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Identification of a Herbicide Safener AD-67 Inducible cDNA in Rice

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Abstract: A herbicide safener AD-67 inducible cDNA was identified in an indica rice variety 9311 by mRNA differential display. The transcript was increased 6 h after sprayed with the safener solution, and 4 days later, the expression still could be detected. The fragment was recycled from the poly-gel and sequenced, and homologous analysis revealed the cDNA was 100% identical to some ESTs and cDNAs in rice database, and the amino acid sequence was 60-84% homologous to those of the *Yippee* genes in several eukaryotes. The fragment was extended to the whole long cDNA, and thus a primer pair was designed. RT-PCR analysis for the designed primer supported the induction result.
Key words: rice; chemical induction; cDNA; mRNA differential display; herbicide safener; inducible gene

Chemical inducible promoter, as a tool of controlling gene expression, is important for plant research in laboratory and field application. The chemical inducible system can control transgene expression both temporally and spatially, which has an advantage to the old systems including the constitutive promoter and tissue specific promoter systems. This made the system become a new and powerful strategy for gene analysis [1-2]. After Gatz and Quail first reported the tetracycline induction system in 1988 [3], several induction systems were established and developed, such as copper, ethanol, glucocorticoid, estrogen, ecdysone agonist, benzothiadiazole (BTH) and safener inducible systems [3-10]. These systems have some applications in laboratory, but not yet in field due to existing defects [11].

Herbicide safeners selectively protect crop plant from herbicide damage without reducing activity in target weed species. The working mechanism of safener is not very clear, several hypotheses were established to explain how the safeners prevent the detrimental effects of herbicide, but many studies produced inconsistent results. Generally, safener could increase crop tolerance to herbicide [12]. The activities of some genes were increased when plant was treated with safener, such as the cytochrome P450 and glutathione S-transferases (GST) genes, but these genes were constitutively or temporally expressed with development [13]. In 1991, Hershey's group isolated two genes induced by benzenesulfonamide herbicide safener in maize, and the safener was used as a promoter to develop a new inducible gene expression system for plant [14]. This suggests that searching of compounds capable of inducing specific gene expression in plant without affecting growth and development maybe still an effective way for discovering new induction system.

AD-67 is a safener widely used in maize herbicide EPTC (S-ethyl dipropylcarbamothioate). However, few reports revealed its mechanism and inducible gene. In this study, the safener was used as the inducer to detect inducible gene.

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MATERIALS AND METHODS

Growth of rice seedlings

Seeds of rice variety 9311 were sterilized in 0.1% $HgCl_2$ for 5 min and rinsed with sterile water, and then planted into growth chamber with cool-white fluorescent lamps under a photoperiod of 16 h/8 h (light/dark), 28℃ and a humidity of 85%. The seedlings were cultured in MS liquid medium and grown over three weeks when the third leaves fully expanded. At this stage, the plants are more sensitive to exogenous inducer.

Treatment with AD-67 and extraction of total RNA

Rice seedlings were treated by foliar spray with solution of AD-67 (3 g/L) in acetone, with acetone as the control. All of the leaves were collected at 6 h, 12 h, 1 d, 2 d, 3 d, 4 d and 5 d after the treatments and stored at -80℃. Total RNA was extracted using Trizol agent (TaKaRa) according to the manufacturer instruction. After digestion by DNase, the RNA was resuspended in DEPC water and diluted to 1 μg/μL, then stored at -80℃.

Synthesis of the first strand cDNA

Synthesis procedure was according to Promega M-MLV

manufacturer instructions. The 25 μL reaction system contained 2 μg RNA, 40 pmol anchor primer, 5 μL 5×RT buffer, 10 mol/L dNTPs of 5 μL, 25 U RNasin, 200 U M-MLV. The total RNA and primer were incubated at 70℃ for 5 min, followed by adding other components. The mixture was incubated at 42℃ for 1 h to complete the reaction, then at 95℃ for 10 min to end the reaction. Finally, the mixture was diluted to 100 μL for use.

Differential display

The first strand cDNA products were used for PCR amplification with three anchor primers and twelve arbitrary primers. The anchor primers were: XTH_1, 5'TGCCGAAGC TTTTTTTTTTA3'; XTH_2, 5'TGCCGAAGCTTTTTTTTTTG G3'; XTH_3, 5'TGCCGAAGCTTTTTTTTTTC3'. The arbitrary primes were: DD10, 5'TGCCGAAGCTTTGGTAGC3'; DD12, 5'TGCCGAAGCTTGGAGAGT3'; DD18, 5'TGCCGAAGCT TTGGTCAC3'; DD20, 5'TGCCGAAGCTTTGGTCAT3'; DD31, 5'TGCCGAAGCTTTGGTCTG3'; DD33, 5'TGCCGAAGCTT GGAGGAA3'; DD34, 5'TGCCGAAGCTTTGGTGAC3'; DD35, 5'TGCCGAAGCTTTGGTGAG3'; DD36, 5'TGCCGAAGCT TGGAGGAT3'; DD38, 5'TGCCGAAGCTTGAT TGGC3'; DD54, 5'TGCCGAAGCTTTGGTTCC3'; DD60, 5'TGCCGAA GCTTCGACTGT3'.

PCR was carried out in a 20 μL reaction system including 2 μL cDNA products. The reaction mixture was subjected to one cycle of denaturation at 94℃ for 5 min, annealing at 40℃ for 4 min and extension at 72℃ for 2 min; 15 cycles of denaturation at 94℃ for 45 s, annealing at 54℃ for 2 min and extension at 72℃ for 1 min; and 20 cycles of denaturation at 94℃ for 45 s, annealing at 60℃ for 2 min and extension at 72℃ for 1 min; and final extension at 72℃ for 7 min.

The PCR products were denatured with formamide and xylene at 95℃ for 10 min, then 4 μL products were separated on 6% denaturing polyacrylamide gels. After silver staining [15], the bands showing differences between the control and induction treatment were excised and recovered by boiling. The cDNA fragments were re-amplified using the same PCR conditions and primers as above, and recovered with agarose gel electrophoresis. The purified fragments were linked to Pmd18-T (TaKaRa) vector, and the positive clones were subjected to check and sequence.

Sequencing and RT-PCR analysis

Nucleic acid and protein homology searches were performed using the BLAST program at National Center of Biotechnology Information. Some data scanning were conducted with DNAMAN6.0 bioinformatics software. Primers responding to the cDNA were designed by Primer5.0, and RT-PCR was carried out to check the expression of cDNA. Two primers designed for RT-PCR were: Yipa, 5'CAGCAAAC CCATCAATC 3'; Yips, 5'TGGTCCTCGCCA GATA 3'.

RESULTS

Differential display and sequencing

The fragments with significant differences between the control and treatment were selected, and one cDNA designed as ADT3-8 (Fig. 1) was used to sequence. After the sequence being edited manually to remove vector and ambiguous sequence, a fragment with final length of 256 bp was obtained (Fig. 2).

Homologous analysis of nucleotide and protein sequence

Nucleotide sequence search by BLASTN revealed that the cDNA was homologous to multiple ESTs, mRNAs and genomic sequences of rice with high identities (Table 1). Interestingly, the homologous ESTs are mostly isolated from the induced tissues and organs of rice, for example, the CI491559 came from rice being induced by ACC, CI368508 and CI366717 were identified in the callus, the other ESTs were reported in the stem treated by γ ray. This suggested that the cDNA was a AD-67 inducible gene in rice.

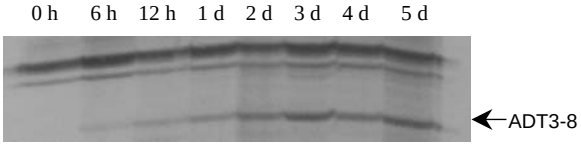

Fig. 1. The result of differential display (silver staining).

The arrow shows the differential display band ADT3-8. The induction time was from 0 h (CK) to 5 days.

>ADT3-8

5'*TGCCGAAGCTTTTTTTTTTC*ACATGACATTATATTTCATAAATGCAATTTAATGACGATGGTCCTCGCCAGATAATCAATTT

TGGGGAGAATTGAAACCCAATATTCAGCGATATTTTACATGGTTTCATATGCCTGCATTTGCAGACTTAATAGTTCAGCGGTT

TTCCAATCACTATCCTTCTTTCAGCATCATGTGCTTCTCTAATATATACTTGCCTTCTTTGTACTTCTGGTC***CTCACCAAAGCTT***

***CGGCA* 3'**

Fig. 2. Sequence of the differentially expressed fragment.

Black italic shows the primers of PCR, 5' is the anchor primer and 3' is the arbitrary primer.

Table 1. Nucleotide sequences homologous with ADT3-8 in rice.

GenBank accession No.	Clone type	Chromosome	Identity (%)	Length (bp)
CI491559.1	EST		100	380
CI552836.1	EST		100	422
CI543723.1	EST		100	385
CI550667.1	EST		100	388
CI551477.1	EST		100	425
CI368508.1	EST		100	442
CI366717.1	EST		100	483
XM_478909	mRNA		100	826
AK073870.1	cDNA		100	826
AK068081.1	cDNA		100	2 159
AP003747.5	Genomic DNA	7	100	123 787
AP008213.1	Genomic DNA	7	100	29 644 043

By BLASTX program, protein sequence analysis showed that many *Yippee* genes in different species including yeast and *Tetrahymena thermophila* shared the same function domain with the cDNA (Table 2). Thus, we identified the mRNA (GenBank accession No. XM_478909) as the transcript of the ADT3-8 according the homologous sequences of the fragment.

RT-PCR analysis

Using the primers designed for the mRNA of rice *Yippee* gene, RT-PCR result proved that the gene transcripts increased after induced by AD-67 (Fig. 3). The significant increase was found at 6 h after treatment, with maximum at 12 h after treatment. At 5 days after treated by AD-67, the expression could also be detected. Moreover, sequencing of the RT-PCR products showed the nucleotide sequence of the 731 bp PCR product was identical to the mRNA (GenBank accession No. XM_478909) in rice.

DISCUSSION

Rice is the staple food crop, as well as the valuable model plant in cereal crops. The genome draft sequence of rice had been completed early in 2002, and the whole genome fine map had also been published by different groups. The rice genome

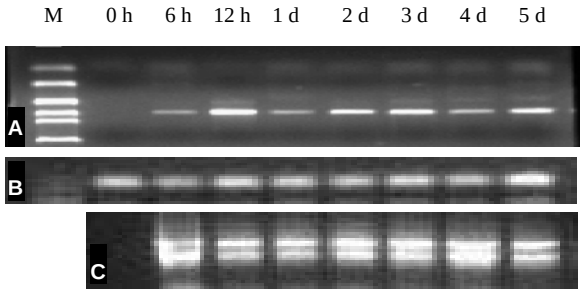

Fig. 3. RT-PCR analysis of inducible gene.

A. *Yippee* gene, the left is Marker DL2000; B. *actin* gene as control; C. rRNA.

database can provide mass bioinformation to scientists, which promote the rice research and release the researchers from the laborious experimental work to the efficient computational analysis.

Yippee is an intracellular protein in *Drosophila*, which is conserved in eukaryote according to the reports [16]. This suggested that *Yippee* gene is an important gene. The gene expressed when the *Drosophila* was infected by pathogens, functioning as an immune related protein. The *Yippee* gene of rice we identified according to the cDNA fragment is induced by safener AD-67, this is consistent with the results of previous studies, which concluded that safener could induce the expression of *R* gene [12]. Therefore, we deduced that the mechanisms of immune responses in rice and *Drosophila* might be similar.

Although the function of *Yippee* gene was discussed in some studies in *Drosophila*, its respondent in rice is still unknown and should be studied. Moreover, if there is a regulator element in the 5' upstream of the gene will also need to be done.

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Table 2. Protein sequences homologous with ADT3-8.

Genbank accession No.	Gene name	Organism	Similarity (%)	Peptide length (aa)
XP_478909	*Yippee*	*Oryza sativa* ssp. *japonica*	84	110
BAC82961	*Yippee*	*Oryza sativa* ssp. *japonica*	84	110
ABA94243	*Yippee*	*Oryza sativa* ssp. *japonica*	62	106
EAR94563.1	*Yippee*	*Tetrahymena thermophila*	62	98
AAW44764.1	*Yippee*	*Cryptococcus neoformans* var. *neoformans*	60	134
XP_572071.1	*Yippee*	*Cryptococcus neoformans* var. *neoformans* JEC21	60	134

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