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Isolation and Characterization of a Wound or UV Induced cDNA Fragment from *Pleurotus sajor-caju*

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상처 및 자외선 자극에 반응하는 여름느타리 cDNA 단편의 분리 및 그 발현 특성

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ABSTRACT: A 0.4 kb cDNA fragment was isolated from mRNA of UV or mechanical wound damaged *Pleurotus sajor-caju* by the differential display method. Expression of the gene corresponding to this cDNA fragment was highly induced by mechanical wound damage or UV treatment. This gene showed only basal level expression in mycelia, stipe, and cap under normal growth conditions. Sequencing analysis showed that this cDNA fragment contains partial open reading frame. Homology search using genbank database revealed that although this gene do not have homology with already reported wound induced genes, it has a significant sequence homology in defined region with the cdc2-related protein kinase gene which is known to be involved in negative regulation of meiotic maturation in *Xenopus oocytes*.

KEYWORDS: *Pleurotus sajor-caju*, Gene isolation, Wound induction, UV induction

Higher organisms have developed their own defence mechanisms against attacking viruses, bacteria, fungi, and insects (Dixon and Lamb, 1990; Dixon *et al.*, 1994). Biochemical and molecular studies revealed that kinds of plant genes are induced as part of the general defence response when plants are challenged by microorganisms or wound by chewing insects (Baron and Zambryski, 1995; Ryan, 1990). The genes encoding enzymes in phenyl-propanoid metabolism such as Phenylalanine ammonium-lyase (PAL), Chalcone synthase (CHS), and Chalcone isomerase (CHI) are most well known defence response genes (Baron and Zambryski, 1995; Lawton. and Lamb, 1987). The end products of these genes function in cell wall reinforcement, like lignin, or as direct antimicrobial compounds, like phytoalexin (Baron and Zambryski, 1995; Dixon, 1986; Higuchi, 1985). On the other hand, many enzymes including chitinase, pectinase, β-glucanases, protease inhibitors, and other PR-proteins are synthesized as a result of defense response and function in degradation of the cell walls of invading fungi or releasing endogenous elicitors from the cell wall to stimulate the defense response (Baron and Zambryski, 1995; Rogers and Ausubel, 1997; Ryan, 1990; Ward *et al.*, 1991). In addition, the genes encoding structural protein such as Hydroxyproline-rich glyco-proteins (HRGPs) are also induced to form a structural barrier against pathogens invasion (Hedrick *et al.*, 1988; Lawton and Lamb, 1987;

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Mehdy and Lamb, 1987). In plants attacked by chewing insect, as same as pathogen invasion, these defense related genes are induced at the site of physical damage first and then systemically induced in whole plant body (Baron and Zambryski, 1995; Higuchi, 1985; Ryan, 1990). It was strongly suggested that a peptide hormone, systemin, is a signal molecule of such signal transduction pathways in plant wounding response (Baron and Zambryski, 1995; MeGurl *et al.*, 1992; Orozco-Cardenas *et al.*, 1993). Lots of experiments indicate that systemin induces above mentioned PR-genes through the synthesis and action of jasmonic acid (Baron and Zambryski, 1995; Constabel *et al.*, 1995, McConn *et al.*, 1997; Pena-cortes *et al.*, 1995). In addition of systemin, abscisic acid and electrochemical potentials have also been proposed as long-distance signals for systemic acquired resistance in wounded plants (Pena-cortes *et al.*, 1995; Wildon *et al.*, 1992). Edible mushroom, which is one of the most profitable product in farm, is also severely damaged by chewing insects and even in mechanical damage. However, molecular studies to find self defense mechanism in mushroom are not well established yet. As a first step to study mushroom defense mechanism against wound damage, we tried to isolate and characterize wound induced genes from *Pleurotus sajor-caju*. By the differential display method (Liang and Pardee, 1992) with simple modification, we have isolated a cDNA fragment of the gene which is induced by wound or UV treatment. Induction of this gene under these condition was confirmed by RT-PCR analysis. Furthermore, we have tested whether this gene is organ-specifically expressed.

Materials and Methods

Strains and culture condition

Pleurotus sajor-caju, ASI 2070, was grown in culture bottles with sawdust substrates at 30°C. Mature fruitbody was used in wound treatment and RNA isolation. pBS^+ (KS) and *E. coli* DH5α strain were used as a cloning vector and host cell, respectively.

RNA isolation

Total RNA from mycelia, and stipe and cap of the fruitbody was isolated by a LiCl precipitation protocol adapted from the procedures of Sambrook *et al.* and described in detail by Conley *et al.*. For RT-PCR reactions, RNA samples were treated with DNAse I twice to deplete contaminating genomic DNA (Simpson *et al.*, 1992). RNA concentration was measured by spectrophotometer and confirmed by electrophoresis in formaldehyde agarose gel.

Recombinant DNA Techniques and Sequencing

Standard enzymatic manipulations and purification procedures were performed essentially as described by Sambrook *et al.*. Restriction and DNA modification enzymes were purchased from Promega (Madison, WI, USA). For DNA sequencing, the isolated 0.4 kb cDNA fragment was subcloned into pBS^+ (KS) (Stratagene). DNA sequencing was performed by the dideoxy sequencing method using a Sequenase kit (US Biochemical Corp.). DNA sequence analysis was carried out with DNASIS program (HITACH) and Genbank database (NIH).

Wound and UV treatment

The cap of mature fruitbody was cut by razor blade from the edge to inside about 5 cm in length to make effect by wound mechanically. Ultraviolet light (UV) treatment of cap was performed under UV light in laminar flow cabinet. After 4 hours, the tissue adjacent to the mechanically wounded site was collected and immediately frozen in

liquid N_2. Total RNA was extracted from the wounded and unwounded cap tissues at similar developmental stages for mRNA differential display analysis.

Differential display analysis

Poly dT primer (5'-CGAGGAATTCTTTTT-TTTTTTTTTTTTTTTTTTTTTTTTT-3') was synthesized to be used as antisense primer in reverse transcription (RT) reaction. First strand cDNA synthesis was performed in a 20 μl reaction mixture containing 2 μg DNAse I treated RNA, 0.5 μg of poly dT primer, 40 mM each of the four deoxynucleotides (dNTPs), 200 units of M-MLV reverse transcriptase and 20 units of ribonuclease inhibitor. The reaction mixture was incubated at 42°C for 30 minutes and stopped by heating to 65°C for 10 minutes. For PCR, 20 μl of the RT reaction mixture was combined with an additional 0.25 μg poly dT primer, each one of 30 different 10 bp length of arbitrary sense primer (opa 1-30), 10 μl of 10x DNA polymerase buffer, 10 μl of 25 mM $MgCl_2$, dNTPs to final concentrations of 0.25 mM each, and 2.5 units of Taq DNA polymerase. Final reaction volumes were brought up to 100 μl by addition of H_2O. The PCR was performed in a Perkin Elmer/Cetus DNA Thermal Cycler for 40 cycles of 30 sec at 94°C, 30 sec at 48°C and then 1 min at 72°C. Reaction samples of wound treatment and control were separated in parallel by electrophoresis on an 1.2% agarose gel to compare differences in their mRNA level. The cDNA fragment of differentially expressed mRNA from wound treated samples was then extracted from agarose gel by using a Gel extraction kit (Qiagen).

RT-PCR procedures

RT-PCR reaction was performed as described in the differential display analysis except that the specific primer, 5'-CCAAGCACTTT-ACTCT-3', correspond to nucleotide sequences of the internal region of the gel extracted cDNA fragment, was used as sense primer in PCR reaction instead of arbitrary primer. The same reactions with same amount of RNA were performed using 18S rRNA specific sense and antisense primers as a standard to normalize for the amount of total RNA per lane.

Results and Discussion

Isolation of a wound or UV induced gene fragment

To isolate stress induced genes from *Pleuro-*

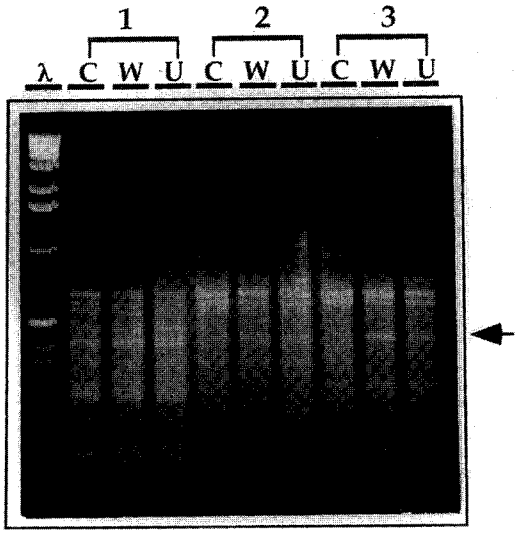

Fig. 1. Isolation of a wound or UV induced cDNA fragment from *P. sajor-caju*.
Total RNA (2 ug) from wound (W) or UV (U) treated and untreated (C) cap tissue was subjected to differential display analysis to isolate stress induced genes. RNA from each treatment was used in cDNA synthesis using poly dT primer and then amplified by PCR with combination of various random 10 mer primers, opa1 (1), opa3 (2), and opa10 (3), and poly dT primer. The PCR products were separated by electrophoresis on an 1.2% agarose gel with 1 kb ladder DNA size marker (λ). Arrow indicates position of the PCR product which is only generated by wound or UV treatment.

tus sajor-caju, mature fruitbodies in culture bottles were wounded or UV treated for 4 hours (see Materials and Methods). Total RNA from wounded or UV treated cap tissue was isolated, and then compared with those from non-treated control cap tissue by the differential display method. After reverse transcription of mRNA by poly dT antisense primer, PCR reactions were performed by using poly dT antisense and each of 30 different arbitrary sense primers in each reaction. Although most bands generated by PCR were the same, one band was seen only in wounded and UV treated samples when opa 10 primer was used as sense primer (Fig. 1). This around 0.4 kb cDNA fragment was extracted from agarose gel and then sub-cloned into pBS$^+$ for sequencing.

DNA sequencing and analysis

Nucleotide sequencing of the 0.4 kb cDNA fragment shows that this fragment contains a partial ORF of 136 nucleotides started from the 5' end, a 3' UTR region with two consensus AATAA polyadenylation signals, and poly A tail at the 3' end of the fragment. Deduced amino acids sequences of the ORF shows a significant sequence similarity with those of cdc2 related protein kinase from *Xenopus oocytes* (Fig. 2). Since this gene was known to be functioned in cell division, it is of interesting that whether the product of the cloned gene (designated *Wun* gene) function in healing of wounded tissue by making fast cell division (Shuttleworth *et al.*, 1990). Indeed, it was well known that cell division in higher organisms is rapidly induced by wounding (Baron and Zambryski, 1995).

Expression of the *Wun* gene

To confirm that this *Wun* gene isolated by the differential display method is a real damage induced gene, the levels of *Wun* mRNA under wound, UV, and normal growth

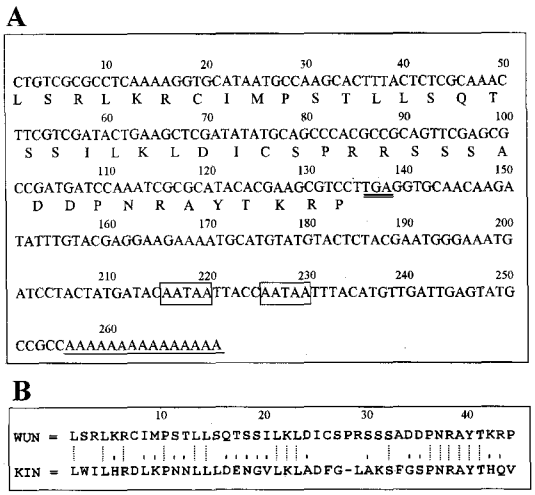

Fig. 2. Nucleotide and deduced amino acid sequence of the partial cDNA fragment of the wound or UV induced gene.
A) Nucleotide and deduced amino acid sequence of the partial cDNA fragment isolated by mRNA differential display of wound or UV treated *Pleurotus sajor-caju*. The translation stop codon (TGA), putative poly A tail signals (AATAA), and a poly A tail (AAAA) are indicated.
B) Comparison of the deduced amino acid sequence of the partial cDNA fragment of the wound or UV induced gene (Wun) with the cdc2-related protein kinase of Xenopus oocytes.

condition were compared by RT-PCR analysis. Total RNA isolated from wound, UV, and non-treated cap tissue was used again in this experiment. After reverse transcription of mRNA by poly dT antisense primer, PCR reactions were performed by using poly dT antisense and the *Wun* gene specific primer, corresponding to nucleotide sequences of the *Wun* coding strand as a sense primer in each reaction. Result of this experiment is shown in Fig. 3. The *Wun* mRNA level was highly increased by wound or UV treatment, while it showed only basal level expression under normal growth condition. Induction level of the *Wun* gene was approximately same in wound and in UV treated sample. This result strongly suggests that this *Wun* gene is a

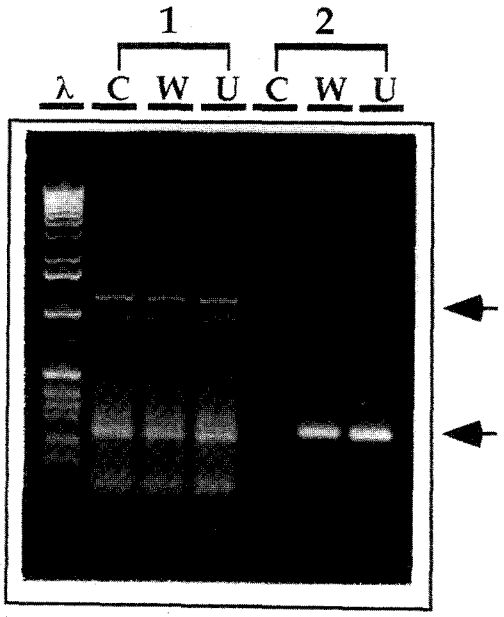

Fig. 3. Induction of the *Wun* gene expression by wound or UV treatment.
Total RNA (2 ug) from wound (W) or UV (U) treated and untreated (C) cap tissue was subjected to RT-PCR reaction to analyze the *Wun* gene induction by wound or UV (2). RNA from each treatment was used in cDNA synthesis using poly dT primer and then amplified by PCR with the redesigned sense primer, corresponding to nucleotide sequence of the isolated cDNA fragment, and poly dT oligimer as a antisense primer. The RT-PCR products were separated by electrophoresis on an 1.2% agarose gel with 1 kb ladder DNA size marker (λ). Same amount of RNA from each treatment were also subjected to RT-PCR reaction using 18S rRNA specific primers as a control (1). Arrows indicate position of the RT-PCR products generated by 18S rRNA specific primers (upper one) and the *Wun* gene specific primer and poly dT primer (lower one).

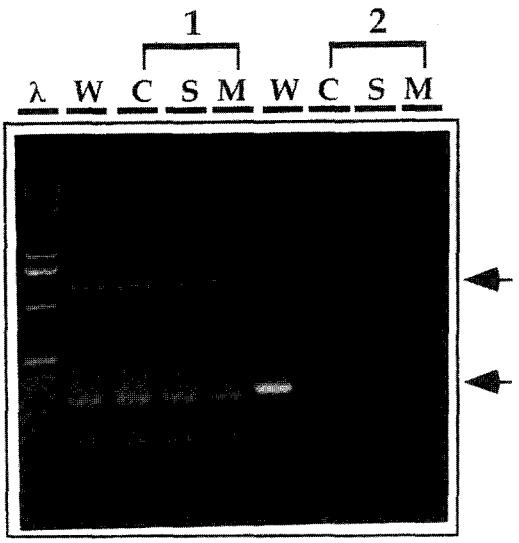

Fig. 4. Expression of the *Wun* gene in unwounded cap, stipe, and mycelium of *P. sajor-caju*.
Total RNA (2 ug) from wound (W) treated cap tissue and untreated cap (C), stipe (S), and mycelium (M) was subjected to RT-PCR reaction to analyze the *Wun* gene expression pattern in various stages or organs (2). RNA from each treatment was used in RT-PCR reaction as same as explained in Fig. 2. Same amount of RNA from each treatment was also subjected to RT-PCR reaction using 18S rRNA specific primers as a control (1). Arrows indicate position of the RT-PCR products generated by 18S rRNA specific primers (upper one) and the *Wun* gene specific primer and poly dT primer (lower one).

kind of real damage induced gene. It is also suggested that *Wun* gene product might function in upstream of damage inducing signal transduction pathway because this gene showed high induction in almost same level by either wound or UV treatment.

Since we used cap mRNA only for the *Wun* gene expression experiment, we next check whether this gene has organ specific or stage specific expression pattern under normal growth condition. Total RNA isolated from cap, stipe, and mycelium of *P. sajor-caju* was subjected to RT-PCR reaction to be analyzed the *Wun* gene expression pattern. The result of this experiment (Fig. 4) reveals that the *Wun* gene has only basal level expression under normal growth condition not only in all organs such as cap, stipe, and mycelium but also in both vegetative and reproductive stage.

Taken together it is suggested that the *Wun* gene is kept up its expression in basal

level under normal condition. However, when mushroom is damaged by wound or UV, this gene is highly induced in transcriptional level to trigger downstream events in self defence signal pathway of *P. sajor-caju*. As a next step of these researches, cloning of full gene and more various gene expression kinetic studies should be followed to elucidate the function of this gene in defence mechanism.

적 요

mRNA의 differential display 방법에 의해 여름느타리 자실체의 상처 또는 자외선 처리시 발현되는 약 0.4 kb의 cDNA 단편을 분리하였다. 이 cDNA 단편의 염기서열 분석결과 세포분열 촉진에 관여하는 cdc2-related protein kinase gene과 상당부분 유사성을 보였으며 RT-PCR 방법을 이용한 분리 유전자의 발현 실험을 통해 이 유전자가 정상 생장 환경에서는 갓, 대, 균사 등 모든 부위에서 기본적인 발현상태를 유지하고 있으며 기계적 상처 또는 자외선 처리에 의해 그 발현이 증폭됨을 확인하였다. 이러한 결과를 통해 향후 분리된 유전자의 연구를 통한 버섯 병 방어 관련 신호 전달 체계 분석에 대한 가능성을 검토해 보았다.

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