel stained with ethidium bromide.

BLAST searching

DEG sequences were analyzed using Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information (NCBI), USA. The first step of the analysis using blast nucleotide to nucleotide (blast-n) was based on BLAST search with "nr" (non redundancy; which is one of the databases). If the result was "no significant similarity found", another BLAST search was carried out using "EST" (Expressed Sequences Tag) in the database category. Blast protein to protein (blast-x) was also performed after translated nucleotides to protein to confirm the blast-n result and to find out the gene function of protein resulted.

Results and Discussion

DEG pre-screenings

The number of annealing control primer (ACP, then called GP, GeneFishing primers) used for screening differentially expressed genes of double strand amplicon derived from cDNA

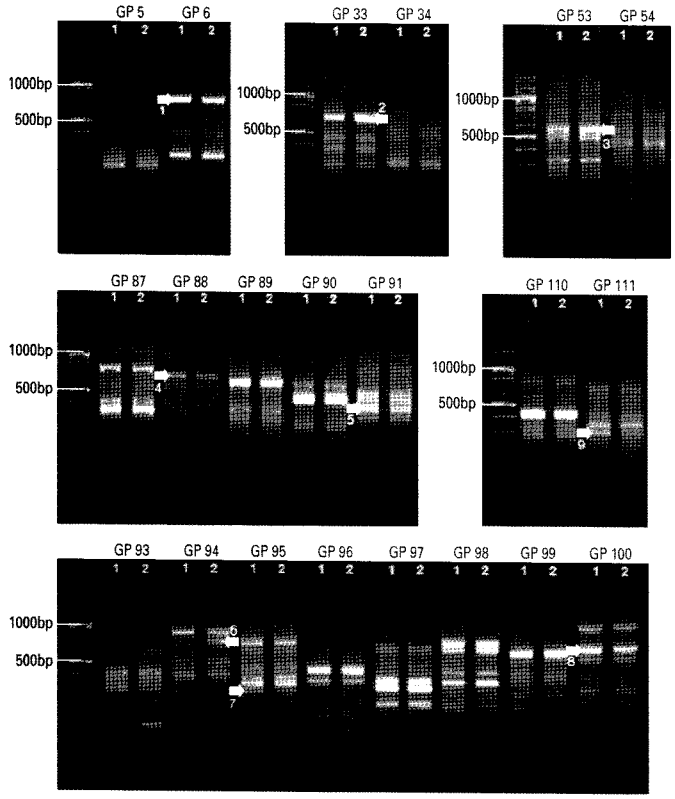

Fig. 1. Gel photograph of GeneFishingTM DEG screening result using 120 primers set (represented by GP primer show difference expression)
Note : GP: GeneFishing Primer, to be followed by index of set number
1 : RNA sample was taken from rice grown with barnyardgrass
2 : RNA sample was taken from rice grown without barnyardgrass
⇨ : Indicated different expression, more expressed in 1
⇦ : Indicated different expression, more expressed in 2

was 120 pairs. Figure 1 shows gel electrophoresis results of selected GP for the expression of Sathi variety grown with barnyardgrass (1) and without barnyardgrass (2). From tested 120 GP, nine GP showed different expression of bands. Among nine GP showing differential expression, six bands from the GP number 6, 88, 91, 95, 100, and 111 (named as DEG-1, 4, 5, 7, 8, and 9, respectively) showed higher expression; and three bands from the GP number 33, 53, and 94 (named as DEG-2, 3, and 6, respectively) showed lower expression in rice plants grown with barnyardgrass as compared to rice plants grown without barnyardgrass.

DEG sequences

Result of DEG sequences showed that DEG-1 to 9 consisted of 561, 531, 491, 528, 290, 511, 207, 531, and 209 nucleotides, respectively. From DEG pre-screening, DEG-7 and DEG-9 seemed to have the same nucleotides length, and from the sequences found that DEG-7 which had 207 nucleotides was totally homologous to DEG 9 which had 209 nucleotides. In DEG-9, guanine and adenine were added in the end of the sequences of DEG-7. Therefore, DEG-7 and DEG-9 were considered as homologous sequences. Consequently, the numbers of DEG found became eight.

Table 1. List of GeneFishing Primer (GP) shown different expression in electrophoresis gel of amplicon and the primer derived from the DEG.

GP number	DEG number	Primer derived and specific sequence of primer
GP6	DEG-1	Z_1, size: 490bp Z1-5'(22mer): *AACGATGAGATTGCTGCTGACC* Z1-3'(20mer): *CCGTCTTGAGGTAGCGTCCG*
GP33	DEG-2	Z_2, size: 597bp Z2-5'(19mer): *TTATGCGTTGGAGGGACCG* Z2-3'(23mer): *GATGTCCTAAAGTTCCTCCACCA*
GP53	DEG-3	(primer was not designed)
GP88	DEG-4	Z_4, size: 429bp Z4-5'(18mer): *CGGAGGAGGTGAGGTCGG* Z4-3'(19mer): *CCTCCTCCGTTCGCACAGA*
GP91	DEG-5	Z_5, size: 177bp Z5-5'(17mer): *CGTCCGCCTCGTCCTCC* Z5-3'(22mer): *CCAACATCCATTTCCCATCATT*
GP94	DEG-6	Z_6, size: 248bp Z6-5'(21mer): *GCGGACAAGAACACTACTGCG* Z6-3'(19mer): *CATCCCGGCACTATCCACC*
GP95	DEG-7	Z_7, size: 159bp Z7-5'(22mer): *GTACCACGAGAACTATGACAAG* Z7-3'(18mer): *CGCCAAATTCATGTCTTC*
GP100	DEG-8	Z_8, Size: 400bp Z8-5'(23mer): *CGATTGAGGCCTGATAGATATGC* Z8-3'(21mer): *AGATGAATCAGCGTGCAAAGC*
GP111	DEG-9	Same sequence with DEG-7

RT PCR confirmation

Seven pairs of primers were constructed from DEG-1, 2, 4, 5, 6, 7, and 8 as shown in Table 1. These primers were then used for RT PCR for confirmation of the DEG expression using *Oryza sativa* ß-actin (520 bp, 5': AGCTTCCTGATGGACAGGTT and 3': GGCCGGTTGAA AACTTTGTC) as a standard. Figure 2 shows gel electrophoresis result of RT PCR for each DEG. primer.

According to Figure 2, RT PCR using primer pairs constructed from the DEG sequences found that different expression showed clearly for DEG-6 and DEG-7, whereas for others DEG seemed unclear. A more accurate investigation of these differential expression, will require the use of the real time quantitative RT PCR. Alternatively, the differential expression of DEG could be investigated visually. From this result, DEG-6 and DEG-7 are considered as true DEG, whereas DEG-1, 2, 4, 5, and 8 are considered as putative DEG.

BLAST search

Table 2 shows BLAST nucleotide (blast-n) search result of DEG sequence using quick search for highly similar sequence. Table 3 shows BLAST nucleotide translated query to protein database (blast-x) search result of DEG sequence using quick search for highly similar sequence. BLAST search showed that DEG-1, 2, 5, 6, 7, and 8 were homolog with nr blast-n (note: in DEG-5 and DEG-7 not include A-tail) in data bank of NCBI. DEG-3 showed highly significant similarity with nr blast-n, showing 485 nucleotides homolog among 491 nucleotides (98.8%); and also showed highly significant similarity with EST blast-n, showing 491 nucleotides homolog among 492

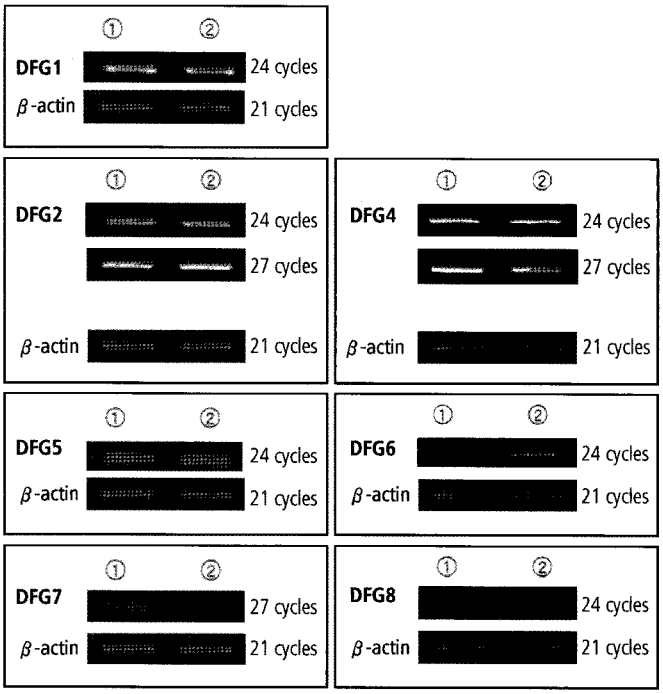

Fig. 2. Gel photograph of RT-PCR result using 7 primers derived from the DEG

Table 2. BLAST nucleotide (blast-n) of nr (non redundancy) searching result of DEG sequence using quick search for highly similar sequence (mega blast)[§].

DEG, Length	Accession, plant source gene, product, length	Description of homology
DEG-1 561	gi|17529620|emb|AJ296743.1|OSA296743 *Oryza sativa* mRNA for S-adenosylmethionine synthetase (SAMS gene), Length = 1594	Score = 1112 bits (561), Expect = 0.0 Identities = 561/561 (100%), Gaps = 0/561 (0%), Strand = Plus/Plus
DEG-2 531	gi|42795537|gb|AY522330.1| *Oryza sativa* (*japonica* cultivar-group) cultivar Nipponbare chloroplast, complete genome Length = 134551	Score = 1053 bits (531), Expect = 0.0, Identities = 531/531 (100%), Gaps = 0/531 (0%), Strand = Plus/Plus
DEG-3 491	gi|116639386|emb|CT829547.1| *Oryza sativa* (*indica* cultivar-group) cDNA clone:OSIGCPI103J03, Length = 1334	Score = 961 bits (485), Expect = 0.0, Identities = 485/485 (100%), Gaps = 0/485 (0%), Strand = Plus/Plus
DEG-4 528	gi|116637518|emb|CT834850.1| *Oryza sativa* (*indica* cultivar-group) cDNA clone:OSIGCPI016N06 Length = 640	Score = 668 bits (337), Expect = 0.0, Identities = 337/337 (100%), Gaps = 0/337 (0%), Strand = Plus/Plus Score = 299 bits (151), Expect = 6e-78, Identities = 151/151 (100%), Gaps = 0/151 (0%), Strand = Plus/Plus
DEG-5 290	gi|116636996|emb|CT828153.1| *Oryza sativa* (*indica* cultivar-group) cDNA clone:OSIGCSA054F24, full insert sequence Length = 762	Score = 543 bits (274), Expect = 1e-151 Identities = 274/274 (100%), Gaps = 0/274 (0%), Strand = Plus/Plus
DEG-6 511	gi|116012813|dbj|AK243448.1| *Oryza sativa* (*japonica* cultivar-group) cDNA, clone: J100069E13, full insert sequence Length = 2387	Score = 1013 bits (511), Expect = 0.0, Identities = 511/511 (100%), Gaps = 0/511 (0%), Strand = Plus/Plus
DEG-7 207	gi|21104877|gb|AC122144.1| *Oryza sativa* (*japonica* cultivar-group) chromosome 10 clone OSJNAa0011L09, complete sequence Length = 121055	Score = 377 bits (190), Expect = 1e-101, Identities = 190/190 (100%), Gaps = 0/190 (0%), Strand = Plus/Minus
DEG-8 531	gi|115452200|ref|NM_001056236.1| *Oryza sativa* (*japonica* cultivar-group) Os03g0274000 (Os03g0274000), mRNA, complete cds, Length = 1898	Score = 1053 bits (531), Expect = 0.0, Identities = 531/531 (100%), Gaps = 0/531 (0%), Strand = Plus/Plus

[§] Only the top of entry result is shown.

nucleotides (including 1 nucleotide gap). DEG-4 both in nr and EST blast-n showed homology for 337 and 151 nucleotides, equal to 63.8 and 28.6%, respectively, or total 92.4% homolog and classified as similar.

From blast-x and blast-n search, it was observed that DEG-1 was the gene for S-adenosylmethionine synthetase (SAMS gene) and DEG-2 was the chloroplast gene for ribulose 1,5-bisphosphate carboxylase large subunit. DEG-3 showed 92.7% similarity to oxygen evolving complex protein in photosystem II. DEG-4 was unknown protein. DEG-5 had highest relation (47.9% similarity) with histone 2B protein. DEG-6 had highest relation (33.1% similarity) with nicotineamine aminotransferase. DEG-7 showed no significant similarity because of short sequence, but it is suggested that DEG-7 had relation with putative serin/threonine protein kinase and putative transposable element. DEG-8 showed 85.7% similarity with oxysterol-binding protein.

S-Adenosylmethionine (AdoMet or SAM) plays a pivotal role as a methyl donor in a myriad of biological and biochemical events. The methylation of 5'-terminal cap plays an important role in mRNA export from the nucleus, efficient translation, and protection of the integrity of mRNAs (Chiang et al. 1996). Overexpression of SAMS-gene in *Streptomyces coelicolor* has significant involvement in initiating the onset of secondary metabolism of antibiotic production (Okamoto et al. 2003). More expression of SAMS gene in Sathi rice plant treated with barnyardgrass may relate with production of more secondary metabolites as allelochemicals.

The bifunctional enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) catalyses the primary reactions of carbon fixation and photorespiration by either carboxylation or oxygenation of ribulose 1,5-bisphosphate (RuBP). Under various ecophysiological conditions, the catalysis by RuBisCO is a bottleneck in both metabolic pathways due to the inherent inefficiency of the enzyme (Marcus and Gurevitz 2000). Less expression of RuBP carboxylase/oxygenase gene in Sathi rice plant treated with barnyardgrass may relate with less activity of photosynthesis compared with that in Sathi without barnyardgrass.

The oxygen-evolving complex of eukaryotic photosystem II (PSII) consists of four extrinsic nuclear-encoded subunits, PsbO (33 kDa), PsbP (23 kDa), PsbQ (17 kDa) and PsbR (10-kDa) protein (Suorsa et al. 2006). Lower expression of this gene in Sathi rice plant treated with barnyardgrass may relate with lower activity of photosynthesis compared with that in Sathi without barnyardgrass. This situation agrees with RuBP gene expression.

Nicotinamine aminotransferase (NAAT) catalyzes the amino group transfer of NA in the biosynthetic pathway of phytosiderophores and is essential for iron acquisition in graminaceous plants (Takahashi et al. 2003). Nicotianamine synthase overexpression in Arabidopsis transgenic plants resulted in increased biosynthesis of nicotianamine, which conferred enhanced tolerance of high levels of metals, particularly nickel. Nicotianamine plays a critical role in metal detoxification, and this can be a powerful tool for use in phytoremediation (Kim 2005). NAAT activity was reported to be dramatically induced by Fe deficiency and suppressed by Fe re-supply (Takahashi et al. 1999). DEG-6 that was considered as NAAT shows lower expression when Sathi rice plants were grown with barnyard-

Table 3. BLAST nucleotide translated query to protein database (blast-x) searching result of DEG sequence using quick search for highly similar sequence (mega blast)[§].

DEG	Accession, plant source, protein, length	Description of homology
DEG-1	gi|17529621|emb|CAC82203.1| S-adenosylmethionine synthetase [*Oryza sativa* (*indica* cultivar-group)] Length=396	Score = 254 bits (648), Expect = 2e-66 Identities = 139/139 (100%), Positives = 139/139 (100%), Gaps = 0/139 (0%), Frame = +1
DEG-2	gi|109156602|ref|YP_654221.1| ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Oryza sativa* (*indica* cultivar-group)] Length=484	Score = 370 bits (951), Expect = 1e-101 Identities = 177/177 (100%), Positives = 177/177 (100%), Gaps = 0/177 (0%), Frame = +1
DEG-3	gi|15408655|dbj|BAB64069.1| putative 33kDa oxygen evolving protein of photosystem II [*Oryza sativa* (*japonica* cultivar-group)] Length=333	Score = 179 bits (455), Expect = 4e-44 Identities = 89/89 (100%), Positives = 89/89 (100%), Gaps = 0/89 (0%), Frame = +1
DEG-4	gi|56784386|dbj|BAD82425.1| unknown protein [*Oryza sativa* (*japonica* cultivar-group)]	Score = 132 bits (332), Expect = 8e-30 Identities = 81/97 (83%), Positives = 82/97 (84%), Gaps = 7/97 (7%), Frame = +3
DEG-5	gi|122894114|gb|ABM67703.1| histone 2B [*Nicotiana benthamiana*] Length=147	Score = 58.2 bits (139), Expect = 1e-07 Identities = 29/29 (100%), Positives = 29/29 (100%), Gaps = 0/29 (0%), Frame = +1
DEG-6	gi|48716886|dbj|BAD23582.1| putative nicotianamine aminotransferase A [*Oryza sativa* (*japonica* cultivar-group)] Length=444	Score = 348 bits (894), Expect = 5e-95 Identities = 169/169 (100%), Positives = 169/169 (100%), Gaps = 0/169 (0%), Frame = +3
DEG-7	No significant similarity found.	It is suggested has relation with putative serin/threonine protein kinase and putative transposable element
DEG-8	gi|108707445|gb|ABF95240.1| Oxysterol-binding protein, expressed [*Oryza sativa* (*japonica* cultivar-group)] Length=463	Score = 179 bits (455), Expect = 4e-44 Identities = 104/104 (100%), Positives = 104/104 (100%), Gaps = 0/104 (0%), Frame = +1

[§] Only the top of entry result is shown.

grass. It may need to be clarified whether the lower expression of NAAT in Sathi rice is a consequence from the use of the same substrate to produce allelochemical.

Protein kinases play a key role in signal transduction pathways in both eukaryotic and prokaryotic cells. Activity of these protein kinases can be regulated by specific events, e.g. DNA damage, as well as numerous chemical signals, Diacylglycerol and Ca^{2+}/calmodulin (Motley and Lory 1999; Nováková et al. 2005). More expression of this gene in Sathi rice plant treated with barnyardgrass may relate with signal transduction to affect down regulated genes.

Oxysterols play several roles in lipid metabolism. Members of this class regulate the expression of genes that participate in both sterol and fat metabolism, serve as substrates for the synthesis of bile acids, and are intermediates in the transfer of sterols (Russell 2000). More expression of oxysterol-binding protein in Sathi rice plant treated with barnyardgrass may relate with sterol compound metabolism as allelochemicals. Mattice (1998) reported that stearic acid released by rice root showed allelopathic activity (see also Noguchi 2005).

In conclusion, DEG in Sathi rice plants grown with barnyardgrass are suggested to have allelopathic potential. GeneFishing PCR and sequencing allowed for the identification of six genes, namely DEG-1, 4, 5, 7=9, and 8, that showed higher expression, and three genes, namely DEG-2, 3 and 6, that showed lower expression, when rice plants were grown with barnyardgrass. BLAST search results showed that DEG-1, 2, 5, 6, 7, and 8 have homology with nr blast-n, DEG-3 has significant similarity with nr blast-n, and DEG-4 has similarity both in nr and EST blast-n. The results from blast-x and blast-n search showed that DEG-1 encodes for S-adenosylmethionine synthetase, DEG-2 is a chloroplast gene for ribulose 1,5-bisphosphate carboxylase large subunit, and DEG-6 encodes for nicotineamine aminotransferase, DEG-3 is similar to oxygen evolving complex protein in photosystem II, and DEG-4 is an unknown protein. Whereas DEG-5, DEG-7 and DEG-8 appear to be related with histone 2B protein, putative serin/threonine protein kinase and putative transposable element, and oxysterol-binding protein, respectively.

Acknowledgements

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