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Genes of Wild Rice (*Oryza grandiglumis*) Induced by Wounding and Yeast Extract

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Oryza grandiglumis (CCDD, 2n=48), one of the wild rice species, has been known to possess fungal-, bacterial-, and insect-resistance against sheath blight, rice blast, bacterial leaf blight and brown plant hopper (*Nilaparvata lugens*). To rapidly isolate differentially expressed genes responding to fungal and wounding stress, wounding and yeast extract were treated to *O. grandiglumis* for 24 hrs. Suppression subtractive hybridization (SSH) method was used to obtain differentially expressed genes from yeast extract and wounding treated plants. Seven hundreds and seventy six clones were obtained by subcloning PCR product, and colony array and screening were carried out using radio-isotope labeled cDNA probes prepared from the wounding and yeast extract treated plants. One hundred and fifteen colonies were confirmed as true positive ones. Average insert size of the clones were ranged from 400 bp to 700 bp and all the inserts were sequenced. To decide the identity of those clones, sequences were analyzed by sequence homology via GenBank database. The homology search result showed that 68 clones were matched to the genes with known function; 16 were related to primary metabolism, 5 to plant retrotransposons, 5 to defense related metallothionein-like genes. In addition to that, others were matched to various genes with known function in amino acid synthesis and processing, membrane transport, and signal transduction, so on. In northern blot analysis, induced expressions of ogwfi-161, ogwfi-646, ogwfi-663, and ogwfi-695 by wounding and yeast extract treatments were confirmed. The result indicates that SSH method is very efficient for rapid screening of differentially expressed genes.

Key words – *Oryza grandiglumis*, SSH, yeast extract, wounding, colony array

Oryza grandiglumis, which is originated from Central or South America based on the phylogenetic tree constructed by restriction fragment length polymorphism (RFLPs), is one of members of CCDD genome allotetraploid wild rice [6]. *O. grandiglumis* is a species of the *O. officinalis* complex and very closely related to *O. alta* and *O. latifolia* with which it shares the same chromosome number (2n=48) and tetraploid genome (CCDD). Even though most of wild rice is thought to be inferior to modern cultivars, it has been tried for long time to improve modern cultivars for both yield and quality using these wide varieties of germplasm [13]. In their study, two quantitative traits loci (QTL) alleles of *O. rufipogon* on chromosomes 1 and 2 were identified as genetic sources for increasing grain yield per plant. Not only a gene source for trait-improvement but the safety of wild rice for human consumption was also evaluated [14]. However, few researches have been focused to obtain agriculturally important genes from wild rice. So far, most studies have been concentrated on molecular genetics to find polymorphism and its application for mapping [5,6,10].

In terms of gene cloning, very efficient gene cloning methods and strategies have been rapidly developed and published [2,8,9]. Especially, a method for massive screening of temporally and spatially expressed genes demonstrated its efficiency and wide applicability [7]. Considering that only certain proportion of genes is expressed in any given condition and development stage, temporal and spatial classification of cloned genes are a very important step to comprehend gene expression and regulation in the living cells of higher organism. The suppression subtractive hybridization (SSH) method is based on suppression PCR [12], and combines normalization and the subtraction in a single step. The normalization step equalizes the abundance of cDNA with the target population and subtraction step excludes the common molecules between the tester and the driver population [2]. As a result, 1000 fold enrichment for differentially expressed cDNAs can be

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easily achieved. Proper application of this method is very efficient to screen and clone large amount of differentially expressed genes from a specific species, such as wild rice species.

In this study, various genes of wild rice *O. grandiglumis* responding to wounding and yeast extract were cloned by SSH, and screened and characterized by dot blot and northern blot analysis followed by sequence alignment. The successful cloning and functional characterization of the genes from wild rice will lead to the later development of robust rice variety against stress and disease via genetic transformation of specifically targeted gene.

Materials and Methods

Plant materials and RNA isolation

Wild Rice (*Oryza grandiglumis*: CCDD, 2n=48) seeds were sown and grown for 50 to 100 cm in field of National Crop Experiment Station. For wounding, plant leaves were punched by paper punch and samples were harvested at 24 hrs after wounding. For fungal elicitor treatment, 20% yeast extract solution was sprayed onto the surface of plant and harvested at 24 hrs after treatment [11]. Plant samples were frozen immediately in liquid nitrogen. Total RNA was isolated from the sample harvested at 24 hrs, and by Chomczynski's method [1] and mRNA was extracted using PolyA Tract mRNA Isolation Systems (Promega, USA). Total RNA was need for Northern blot analysis extracted from the plant material treated for 24 hrs and used.

Suppression subtractive hybridization (SSH)

For PCR-based cDNA subtraction method, all the procedure was carried out according to manufacturer's instruction (Clontech, USA). Plant materials, neither wounded nor sprayed with yeast extract solution, were harvested as a control used for driver preparation of cDNA, and plant materials from the treated, wounded and sprayed, for tester preparation. Both ds cDNAs for tester and driver were synthesized from 5 μg of mRNA extracted from control or treated plants. First and second strand cDNA synthesis and blunt ending of DNA ends were carried out according to manufacturer's instruction. The resulting cDNA pellet was digested by *Rsa*I for 2 hrs. Two tester cDNAs were then ligated to adaptor 1 or adaptor 2, but no adaptor ligation was made for driver cDNA. All the cDNAs were denatured, and driver cDNA was added to two separate testers. Then allowed to anneal for 8 hrs at 68℃. After the 1st hybridization, the two samples were combined and a fresh portion of heat denatured driver in hybridization buffer was added. The samples were allowed to hybridize at 68℃ overnight. For each subtraction, two PCR amplifications were performed. PCR was performed with the following parameters: 75℃ for 7 min; 30 cycles at 94℃ for 42 sec, 68℃ for 42 sec, 72℃ for 3 min, and a final extension at 72℃ for 7 min. The amplified products were diluted 10-fold in deionized water. Some of the product were then used as the template in secondary PCR for 15 cycles under the same conditions used for the primary PCR, except that the PCR primer P1 and P2 were replaced with nested PCR primer PN1 and PN2, respectively. Product from the second PCR was inserted into pCRII vector using a T/A cloning kit (Invitrogen, USA).

Colony array screen for positives (dot blot)

The subclones of differentially expressed cDNAs were screened according to the manufacturer's manual (PCR-Select Differential Screening Kit; Clontech, USA). Seven hundred and seventy six colonies were grown in 100 μl of LB liquid + ampicillin medium at 37℃ overnight with shaking. The nylon membranes are placed onto LB/agar plate containing ampicillin. 1 μl of each bacterial culture was placed onto each membrane and incubate at 37℃ overnight. The membranes were saturated colony-side-up with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 4 min and neutralizing solution 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl for 4 min. After that, membranes were air-dried for 30 min and the DNA was fixed by baking for 2 hours at 80℃ in oven. Probe was prepared by ^{32}P-labelling of forward-subtracted cDNA and hybridized to the membranes at 42℃ overnight. Membranes were washed twice with low-stringency washing solution (2×SSC, 0.5% SDS) and high-stringency washing solution (0.2×SSC, 0.5% SDS). After wash-out, membranes were exposed to X-ray film overnight with an intensifying screen at -70℃.

Northern blot analysis

About 25 μg of total RNA from the wounding and fungal elicitor treated plants was electrophoresed on 1% agarose-formaldehyde gel, and transferred to Hybond-N+ filters (Amersham, UK). Northern blot hybridization, with each SSH clone Biotin-Labeled probes (Tropix, USA), was performed at 65℃ for 20 hrs. Membranes were washed twice under high stringency condition (0.2×SSC, 0.5%

SDS), and exposed to X-ray film (Fuji film, Japan).

DNA sequence analysis

The nucleotide sequences of the cloned cDNAs were determined by the dideoxy-chain termination method using the AutoCycle TM Sequencing Kit (Amersham, UK). Electrophoresis was performed on ALFexpress II DNA Analysis System (Amersham, UK). Homology search was carried out through BLAST Network Service (National Center for Biotechnology Information, USA). Nucleotide and deduced amino acid sequences were analyzed using the PC/GENE programs (Intelligenetics, Switzerland).

Results and Discussion

Construction of SSH library for wounding and fungal elicitor-responding genes

A PCR-based cDNA subtraction method, termed suppression subtractive hybridization, has been developed and turned out to be a successful method for rapid screening of differentially induced genes. SSH was used to isolate genes differentially expressed during wounding and fungal elicitor treatment from wild rice, *Oryza grandiglumis*. For SSH, tester (wounding and yeast extract-treated) and driver (non-treated control) double-stranded cDNA were synthesized and *Rsa*I digestion resulted around 500 bp in length. It was confirmed that most of fragments were ranged from 400 bp to 700 bp (Fig. 1). After subtraction by double hybridization, suppression PCR was carried twice to equalize the amount of cDNA from both rare and abundant transcripts. The suppression PCR reaction was subcloned into TA cloning vector and 776 colonies were obtained. With the subtracted clones, colony array with 32P-labeled forward-subtracted cDNA probes was performed. As a result, 115

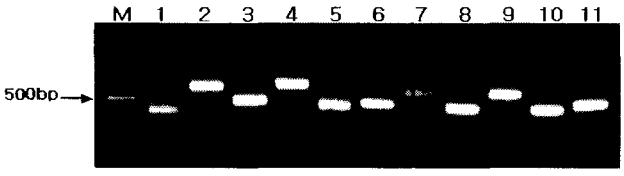

Fig. 1. Confirmation of insert size by colony PCR. After ligation, reaction was transformed into DH5 competent cell. 776 colonies were investigated by colony PCR using M13 forward and M13 reverse primers. The insert size of positive true colony was ranged from 400 bp to 700 bp. Lanes: M, size marker (Promega, USA); lane 1-11, individual positive colony PCR amplification.

out of 776 clones were identified as positive true (data not shown). The observed true colony ratio is relatively high compared to other screening method such as DDRT (Differential Display Reverse Transcription), even though it still needs to be proven further with more delicate experimental tools [2,8]. The sequences of 115 clones were determined and 68 clones were matched to genes with known function (Table 1).

It was known that SSH method has many advantages to overcome the problem found in other screening method like DDRT. Introduction of 4 bp-cut *Rsa*I digestion enhanced the hybridization and the subtraction efficiency and suppression PCR increased the copy number of rarely expressed gene to detectable amount. Although *Rsa*I digestion may be a disadvantage when full-length cDNAs are requested, dividing each cDNA into multiple fragments has two important advantages. First, long DNA fragments may form complex structure that prevents the formation of appropriate hybrids, especially at high concentration required for efficient hybridization. Second, cutting the cDNAs into small fragments provides better representation of whole set of population [2].

Sequence analysis of wounding and fungal elicitor-induced genes

Among 776 clones, 115 positive clones were sequenced. Sequence comparison with the EMBL/GenBank database using the BLAST search revealed that 68 cDNAs showed sequence similarity to genes from other organisms (Table 1). As expected, many of the identified cDNAs were housekeeping genes related to primary metabolism. Twenty three percentage of clones were matched to *Oryza sativa*, *Arabidopsis thaliana*, and *Homo sapiens* genomic DNA. Four clones (ogwfi-6, ogwfi-23, ogwfi-55, ogwfi-611) had homology to plant retrotransposons which are tightly linked to molecular pathways activated by stress, and activation of the retrotransposons are under the control of *cis*-regulatory sequences strikingly similar to those of plant defense genes [3]. Five clones (ogwfi-362, ogwfi-440, ogwfi-460, ogwfi-561, ogwfi-695) had sequence homology (56-69%) to metallothionein-like genes that known to be related to defense mechanism against pathogen. Under the various stress conditions, such as excessive heavy metals and heat shock, significantly elevated expression of some of metallothionein-like gene was reported [4]. In addition, two clones are matched to genes involved in amino acid synthesis and processing, four clones in membrane and transport, ten

Table 1. Cloned wild rice (*O. grandiglumis*) cDNAs induced upon wounding and yeast extractor treatment and their sequence homology to the known function of genes in GenBank database.

Clone ID*	Putative Identity	Species	Score
ogwfi-1	Genomic DNA sequence	*Drosophila melanogaster*	38.5
ogwfi-2	Genomic DNA chromosome 3	*Arabidopsis thaliana*	38.2
ogwfi-6	Dispersed repeat	*Oryza latifolia*	102
ogwfi-7	BAC OSJNBa0056G17	*Oryza sativa*	58.2
ogwfi-8	Squalene epoxidase+B6	*Homo sapiens*	50.6
ogwfi-9	MtN3-like protein	*Dinthus caryopyllus*	54.4
ogwfi-10	MEK kinase	*Arabidopsis thaliana*	46
ogwfi-13	BAC clone RP11-236P2	*Homo sapiens*	36.2
ogwfi-16	BAC OSJNBa0013MD genomic sequence	*Oryza sativa*	34.9
ogwfi-17	DNA chromosome 5 BAC clone F7J8	*Arabidopsis thaliana*	33.3
ogwfi-18	Chromosome 1 BAC T15D22	*Arabidopsis thaliana*	42.3
ogwfi-20	Chlorophyll a/b-binding protein	*Oryza sativa*	87.7
ogwfi-21	Zinc-finger protein	*Arabidopsis thaliana*	36.2
ogwfi-23	Pol polyprotein II bac F15011	*Arabidopsis thaliana*	79
ogwfi-25	Choline binding prtein1 (ccbp1)	*Streptococcus pneumoniae*	36.1
ogwfi-29	Adipose most abundant gene transcript 1 (APM1)	*Homo sapiens*	40.2
ogwfi-30	E2F transcription factor-1 E2F1	*Arabidopsis thaliana*	36.2
ogwfi-31	Hypothetical protein F8J2. 170	*Arabidopsis thaliana*	75.9
ogwfi-37	Light-induced mRNA	*Oryza sativa*	72.7
ogwfi-38	Lipoxygenase-3	*Glycine max*	36.8
ogwfi-39	Actin	*Oryza sativa*	217
ogwfi-44	ABA and salt stress-responsive rice cDNA	*Oryza sativa*	47.8
ogwfi-45	Substance P receptor	*Runa cafesheiana*	36.2
ogwfi-46	Ran-GTP binding protein 5	*Homo sapiens*	50.2
ogwfi-48	Transcription factor E2F	*Xenopus laevis*	34.3
ogwfi-49	ABC transporter protein 1-like	*Arabidopsis thaliana*	149
ogwfi-54	Partial mRNA for hypothetical protein	*Mus musculus*	51.3
ogwfi-55	Putative transposase	*Oryza sativa*	135
ogwfi-56	TU12B1-TY protein	*Homo sapiens*	36.3
ogwfi-57	NADPH-dependent cytochrome P450	*Phanerochaete chrysosporium*	40.7
ogwfi-58	Putative MtN3-like protein	*Dianthus caryophyllus*	42.1
ogwfi-61	Small auxin tip-regulated RNA	*Monodelphis domestica*	40.6
ogwfi-62	Actin mRNA	*Aplysia californica*	160
ogwfi-66	Monocyte/neutrophil elastase ingibitor gene	*Homo sapiens*	41.8
ogwfi-69	RAc1 mRNA for actin	*Oryza sativa*	149
ogwfi-70	Cell expressed developmentally down-regulated 5	*Homo sapiens*	90.3
ogwfi-71	Putative NADPH-cytochrome P450 reductase	*Pisum sativum*	42.4
ogwfi-73	Chloroplast	*Pinus thunbergii*	38
ogwfi-74	MobA-nprE gene region	*Bacillus subtilis*	39.7
ogwfi-76	GcaA gene	*Bacillus subtilis*	38.7
ogwfi-77	Phosphoribulokinase	*Triticum aestivum*	86.3
ogwfi-78	Carbonate dehydratase	*Arabidopsis thaliana*	42
ogwfi-80	ARE1-like protein	*Arabidopsis thaliana*	73.2
ogwfi-84	Polyubiquitin (Rubp1)	*Oryza sativa*	179
ogwfi-91	Photosystem II 10kDa polypeptide	*Oryza sativa*	166
ogwfi-92	Genomic DNA chromosome1	*Oryza sativa*	90
ogwfi-93	MSH-5 mRNA	*Caenorhabditis elegans*	40.5
ogwfi-95	Ribose 5-phosphate isomerase	*Arabidopsis thaliana*	105
ogwfi-99	Photosystem I subunit IV precusor	*Arabidopsis thaliana*	75.5
ogwfi-103	Genomic DNA	*Oryza sativa*	153

Table 1. Continued

Clone ID*	Putative Identity	Species	Score
ogwfi-95	Ribose 5-phosphate isomerase	*Arabidopsis thaliana*	105
ogwfi-99	Photosystem I subunit IV precusor	*Arabidopsis thaliana*	75.5
ogwfi-103	Genomic DNA	*Oryza sativa*	153
ogwfi-104	Sp420/240 protein precusor	*Chironomus tentan*	30
ogwfi-108	Genomic DNA chromosome1	*Oryza sativa*	38.2
ogwfi-112	Glyceraldehtyde-3-phosphate dehydrogenase (NADP+) chloroplast	*Oryza sativa*	51
ogwfi-115	Ribulose bisphosphate carboxylase S	*Oryza sativa*	98
ogwfi-119	Rieske Fe-S precursor protein	*Oryza sativa*	36
ogwfi-123	Genomic DNA ch 3	*Oryza sativa*	81.8
ogwfi-124	Hypothetical protein	*Escherichia coli*	31
ogwfi-161	Na+-D-glucose cotransport regulator gene	*Homo sapiens*	38.4
ogwfi-465	Translation initiation factor 5A (elf-5A)	*Oryza sativa*	62.3
ogwfi-549	Genomic DNA chromosome 6	*Oryza sativa*	44
ogwfi-561	Metallothionein-like protein mRNA	*Oryza sativa*	191
ogwfi-611	Putative gal-pol polyprotein	*Oryza sativa*	106
ogwfi-613	Cell wall proein (Angrp-1) gene	*Oryza sativa*	62.5
ogwfi-646	Pseudo-response regulatior 5	*Arabidopsis thaliana*	40.8
ogwfi-660	3-phosphoshikimate 1-vinyltransferase	*Chlamydia trachomatis*	48
ogwfi-663	Putative integral membrane protein	*Arabidopsis thaliana*	30
ogwfi-686	Transcription factor	*Mus musculus*	32
ogwfi-695	Endo-1,4-beta-glucanse	*Pyrococcus horikoshii*	30

*ogwfi is abbreviated form of *Oryza glandiglumis* wounding and fungal induced gene

clones in signal transduction, three clones in DNA/RNA related gene expression, and two clones in cell division.

Even though limited information is provided in this study due to the small number of sequences data available, our result indicates that *O. grandiglumis* responded environmental stimulus, wounding and fungal elicitor application, and expressed appropriate spectrum of genes to cope with them.

Northern blot analysis

Non-isotopic chemiluminescent detection procedures were applied to confirm the gene expression of several clones identified as positive ones from dot blot. Dot blot method is known to be very rapid and convenient but it could be practiced only for quick screen as a preliminary experiments. More reliable result can be obtained from northern blot analysis. Northern blot analysis of the selected wounding and yeast extract induced genes of *O. grandiglumis* showed that the clones of ogfwi-161, ogfwi-646, ogfwi-663, and ogfwi-695, showing homology to Na^+-D-glucose co-transport regulator gene, pseudo-response regulator 5, integral membrane protein, and endo-1,4,-β-glucanase, respectively, were induced by treatments (Fig. 2).

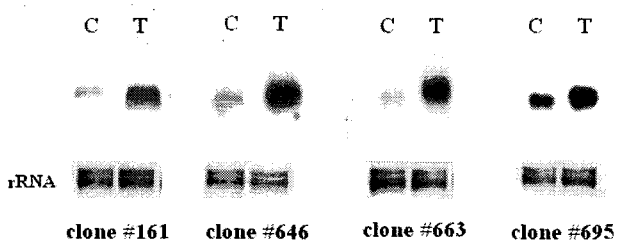

Fig. 2. Northern blot analysis of the wounding and fungal elicitor induced genes of wild rice (*Oryza grandiglumis*) C, control wild rice; T, wounding and yeast extract treated wild rice; clone #161, best homology to Na^+-D-glucose co-transport regulator gene; clone #646, best homology to pseudo-response regulator 5; clone #663, best homology to integral membrane protein; clone #695, best homology to endo-1,4,-β-glucanase.

The result indicated that they are solely responsive genes to external stimulus such as wounding and fungal elicitor. In case of clone ogfwi-646 and ogfwi-668, low level of basal expression were observed in control. Even though the expressions of those genes are detectable under normal condition, the result showed the overall increase of gene expression under the treatment conditions.

In the present study SSH method and dot blot screen were practiced to clone wounding and fungal elicitor

induced genes from wild rice, *O. grandiglumis*. We believed that the massive and rapid screening of differentially expressed genes from useful germplasm resources were essential prerequisite for future use of the genetic material in molecular or conventional breeding fields.

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야생벼의 일종인 *Oryza grandiglumis* (CCDD, 2n=48)는 도열병, 잎집무늬마름병, 흰빛잎마름병, 그리고 벼멸구와 같은 병충해에 저항성을 가지는 것으로 알려져 있다. 이와 같은 곰팡이와 해충에 반응하여 차별 발현하는 유전자를 클로닝 하기 위하여 상처처리와 yeast extract를 *Oryza grandiglumis*에 0시간과 24시간 각각 처리하였다. 유전자의 클로닝을 위하여 희귀 발현유전자의 클로닝에 효율적인 것으로 알려진 Suppression Subtractive Hybridization (SSH) 방법이 처리 후 24 시간된 식물을 재료로 사용되었다. 그 결과, 776개의 cDNA clones이 확보되었으며, 유전자 발현의 진위여부를 빠르게 스크린하기 위하여 colony array가 수행되었다. 115개의 colony가 positive로 판명되었고, 이들의 평균 insert size는 400 bp에서 700 bp에 이르렀고, 이들에 대한 염기서열 분석이 수행되었다. 염기서열 분석 결과, 68개 clone들이 알려진 기능의 유전자와 homology를 나타냈으며, 이중에서 16개 clone이 일차대사에 관련된 것과 유사성을, 5개가 plant retrotransposon과 유사성을, 5개가 식물 방어기작 관련 metallothionein-like gene과 염기서열 유사성을 보였다. 이외에 다양한 유전자들이 아미노산 합성관련, membrane transport, signal transduction등에 관여하는 유전자들과 상동성을 나타내었다. 이들 유전자중에서 4개의 클론(ogwfi-161, ogwfi-646, ogwfi-663, ogwfi-695)들이 선발되었고 이들에 대한 Northern 분석이 수행되었다. Northern 분석결과 ogwfi-161, ogwfi-646, ogwfi-663, ogwfi-695는 wounding과 yeast extract처리에 의한 차별 발현이 확인되었다. 이상의 결과를 종합하여 볼 때, SSH방법은 병충해등과 같은 조건에 의해 차별 발현되는 유전자들을 빠른 시간 내에 다량으로 발굴할 수 있는 매우 효율적인 방법이라고 생각된다.