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Characterization of Fatty Acid Desaturase, Glutathione S-Transferase and Blue Copper-Binding Protein Genes in *Arabidopsis thaliana*

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Mechanical wounding of plant tissues leads to drastic changes in many plant physiolgical processes, so called plant wound responses, including reestablishment of the diffusion barrier, repair of damage, and stimulation of new growth and differentiation around the wound region, etc (reviewed in Davies 1987). Wounding has been shown to lead to changes in the patterns of cellular RNAs and proteins and, at least in part, the physiologcal responses of plants to wounding is a consequence of expression of genes that are induced or reduced upon woundng. Many plant genes were known to be induced upon wounding of plant tissues with increased synthesis of gene products that either lead to certain developments or counteract the harmful effects of stress conditions (Bowles, 1990). However, the molecular mechanisim responsible for activation of those gene expression in response to wounding stress remains largely unknown.

Recently, Seo *et al.* (1995) suggested that a mitogen-activated protein (MAP) kinase can be a part of the initial response to mechanical wounding in tobacco plants. Transcripts of the MAP kinase gene began to accumulate very early reaching a maximum level in approximately 1 hour after wounding. In tobacco plants transformed with the MAP kinase cDNA, transinactivation of the endogenous homologous gene occurred, and both production of wound induced jasmonic acid and accumulation of wound-inducible gene transcripts were inhibited. In contrast, the levels of salicylic acid and transcripts for pathogen-inducible acidic pathogenesis-related proteins were increased upon wounding (Seo et al., 1995). Movements of calcium ion may be one of the first consequences of the plant perception of a stimulation (Braam and Davis,1990; Haley, 1995; Julien *et al.*, 1991). A calmodulin gene expression was also induced very early reaching a maximum level in an hour after wounding in *Bidens pilosa* L. (Vian *et al.*, 1997). The purpose of this study was to identify *Arabidopsis* genes that are activated early in the wound response.

ISOLATION OF WOUND-INDUCIBLE cDNA CLONES

We initially set up the conditions for the wounding treatment to avoid variability on the degree of wounding, which is likely to occur during the manual wounding of the plants by slicing or by pressing the tissue with a tweezer, etc. The wounding treatment we have employed in this experiment, spraying carborundum particles with a defined air pressure from an air compressor, resulted in a fairly consistant wounding injury on plant surface as examined by the number of the wound sites under a dissecting microscope. Many of the wound-inducible genes studied so far are induced at rather later time points. In this experiment, we wanted to isolate and characterize the genes that are induced early in the wound response of the plant cells. A wound-induced cDNA library was constructed from the RNA extracted from the plant tissues harvested 1 h after the wounding treatment and was differentially screened to isolate the cDNAs whose corresponding genes are induced early by wounding in *A. thaliana*. Differential screening of approximately 2,000 recombinant cDNA clones of this cDNA library resulted in several cDNA clones that show stonger hybridization signals with the first strand cDNA probe from the wounded plants than with the cDNA probe from the control plants. The clones were designated as AWI (indicating *Arabidopsis* wound-inducible genes).

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EXPRESSION OF THE WOUND-INDUCIBLE cDNA CLONES

All the 10 different cDNA clones we isolated may function in the rather early stages of the wound response (Yang *et al.*, 1997a). However, the degree of induction and the kinetics of accumulation of the transcripts corresponding to each clone was different among the clones. All of the clones showed detectable changes of accumulation of the transcripts earlier than 0.5 h after wounding treatment. Another feature of the expression of these genes is that all of the genes show transient accumulation of the transcripts. The clones we have isolated can be broadly classified into two groups according to the expression time of their transcripts. Nine clones out of 10 were induced rapidly upon wounding and the expression level stayed relatively constant until 1-1.5 h time points and then started to decline. Two of the cDNAs, named AWI 16 and AWI 24, proved to encode S-adenosylmethione synthase and glutathione S-transferase, respectively (Kim *et al.*, 1994). The clone AWI 31 showed steady accumulation of the transcripts and reached the maximum value at a later time point of 2.5 h and then started to decline. According to the degree of decline in the amount of the transcripts, we could also classify these clones into two groups. While most of the clones show rather early decline and reach the pre-wounding level within 3 h after wounding, the transcripts of the clones AWI 20, 31 and 32 are notably persistent until later time points, suggesting the function of these genes may be continually required for plants to wound response.

CHARACTERIZATION OF THE AWI 23 cDNA ENCODING A CHLOROPLAST ω-3 FATTY ACID DESATURASE

To characterize the nature of the gene, we have performed the nucleotide sequence analysis of the AWI 23 cDNA clone. The total sequence of AWI 23 cDNA was 1,675 bp long and had an open reading frame which predicted a protein of 446 amino acids. The nucleotide and deduced amino acid sequence were used for computer-aided search for similar sequences in the databases. This search revealed that the AWI 23 cDNA encodes a chloroplast ω-3 fatty acid desaturase (FAD 7), the gene of which had already been cloned in *A. thaliana* (Iba *et al.*, 1993).

Chloroplast membranes of higher plants have highly polyunsaturated fatty acids and are generally composed of about 70% trienoic fatty acids (TAs, 16:3 and 18:3)(Browse and Somerville, 1991). ω-3 fatty acid desaturase (FAD), catalyzing the desaturation of lipid-linked dienoic fatty acids, is a key enzyme for the formation of TAs. Four loci (fad4, fad5, fad6 and fad7) have been identified in *A. thaliana* that control fatty acid desaturation in the chloroplast, and two additional loci (fad2 and fad3) encode endoplasmic reticulum-localized desaturases (Browse and Somerville, 1991). The fad7 mutant is characterized by strongly reduced levels of linolenic acid and hexadecatrienoic acid and correspondingly increased levels of dienoic fatty acids in the chloroplast membrane lipids of leaf cells (Browse *et al.*, 1986). The mutation has no readily apparent effect on growth or development of the plants but leads to a reduction in the average size and a corresponding increase in the number of chloroplasts in plants grown at elevated temperatures (McCourt *et al.*, 1987). A FAD cDNA (FAD7) from *A. thaliana* that complements the fad7 mutation was isolated (Iba *et al.*, 1993). The introduction of the FAD7 gene into tabacco under the control of the CaMV 35S promoter resulted in a significant elevation of TA levels (Komada *et al.*, 1994). In such transgenic tobacco plants, chilling injury was reduced indicating the importance of highly polyunsaturated fatty acids in cold tolerance of higher plants.

Low temperature is one of the major environmental factors that limit plant growth. An increased production of highly unsaturated fatty acids at low temperature is observed in connection with cold acclimation in many plants (Graham and Patterson, 1982). The fad mutants, fad2, fad5, and fad6, have also reduced amounts of polyunsaturated fatty acids and are more susceptible to chilling (Hugly and Somerville, 1992; Miguel *et al.*, 1993). We are currently in the process of evaluating the individual function of known FAD genes in connection with plant cold acclimation by the gene-specific antisense expression in transgenic plants.

CHARACTERIZATION OF TWO GLUTATHIONE S-TRANSFERASE GENES

A wound-inducible *Arabidopsis* cDNA (AWI 24) synthesized from the mRNA species of approximately 1.0 kb, was proven to encode a glutathione S-transferase (GST) by partial sequence analysis (Kim *et al.*, 1994). We screened a premade *Arabidopsis* genomic DNA library to clone the corresponding gene using the GST cDNA probe. A recombinant

phage was obtained by plaque hybridization method. The purified phage DNA was partially digested with *Sac*I resriction endonuclease, separated on a 0.8% agarose gel, blotted onto a nylon membrane and probed with the cDNA. *Sac*I fragments of approximately 6, 3.5 and 2.5 kb were strongly hybridized with the cDNA probe. The three DNA fragments were eluted from the agarose gel and subcloned into the plasmid pUC 19, respectively. Nucleotide sequence analysis of the three DNA fragments revealed that two closely related GST genes, designated *ATGST 1* and *ATGST 11* in order, were tandemly arranged in the genomic DNA fragment. The 5' upstream region of the *ATGST 1* gene missed in the 6 Kb DNA fragment was analyzed as described previously (Yang *et al.*, 1997b). The nucleotide sequence appeared in the EMBL, GenBank and DDBJ nucleotide Sequence Database under the accession number Y11727. The *ATGST 11* gene, the corresponding gene of the AWI 24 cDNA, was located just next to the *ATGST 1* in the *Arabidopsis* genome.

The GSTs are a family of enzymes that have been found as homodimers or heterodimers of subunits, and are believed to play an important role in the protection of cellular macromolecules from attack by a range of toxic xenobiotics in most forms of life (Daniel, 1993). Considerable effort has been invested to characterize the GST genes in various plant species (reviewed by Marrs, 1996) and the plant GST genes are known to form a group that is distinct from animal GSTs. In *Arabidopsis thaliana,* six different members of the GST genes were distinguished by their nucleotide and deduced amino acid sequence homology. The gst 1 cDNA was induced by pathogen infection (Greenberg *et al.*, 1994) and dehydration (Kiyosue *et al.*, 1993). The gst 2 cDNA was regulated by exogenous ethylene treatment (Zhou and Goldsbrough, 1993). Furthermore, the gst 2 gene product also had an auxin binding property and was located in microsomes as well as in plasma membrane vesicles (Zettl *et al.*, 1994). The ERD 13 cDNA was induced by dehydration (Kiyosue *et al.*, 1993). The PM239×14 cDNA encoded a protein with glutathione-peroxidase activity which did not use H_2O_2 as substrate (Bartling *et al.*, 1993). The GST5 cDNA was capable of binding auxin and related substances at their GSH-binding site (Watahiki *et al.*, 1995), and the corresponding gene structure has been reported (Van der Kop *et al.*, 1996). The GST6 gene expression was under tissue-specific control and was induced following treatment with auxin, salicylic acid and H_2O_2 (Chen *et al.*, 1996).

The *ATGST 1* gene contained the entire transcription unit in three exons interrupted by two introns. The combined sequence of three exons had an open reading frame which predicted a protein of 208 amino acids. The ATGST 1 was the corresponding gene of the gst 1 cDNA (Greenberg *et al.*, 1994) and the ERD 11 cDNA (Kiyosue *et al.*, 1993). The amino acid sequence of *ATGST 1* gene product shared 71% homology with that of the gst 2, 52% with the GST 6, 31% with the PM239×14, 25% with the ERD 13 and 10% with the GST5. The transcriptional initiation site of the *ATGST 1* gene lies 50 bp upstream from the translational initiation codon. The *ATGST 11* gene predicted a GST of 206 amino acids. Although the *ATGST 11* gene predicted a GST that has one more amino acid than the *ATGST 1*, both genes were very similar with complete conservation of the intron position and the highly conserved nucleotide sequence. Deduced amino acid sequences from both genes showed 93.8% similarity, and the nucleotide sequence including noncoding region was also highly conserved between the genes suggesting that they likely arose by duplication of a single primordial gene. We could find the very similar case of this in the literature. Structural analysis of a region of the carnation genome which contains two tandemly arranged GST genes (*GST1* and *GST2*) was reported (Itzhaki *et al.*, 1993).

Expression of the gst 1 and ERD 11 cDNAs, which was proven to be the same product of the *ATGST 1* gene, have been known to be induced by pathogen attack (Greenberg *et al.*, 1994), ozone (Sharma and Davis, 1994) and dehydration (Kiyosue *et al.*, 1993) in the literature. Northern hybridization study using a specfic DNA probe at high stringency washing condition, revealed that the *ATGST 1* gene was also rapidly induced by other environmental stresses such as wounding, high salt, low temperature and a DPE herbicide treatment (Yang *et al.*, 1997b). Therefore, it was suggested that the *ATGST 1* gene may be a member of the class of general stress-responsive genes activated by many different kinds of stimuli. Since plants are constantly subjected to adverse environmental conditions, they have to make metabolic adjustments to cope with the stressful conditions. The genetic program in plants is altered by stress stimuli to produce specific proteins and activate biochemical pathways that ensure survival. The *ATGST 1* gene may be a gene involved in such a category. Some sequence motifs, ATTTCAAA (Itzhaki *et al.*, 1994; Sessa *et al.*, 1995), TCA (Goldsbrough *et al.*, 1993), and G-box (Dolferus *et al.*, 1994), that have

been known to be conserved in various genes regulated by ethylene and environmental stresses were found in the promoter region of the *ATGST 1* gene.

As mentioned earlier, the *ATGST 11* is the corresponding gene of the AWI 24 cDNA. To determine the specific regulation pattern of the *ATGST 11* gene, we prepared the specific DNA probe from the 3' untranslated region of the gene by PCR. Northern hybridization study using the DNA probe revealed that the *ATGST 11* gene is not regulated by environmental stresses. The sequence motifs that has been found in the promoter region of the *ATGST 1* were not existed in the *ATGST 11*. However, the promoter region of the *ATGST 11* gene contained inversely-repeated ACCAACTA motif which may be recognized by the myb-homologous P gene product in plants. The P gene controled phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset (Grotewold *et al.*, 1994). Therefore, we are currently in the process of evaluating the tissue specific expression pattern of the ATGST 11 gene.

FUNCTIONAL ANALYSIS OF THE BLUE COPPER-BINDING GENE PROMOTER

The total sequence of the AWI 32 cDNA including a 11 bp poly dA track defining the 3' end of the messenger, was 749 bp long. The sequence predicted a polypepyide of 196 amino acids. The product has been known to encode a protein showing high similarity to blue copper-binding (bcb) proteins (Van Gysel *et al.*, 1993). In mature plants, a 20-fold increase in the amount of steady-state bcb mRNA can be detected upon 48 h of dark adaptation. The expression level of the gene is also dependent upon the developmental stage of the plant. The 21.5-kDa gene product shows extensive similarity with blue Cu^{2+}-binding proteins such as plastocyanin and stellacyanin (Engeseth *et al.*, 1984: Vorst *et al.*, 1988). The corresponding gene was also proven to be rapidly induced by dehydration, high salt, and low temperature conditions in this experiment. An interesting feature in plant stress response is the similarity among proteins induced by different stress conditions. For example, heat shock protein can be induced by various stress conditions in addition to heat stress (Czarnecka *et al.*, 1984; Edelman *et al.*, 1988). It was thus suggested that the diverse stress responses may share some common regulatory steps (Ho and Sachs, 1989) and/or one gene product may have diverse functions leading to several physiological responses of the plants.

We screened premade genomic DNA libraly (*A. thaliana* ecotype Landsberg) to clone the corresponding gene. A positive genomic DNA clone was selected by plaque hybridization using the AWI 32 cDNA probe. A portion of 4.5 kb *Eco*RI DNA fragment including the bcb gene was constructed into the plasmid pUC 19. Nucleotide sequence of the bcb gene in the genomic DNA fragment was determined using synthetic primers designed from the cDNA. A promoter region of the bcb gene containing the sequence from -1294 to +247 (about 1.5 kb) was fused to the GUS gene in pBI101. This constructs was used to transform tobacco, and transgenic T_1 plants that expressed the selectable marker gene (NPTII) were regenerated. Eight progeny seed lines (T_2 generation) obtained from the kanamycin-resistant T_1 plants were examined for inheritable genetic transmission of the T-DNA. T-DNA inheritance was monitored by screening T_2 seeds on media containing 50 $\mu g\ ml^{-1}$ kanamycin. From the eight T_2 lines, two T_2 lines were segregated as 3:1 and others were 15:1. The two T_2 plants (3:1) were used for the detection of the wound-inducible GUS activity. The presence of a T-DNA in the T_2 plant was futher confirmed by genomic southern blot analysis. The 1.5 kb transcriptional regulatory region contained all of the 5'-regulatory elements necessary for wound-inducible GUS expression in the transgenic tobacco plants. The young seedlings of the T_2 plants were subjected to other environmental stresses such as dehydration, high-salt and low- temperature conditions and GUS actvity was measured at 12 h after treatments. The GUS activity of the T_2 plants under dark condition was measured at 36 h after treatment because 12 h-induction period was too short to detect the GUS activity. The GUS activity was clearly detected in the all treatments. Stable integration of the single chimeric gene into the tobacco genome led to strongly inducible GUS activity upon such environmemtal stresses in the young seedlings of T_2 generations. This means that the promoter region of the gene may have responsible *cis*-elements to such environmental stresses. Transcriptional regulatory region of the gene contained the anaerobic responsive element-like sequence known to be important for response to various stress conditions (Dolferus *et al.*, 1994).

PROSPECT

One of the most severe environmental stresses to which plants may be subjected is wounding, which

may come out through such diverse causes as mechanical injury, pathogen and herbivore attack etc. Therefore, plants have developed highly functional mechanisms for perceiving and responding appropriately to wounding stress. However, the molecular mechanisms for the plant wound responses, especially for the wound signal transduction, are largely unknown. We have cloned several genes whose expression are likely to be in the upstream of the cellular cascade of wound response and whose functions are mostly unidentified. Functional analysis of those gene expression would be valuable for evaluating the signal transduction mechanism of the plant wound response.

On the other hand, genetically engineered resistant plants have been dependent on the use of continuously expressed promoters driving dominant single gene traits. However, the resistance gene expression should be regulated temporarily and spatially in transgenic plant not only for energy economy but for minimizing possible detrimental effects of the product to normal plant growth and development. The transcriptional control region of wound inducible genes we have isolated may allow accurate temporal expression of chimaeric resistance gene within transgenic plants in response to wounding by biological invaders such as pathogen, nematodes and insects.

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