Molecular Mechanism of Early Wound Response in Tobacco Plants

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植物は移動できないため、さまざまな環境ストレスに対して常に受け身であり、素早い対応をしなければならない。なかでも、昆虫や動物による食害や暴風雨などの物理的作用によって起きる傷害は大きなストレスであり、放置すれば病原菌感染や壊死の広がりにより個体の死にもつながる。このため、植物は傷害に対する防御機構を発達させてきた。傷害応答は、細胞・組織・器官の修復・再生、傷害のシグナル伝達、感染・食害に対する予防など、さまざまな遺伝子が関与する複雑な系である。特に、被害を確実に認識し、一刻も早くその情報を全身に伝達することが重要である。傷害シグナルはジャスモン酸合成を通じて、プロテアーゼインヒビター(PI)などの防御遺伝子の発現を誘導することがこれまでに明らかになっている。しかし、ジャスモン酸合成に至る伝達経路の初期過程で働くタンパク質や全身性シグナルなどはほとんど分かっていない。本研究では、全体としてこの傷害応答の初期過程で起きる現象を分子レベルで明らかにすることを目的とした。

本研究の前半では、傷害応答の初期過程で発現が制御される遺伝子の単離を試みた。そのために、まず、蛍光ディファレンシャルディスプレイ法による傷害応答遺伝子のスクリーニング・クローニング系を確立した。次に、合計64回の独立したPCRによる傷害応答遺伝子の探索により、8種類の傷害応答遺伝子のcDNA断片を得た。このうちクローンA7に対応する転写産物は傷処理により数分以内に蓄積し始め、30分後に極大となる一過的な蓄積パターンを示した。クローンA8、C14、C20に対応する転写産物は傷処理後3時間から12時間の間に蓄積し始めた。これらはいずれも防御関連の遺伝子をコードしていた。PI遺伝子は傷処理後24時間から発現した。これらの傷害応答遺伝子は分・時間・日レベルでの段階的な制御を受けていることがノザン解析によって明らかになった。

このスクリーニングによって、クローンA7以外にも分レベルで傷害に応答する複数の遺伝子の存在が確認されたことから、本研究の後半ではこれらの遺伝子の単離を行った。その結果、すでに単離したクローンA7以外に、クローンC10、C15、6-1を得た。クローンA7とC10は、5'-RACE法によって全長cDNAを得た。*A7*遺伝子産物は513アミノ酸からなるタンパク質をコードしており、C末端側にロイシンジッパードメインを持っていた。驚くべきことに、全アミノ酸のうち70%以上が、リジン(K)、グルタミン酸(E)、アスパラギン酸(D)から構成されていた。このことから、この遺伝子を*ked*と名付けた。相同性を示す遺伝子は報告されておらず、そのアミノ酸組成より、デヒドリン様の機能を持つのではないかと推定した。6-1は、リン酸処理で誘導されるタバコの*phi-1*と同一であることが分かった。

*C10*遺伝子産物は356アミノ酸からなるタンパク質をコードしており、ロイシンジッパードメインとジンクフィンガードメインをもつことから、この遺伝子を*wizz* (wound-induced leu-zipper zinc-finger)と名付けた。アミノ酸配列の相同性検索の結果より、オートムギのABF2やパセリのWRKY3などのよく保存された領域を

持つ蛋白質群に属することが示された。そのアミノ酸配列中にはWRKYを含む約60残基が保存されており、この部分がC_2-H_2型のジンクフィンガードメインとなり転写因子として働くことが推定されている。従ってWIZZも転写因子であると考えられた。GFPとの融合タンパク質をタマネギの表皮細胞で一過的に発現させると、融合タンパク質は核に局在した。ゲルシフトアッセイによって、WIZZはほとんどのWRKYファミリーが結合するDNA配列のTTGAC(C/T)を認識し結合した。この結合はキレート剤の添加によって阻害された。一過的発現実験によって、WIZZはタバコBY-2細胞内では転写活性化能を示さなかった。しかし、この認識配列は多くの防御関連遺伝子の転写制御に関っていることから、WIZZは傷害応答遺伝子の転写を制御していることが示唆された。

*ked*と*wizz*の転写産物は健全な葉ではみられず、傷害により数分以内に蓄積し始めることから、転写レベルでの制御が示唆される。そこで、両遺伝子のプロモーターの下流にレポーター遺伝子としてホタルルシフェラーゼ遺伝子をつなげたプラスミドを作製し、タバコの葉で一過的に発現させた。約0.7kbの*ked*プロモーターを導入した場合、レポーター活性が全く認められなかったことより、約0.7kbの領域では転写に必要な部分を欠いていることが示唆された。 一方、約1.5kbの*wizz*プロモーターを導入した場合、導入後3時間から6時間にかけてレポーター活性は一過的に上昇した。次に、約1.5kbから約0.2kbまで5'末端側から*wizz*プロモーターの長さを変えた場合、導入後5時間目のレポーター活性は、プロモーターが短くなるにつれ低下した。このことから、プロモーター上に分散するエレメントが協調的に働いていることが示唆された。

*ked*と*wizz*の転写にかかわるシグナル伝達経路を明らかにするために、タバコ葉の傷害時にさまざまな阻害剤や作動薬で前処理し、その影響を調べた。また、*phi-1*と*wipk*の転写も同時に調べ、非常に早く一過的に発現する遺伝子群がすべて同様の制御を受けているのかどうかを調べた。*ked*と*wizz*の転写産物は、多くの傷害応答遺伝子の発現を誘導するメチルジャスモン酸、リノレン酸、アブシジン酸で処理しても蓄積しなかった。*ked*と*wizz*の傷害による転写産物の蓄積は、シクロヘキシミド前処理では打ち消されなかったことから、新規の蛋白質の合成を必要としないと考えられた。さらに、シクロヘキシミド処理のみで、これら4種類の遺伝子すべての発現が誘導された。傷害による*wizz*転写産物の蓄積は、スタウロスポリン前処理では打ち消され、オカダ酸前処理で持続した。オカダ酸前処理による持続効果は*wipk*の場合にもみられ、蛋白質のリン酸化・脱リン酸化の関与が示唆された。これら4種類の遺伝子の傷害およびシクロヘキシミドによる転写産物の蓄積は、ジフェニレンヨードニウムクロリド前処理では打ち消されなかったことから、NADPHオキシダーゼによる活性酸素種の産出とは関係ないと考えられた。キレーター、チャネルブロッカー、イオノフォア処理の実験から、カルシウムの流入による発現の誘導も認められなかった。細胞膜に局在するH^+-ATPaseの阻害剤であるエリトロシンB前処理と活性化因子であるフシコクシン前処理は、傷害による転写産物の蓄積を阻害しなかったが、その処理自体で転写産物の蓄積を誘導し、細胞内のpHの変化が関与していることが示唆された。さらに、プロピオン酸処理による細胞内の酸性化によって4種類の遺伝子すべての発現が一過的に誘導された。

これらの結果より、傷害応答の初期過程では、細胞内の酸性化や蛋白質のリン酸化・脱リン酸化による制御が*ked*や*wizz*などの早く一過的に応答する遺伝子群の発現に重要な役割を果たすことが明らかになった。そして、WIZZは転写因子として下流の防御関連遺伝子の発現を誘導し、KEDは細胞内環境の維持・改善にかかわると示唆された。本研究により、いまだ同定されていない傷害応答の全身性シグナルの同定に向けて大きな足がかりができ、新たな展開が期待できる。

Molecular Mechanism of Early Wound Response in Tobacco Plants

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Abbreviations

ABA	abscisic acid
ATP	adenosine 5'-triphosphate
BAPTA	1,2-*bis*-(*o*-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid
BLAST	Basic Local Alignment Research Tool
cDNA	complementary deoxyribonucleic acid
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DPI	diphenylene iodonium chloride
EDTA	ethylene diamine tetraacetic acid
FDD	fluorescent differential display
GST	glutathione S-transferase
MES	2-(*N*-morpholino)ethanesulfonic acid
MOPS	3-(*N*-morpholino)propane sulfonic acid
mRNA	messenger ribonucleic acid
ORF	open reading frame
PCR	polymerase chain reaction
pI	isoelectric point
poly(A)	polyadenylic acid
PR	pathogenesis-related
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RT	reverse transcription
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate (buffer)
TAIL-PCR	Thermal asymmetric interlaced PCR
TMV	tobacco mosaic virus

Introduction

Plants are always exposed to diverse environmental stresses, among which wounding may be the most crucial. To cope with wounding caused by mechanical injury, pathogen attack and damage from herbivores and insects, plants have developed refined self-defense systems in which wound signals are quickly transmitted from damaged tissues (local) to the whole plant (systemic), allowing elaboration of a defense reaction. Over the past few decades, numerous studies have been performed on the wound response mechanism. These studies have investigated signal components and their interactions, and target genes and their functions.

The chemical nature of wound signals has been intensively studied. Several substances have so far been identified, including abscisic acid (ABA) (Pena-Cortes et al., 1989; Hildmann et al., 1992), jasmonic acid (JA) (Farmer and Ryan, 1990), ethylene (O'Donnell et al., 1996), the small peptide systemin (Pearce et al., 1991), oligosaccharides (Bishop et al., 1981) and reactive oxygen species (Bradley et al., 1992). In addition, physical signals such as hydraulic change (Boari and Malone, 1993) and electric currents (Wildon et al., 1992) have also been suggested to play a part.

Wound-responsive genes have also been reported. Their products have various functions, including (i) hydrolysis of microbial cell wall components by PR-2 (β-1,3-glucanase) and PR-3 (chitinase), (ii) phenylpropanoid synthesis by phenylalanine ammonia-lyase and chalcone synthase, (iii) reinforcement of the plant cell wall by extensin and peroxidase, (iv) ethylene synthesis by 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase, and (v) interference of digestion of herbivores by proteinase inhibitors

(PIs) (Green and Ryan, 1972; Mauch et al., 1988; Hahlbrock and Scheel, 1989; Kende, H., 1993; Showalter, A.M., 1993). Expression of the PI genes, in particular, has been widely used as an indicator of the wound response pathway, although the transcripts begin to accumulate several hours to a day after wounding.

In recent years, there has been growing interest in molecular events occurring in very early stages of wound response, which triggers whole series of defense reactions, and several immediate-early responsive genes that are induced within an hour after wounding have been identified (Table 1). Transcripts of the tobacco *WIPK* gene encoding a mitogen-activated protein (MAP) kinase homolog begin to accumulate as early as 1 min after wounding in local and systemic leaves (Seo et al., 1995). Expression of the tobacco *ERF3* and tomato *TWI1* genes encoding an ethylene-responsive transcription factor and a glucosyl transferase, respectively, is induced within 30 min after wounding (O'Donnell et al., 1998; Suzuki et al., 1998). Such immediate-early responsive genes provide valuable tools to understand signal transduction in early wound response pathways. In addition, contribution of novel genes encoding polypeptides with no strong similarity to any established gene products have been reported. *Arabidopsis ATHCOR1* gene transcripts are induced by methyl jasmonate and wounding within 30 min (Benedetti et al., 1998). Although the function of ATHCOR1 with a potential ATP-/GTP-binding site remains unclear, this finding provided additional insights into molecular mechanisms of early wound response.

Table 1. Immediate-early wound-responsive genes

Gene	Plant	Features of encoded polypeptide	Other inducers	Reference
WIPK	tobacco	MAP kinase	TMV infection	Seo et al., 1995
BCM1	*Brassica napus*	calmodulin	touch	Oh et al., 1996
AWIs	Arabidopsis	unknown		Yang et al., 1997
Lox D	tomato	lipoxygenase	systemin, MJ	Heitz et al., 1997
MMK4	alfalfa	MAP kinase		Bogre et al., 1997
AR2	Arabidopsis	P450 reductase	light	Mizutani and Ohta, 1998
ATHCOR1	Arabidopsis	unknown	coronatine, MJ	Benedetti et al., 1998
ERF3	tobacco	transcription factor	CHX	Suzuki et al., 1998
TWI1	tomato	glucosyl transferase	SA, pathogen	O'Donell et al., 1998
AtCBL1	Arabidopsis	calcineurin B-like protein	drought, cold	Kudla et al., 1999
CaM	tomato	calmodulin	systemin, MJ, LNA	Bergey and Ryan, 1999
CMBP	tomato	chloroplast mRNA-binding protein	flaming	Vian et al., 1999

SA: salicylic acid, MJ: methyl jasmonate, CHX: cycloheximide, LNA: linolenic acid

To cast further light on the early stage of the wound response, I have conducted fluorescent differential display (FDD) screening in order to identify as many wound-responsive genes as possible (Figure 1). In this thesis, I describe (i) the isolation and characterization of wound responsive genes by FDD screening, (ii) characterization of two novel immediate-early wound-responsive genes, *KED* and *WIZZ*, which encode a highly hydrophilic protein and WRKY family transcription factor with a zinc finger motif, respectively, (iii) isolation and analysis of promoters of both genes, and (iv) analyses of the early stage of the wound response using immediate-early wound-responsive genes as the marker.

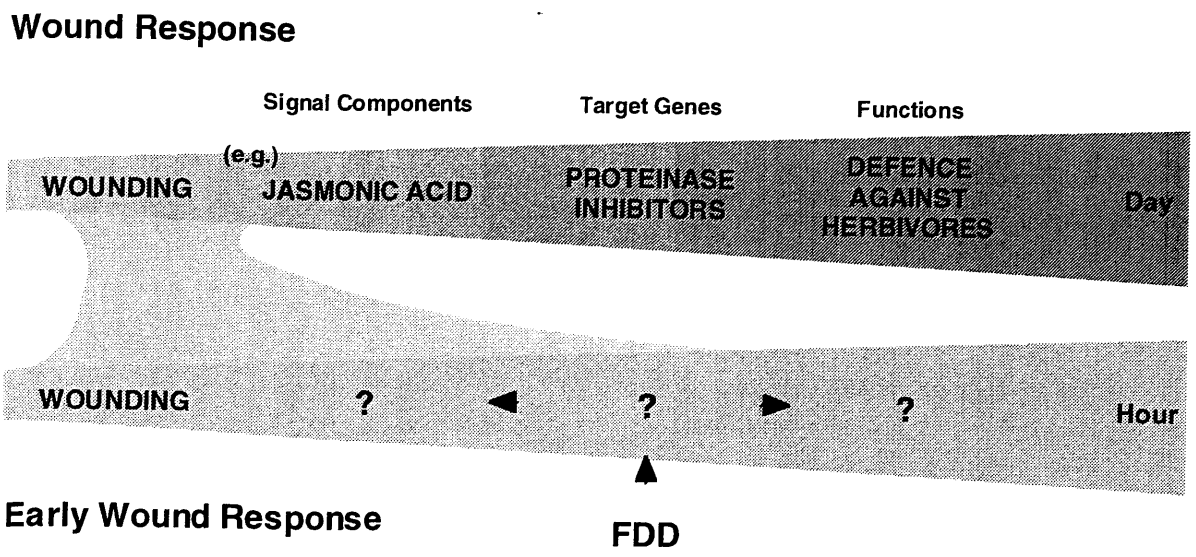

Figure 1. Strategy for this study to understand the early wound response.

Chapter I

Screening of Early Wound Responsive Genes by FDD

In order to understand the general aspects of the early stage of wound response, it is desirable to catalog as many as possible of the genes whose expression is induced at very early stage, and to determine their temporal and spatial expression patterns. Functional interactions among their products can be then hypothesized.

MATERIALS AND METHODS

Plant and Treatments

Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were grown in a greenhouse at 23°C under a 14 h/10 h light/dark photocycle. Wounding was performed by cutting the mature leaves of 2-month-old tobacco plants into pieces with a pair of scissors. The resulting leaf fragments were floated on water. The samples were harvested at an appropriate time point, immediately frozen in liquid nitrogen and stored at -80°C.

Fluorescent Differential Display

The fluorescence differential display (FDD) method was performed described (Ito et al., 1994; Ito and Sakaki, 1996) with a modification. Total RNA was isolated from tobacco plants grown in a controlled environmental room at 23C under continuous light for a week. The first

strand cDNA was synthesized with one of the 3'-anchored oligo(dT) primers (5'-GT_{15}VA-3' or 5'-GT_{15}VC-3' ; V is a mixture of A, C and G). The second strand synthesis and polymerase chain reaction (FDD-PCR) were performed with rhodamine-labeled 3'-anchored primers (TaKaRa Shuzo, Japan) and 10-mer arbitrary primers (Operon Technologies, Inc., USA). The reaction was carried out with 94°C 3 min, 40°C 5 min, 72°C 5 min, followed by 25 cycles of 95°C 15 sec, 40°C 2 min, 72°C 1 min, and 72°C 5 min for a final extension.

The resulting products were fractionated on a denaturing polyacrylamide gel. After electrophoresis, fluorescent image was analyzed on an FM-BIO (TaKaRa Shuzo, Japan). The gel fraction containing the cDNA fragments of interest was excised and suspended in TE buffer. The cDNA fragments were eluted by boiling, precipitated with ethanol and re-amplified by PCR with the same pair of primers as used for the first amplification. The resulting product was subcloned.

To eliminate false positive clones, co-migration test (Ito and Sakaki, 1996) and Southern analysis were carried out. The cloned cDNA inserts co-migrated at the same position as the original wound-responsive products were primarily selected. The gel used in the co-migration test was subjected to semi-dry gel blotting, followed by hybridization with the selected cDNA probe. The cDNA clone exhibiting the same wound-responsive pattern as displayed in FDD was finally selected and further analyzed.

RNA Gel Blot Analysis

Total RNA was isolated by the ATA method (Gonzalez et al., 1980), fractionated on formaldehyde 1% gel and transferred to a nylon membrane (Hybond-N, Amersham). After

crosslinking by UV irradiation, hybridization was carried out at 42 °C for 16 h in a solution containing ^{32}P-labelled cDNA probe, 1 mM EDTA, 0.5% SDS, 50 mM Tris-HCl pH 7.5, 1 x Denhardt's, 3 x SSC, 50% formamide, 10% dextran sulfate, 0.1 mg/ml denatured salmon sperm DNA. The membrane was washed with 0.5 x SSC, 0.1% SDS at 65 °C and autoradiographed. Signal was also visualized with a Fujix BAS-2000 (Fuji Film Co., Ltd, Japan). The cDNA probes were labeled by random labeling method (*Bca*BEST Labeling Kit, TaKaRa). The cDNA for *WIPK* cDNA was provided by Y. Ohashi. The cDNAs for *PI-II* and *actin* were prepared in our laboratory.

5'-RACE

The missing 5' ends of cDNA fragments were obtained using the 5'-RACE (rapid amplification of cDNA ends) system according to the manufacturer's instructions (Marathon cDNA Amplification Kit; Clontech). Total RNA was isolated from tobacco leaves harvested 0.5, 1, 3 and 6 h after wound treatment. Poly(A)+ RNA was isolated according to the manufacturer's instructions (PolyATtract mRNA Isolation System; Promega). After double-stranded cDNA synthesis and adapter ligation, PCR was performed with gene-specific primers as follows: 5'-GAGCAAGAGCAACACGGATACAAC-3' for A1, 5'-CATTACTCCATCGTGTGCCTTCAGGTC-3' for A7, 5'-TTCCTGAAATGGCAGCAGCAAGTG-3' for C10, 5'-CCACTTTGACCCCAATAAATGGCTCC-3' for C13, 5'-CATTGCTCGTCATATGTGCAACGTCC-3' for C20. The RACE products were recovered and subcloned into a cloning vector.

Sequencing Analysis

DNA sequencing was performed using a Dye Deoxy Terminator Sequencing Kit (ABI) and a sequencer (model 373, ABI). Sequences were analyzed using GeneWorks software (IntelliGenetics) and compared in non-redundant databases by using the BLAST program (Altschul et al., 1990).

RESULTS

Screening of Wound-Responsive Genes

To screen and isolate genes that are regulated at the early stage of wound response, fluorescence differential display (FDD) was adopted because of its convenience and suitability for mass screening. Total RNA samples were extracted from tobacco leaves harvested 0, 15, 45, 90 and 180 minutes after wounding, and were converted into cDNAs. Using two fluorescence-labeled anchored primers in combination with 32 arbitrary primers, 64 independent FDD-PCRs were performed. The FDD-PCR products were electrophoretically separated and visualized by an image analyzer. From the differences in displayed patterns, 28 cDNA fragments were initially found to change their levels within 3 h after wound treatment (Figure 2). Among them, eleven cDNA fragments were cloned, sequenced and further characterized by RNA gel blot analysis (Figure 3). Full length cDNAs of five clones (A1, A7, C10, C13 and C20) were obtained by 5'-RACE, and their sequences were determined. Results of sequence analyses are summarized in Table 2.

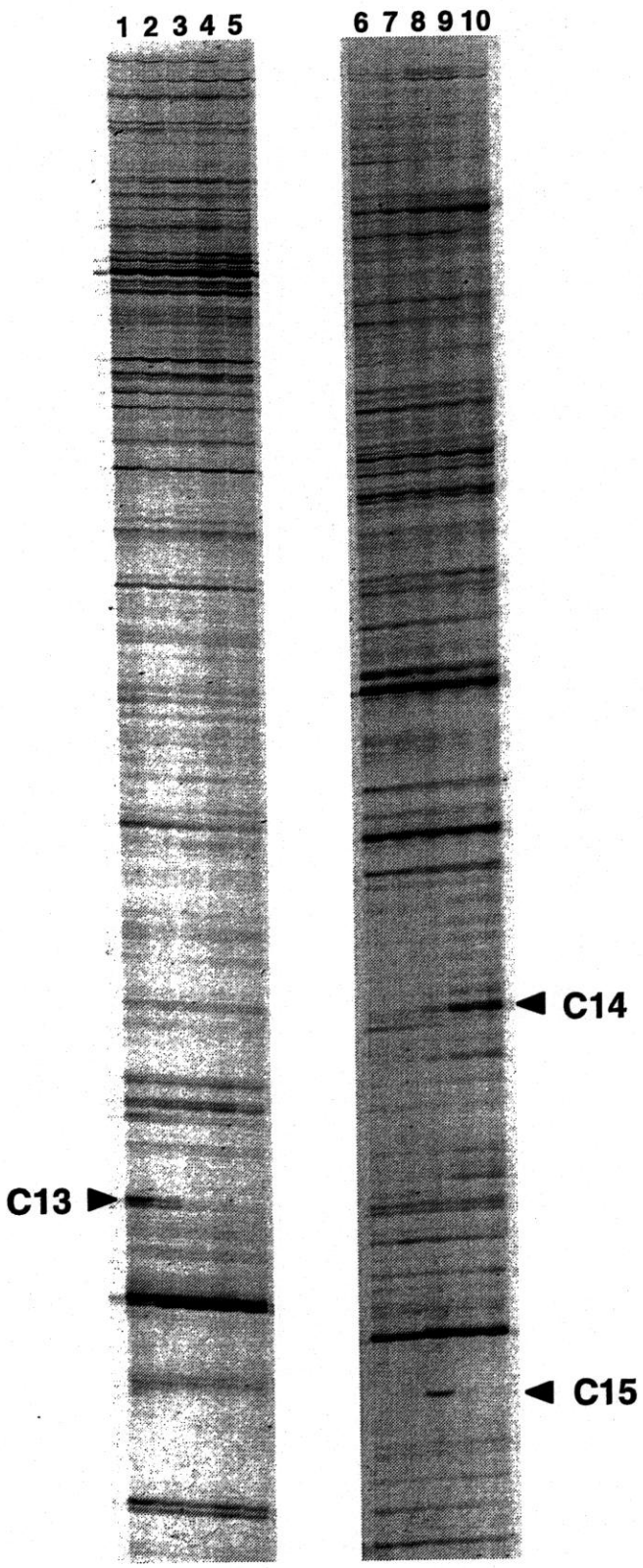

Figure 2. Screening of wound responsive transcripts by FDD.
Sample leaves were harvested at time 0 (lanes 1 and 6), 15 min (lanes 2 and 7), 45 min (lanes 3 and 8), 90 min (lanes 4 and 9), and 180 min (lanes 5 and 10) after wound treatment. After total RNA extraction, cDNAs were synthesized and FDD-PCR using the $GT_{15}VC$ anchored primer with two arbitrary primers, OPA 19 (lanes 1 to 5) and 20 (lanes 6 to 10) was performed. Signals were detected on the FM-BIO image analyzer. Arrowheads indicate the wound responsive transcripts.

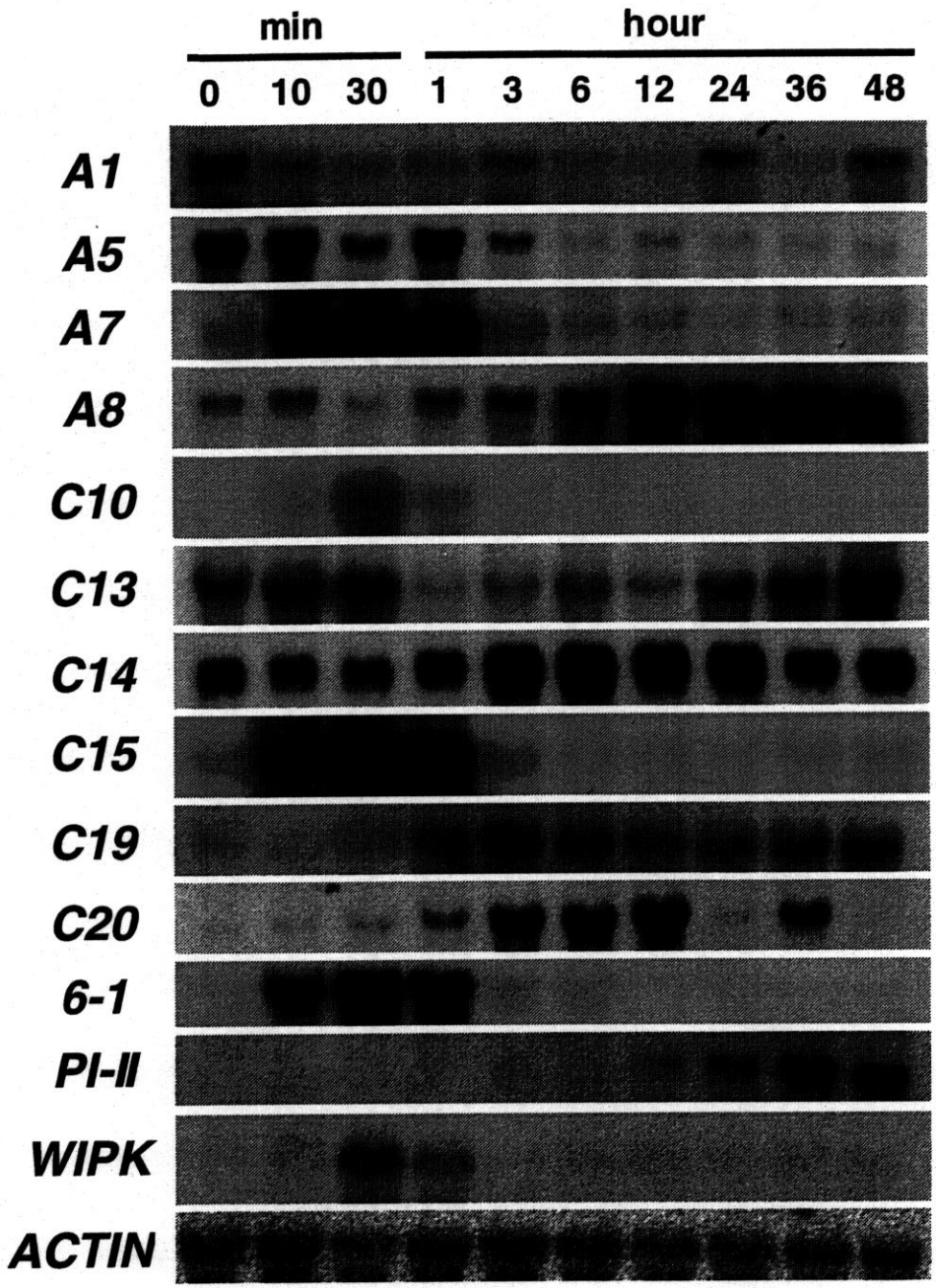

Figure 3. Time course analysis of clones identified by FDD.
RNA blot containing 35 µg RNA per lane was subjected to hybridization with ^{32}P-labeled cDNA probe as indicated.
PI-II encodes proteinase inhibitor II from tobacco, *WIPK* encodes a MAPK homolog from tobacco.

Table 2. Clones identified by FDD

Clone	Size of FDD fragment (bp)	Size of cDNA clone (bp)	Encoded protein (amino acids)	Highest-Scored Homology
A1	195	1,974[a]	510	89% Identity with myo-inositol-1-phosphate synthase of *M. crystallinum* [j]
A5	219[b]	nd	nd	No significant similarity found
KED (A7)	279	1,778[c]	513	No significant similarity found
A8	227[d]	nd	nd	85% Identity with cysteine-rich extensin-like protein of *N.tabacum* [k]
WIZZ (C10)	284	1,251[e]	356	35% Identity with ABF2 of oat[l]
C13	247	1,540[f]	317	No significant similarity found
C14	286[g]	nd	nd	57% Identity with proteinase inhibitor II of *L. esculentum* [m]
C15	227	nd	nd	No significant similarity found
C19	708[h]	nd	nd	90% Identity with chloroplast carbonic anhydrase of *N. tabacum* [n]
C20	408	844[i]	205	44% Identity with 21kD protein precursor of *M. sativa* [o]
PHI-1 (6-1)	607	nd	nd	Identical to phosphate-induced gene, *PHI-1* , of *N.tabacum* [p]

[a-p]Accession number: [a]*A1* AB009881, [b]*A5* AB009882, [c]*KED* AB009883, [d]*A8* AB009884, [e]*WIZZ* AB028022, [f]*C13* AB009885, [g]*C14* AB009886, [h]*C19* AB009887, [i]*C20* AB009888, [j]U32511, [k]L13439, [l]Z48431, [m]X94946, [n]L19255, [o]Y11553, [p]AB018441

nd: not determined

Properties of Cloned cDNAs

The transcript level of the A1 clone decreased upon wounding, but recovered to the basal level at 24 h and 48 h after wounding. The nearly full-length A1 cDNA encodes a protein with 510 amino acids which has a high similarity to *myo*-inositol 1-phosphate synthase (Figure 4A). The level of A5 transcripts gradually decreased within 6 h of wounding and remained at a low level thereafter.

A7, C10, C15 and 6-1 transcripts began to accumulate as early as 10 min after wounding and reached a maximum level within 1 h and decreased 3 h after wounding.

Because of its early temporal response to wounding, A7 and C10 were further characterized in Chapter II.

The transcript level of A8, which encodes a peptide with a high similarity to the C-terminus of cysteine-rich extensin-like proteins from tobacco (Figure 4B) (Wu et al., 1993), gradually increased 1 h after wounding and reached a maximum level at 36 h. The level of C13 transcripts decreased 1 h after wounding and then gradually increased throughout the period examined. The transcript level of C14, which encodes a 44-amino-acid protein with high similarity to proteinase inhibitor class II (PI-II) from tomato and tobacco (Figure 4C) (Balandin et al., 1995; Gadea et al., 1996), increased 3 h after wounding and declined thereafter to the initial level.

Transcripts of C19, which encodes a chloroplastic carbonic anhydrase (CA) (Figure 4D) (accession numbers L19255 and M94135) were not detected in healthy leaves, but gradually increased after wounding. The level of C20 transcripts increased 30 min after wounding and reached a maximum level within 12 h. The deduced amino acid sequence of the C20 transcript showed some similarity with 21 kDa proteins (Figure 4E) (accession numbers X52395, Y11553, X80342 and L12245).

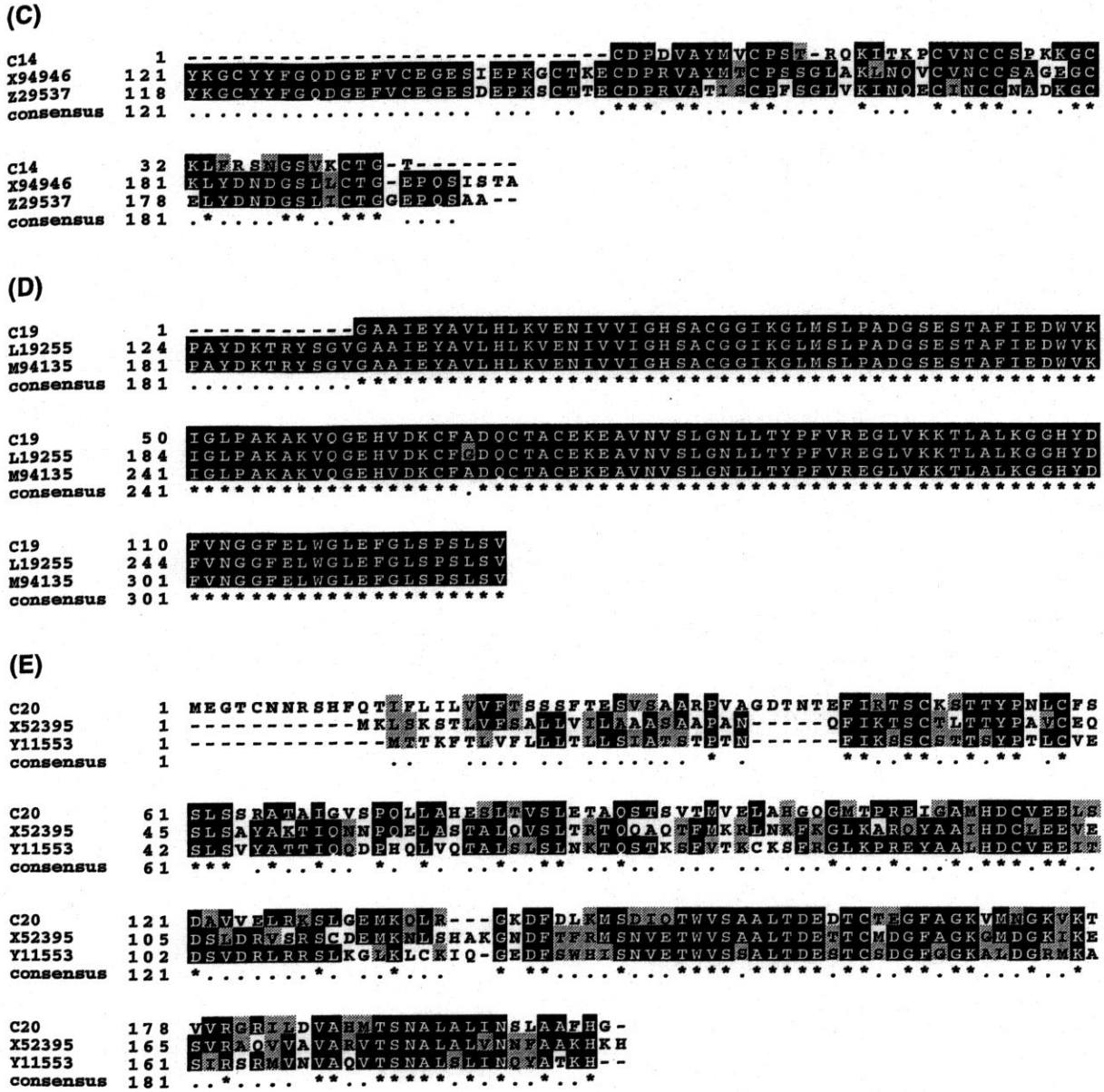

Figure 4. Protein sequence alignment of FDD clones.
Comparison of the derived protein sequences of cDNA clones with sequences from the GenBank/EMBL databases. Filled boxes indicate where the majority of the genes show the same sequence. Shaded boxes indicate where the majority of the genes show the similar sequence. The consensus amino acid residues are marked with astarisk. (A) Multiple alignment of similar proteins to *myo*-inositol 1-phosphate synthase protein sequences showing similarity to A1. Sources: U30250 from *Arabidopsis*; U66307 from *Brassica napus*; U32511 from *Mesembryanthemum crystallinum* (ice plant); Z11693 form *Spirodela polyrrhiza* (duckweed). (B) Multiple alignment of exteinsin-like protein sequences showing similarity to A8. Sources: L13439, L13440, L13441, L13442, L13443 from *Nicotiana tabacum*. (C) Multiple alignment of proteinase inhibitor II protein sequences showing similarity to C14. Sources: X94946 from *Lycopersicon esculentum*, X94946; Z29537 from *N. tabacum*. (D) Multiple alignment of carbonic anhydrase protein sequences showing similarity to C19. Sources: L19255 and M94135 from *N. tabacum*. (E) Multiple alignment of amino acid sequences showing similarity to C20. Sources: X52395 from *Daucus carota*, Y11553 from *Medicago sativa*.

DISCUSSION

Using two anchored primers in combination with 32 arbitrary primers, we performed 64 independent FDD-PCRs, by which approximately 100 individual cDNAs, which are visualized as signal bands, were analyzed per lane. Through this screening, 28 bands were found to show diverse wound-responsive patterns, such as a rapid decrease or a transient increase in transcript accumulation. Assuming that a total of 15,000 individual transcripts are present in the leaf tissues examined, over 450 combinations of primers for FDD-PCR are theoretically necessary in order to screen all the available transcripts with 95% probability under our sampling conditions (Ito and Sakaki, 1996). Since we obtained 28 positive clones from 64 independent FDD-PCRs, which would cover more than 2,100 individual transcripts, it can be estimated that nearly 200 genes are differentially expressed in a tobacco leaf within 3 h after wounding. This is much higher than the number of genes so far known to be involved in the wound response mechanism, but a response of this magnitude may be necessary for plants to cope with wound stress.

The eight clones further characterized were categorized into three groups: (i) genes known to be defense-related (A8, C10, C14 and C20); (ii) genes so far not known to be related to wounding (A1, C19 and 6-1); and (iii) unidentified genes (A5, A7, C13 and C15). The gene products of the first group are apparently involved in defense, because extensins are well known to reinforce cell walls, and proteinase inhibitors cause indigestion in herbivores (Hilder et al., 1987; Johnson et al., 1989; Lagrimini and Rothstein, 1987; Memelink et al., 1993; Mohan and Kolattukudy, 1990; Urwin et al., 1995). Transcripts of *Arabidopsis*

homologs of the C20 gene are reported to be induced by stresses such as pathogen attack (Yu and Ausubel, unpublished; Yang, et al., 1995).

Genes in the second group offer a novel view of the wound response mechanism. The cDNA sequence of A1 has a high similarity to that of the gene encoding *myo*-inositol 1-phosphate (Ins-1P) synthase, which converts glucose 6-phosphate into Ins-1P in the pathway of inositol biosynthesis. Ins-1P, essential for formation of phospholipids, is the substrate for synthesis of plasma membrane phosphoinositides, which are also involved in signal transduction as second messengers (Gross and Boss, 1993). Genes encoding Ins-1P synthase have so far been isolated from ice plant, citrus and duckweed (Smart and Fleming, 1993; Abu-Abied and Holland, 1994; Ishitani et al., 1996). Transcript levels were increased by treatment with ABA in duckweed and with NaCl in ice plant, and were regulated in a diurnal fashion in ice plant and citrus. These observations together with our results suggest that Ins-1P functions in stress response by modulating its level depending on external signals.

The cDNA sequence of 6-1 is identical with that of the phosphate-induced gene, *PHI-1*, from tobacco (accession number AB018441; Sano et al., 1999). The *PHI-1* transcripts are rapidly induced by addition of phosphate in phosphate-starved cells. The predicted *PHI-1* gene product showed no significant homology with anything in the database, indicating that *PHI-1* belongs to a novel class of genes. However, the N-terminal region of PHI-1 has some homology with ATP-binding site of plasma membrane H^+-ATPases of fungi and plants. Although *PHI-1* is not an ATPase in overall structure, *PHI-1* is thought to have some role in phosphorylation. The rapid induction of *PHI-1* upon wounding is discussed in Chapter IV.

The cDNA sequence of C19 is identical with that of the chloroplastic carbonic

anhydrase (CA) gene from tobacco (accession numbers L19255 and M94135). In animal cells, CA plays a critical role in maintenance of homeostasis by pH regulation, by CO_2 and HCO_3 transport, and by water and electrolyte balance (Sly and Hu, 1995). In plants, however, its role is not necessarily clear. In C_4 plants, CA was suggested to couple with the PEPC pathway, which is required for CO_2 fixation (Kurkdjian and Guern, 1989). Within the chloroplast of C_3 plants, CA was postulated to function in buffering the stromal environment by hydration/dehydration reactions (Majeau and Coleman, 1991). Induction of CA upon wounding suggests a new function related to maintenance of plant cellular homeostasis.

In the third group, transcript levels of the A5 and C13 clones decreased on wounding, suggesting that the wound response of the A5 and C13 genes is not direct but rather indirect, caused by, for example, decline of metabolic activities.

Chapter II

Characterization of Immediate-Early Wound-Responsive Genes

The accumulation patterns of A7 and C10 transcripts are distinct, showing a rapid increase within a few minutes after wounding followed by a rapid decrease 1 h later. This suggests that the A7 and C10 gene products may play critical roles in the early stage of wound response. To make clear functions of these immediate-early wound-responsive gene products, both genes were further isolated and characterized.

II-1 Highly Charged Protein Encoded by A7

MATERIALS AND METHODS

Plant and Treatments

Tobacco plants were grown as described in Chapter I. Wounding was performed by cutting mature leaves across the midrib with a pair of scissors. The wounded leaf and upper unwounded leaf were harvested at an appropriate time point, immediately frozen in liquid nitrogen and stored at -80°C.

cDNA Cloning

The almost full-length cDNA was amplified by RT-PCR with the specific primers (5'-

GGATGCCTTGGAGAAATTAAAGGCACATTGACG-3' and 5'-GACAGTGGAAGGGAAA GGTATACACAACGACAG-3') and cloned. The nucleotide sequence was determined by the dideoxy chain termination method (ABI PRISM Dye Terminator). Editing of DNA sequences and amino acid sequences were carried out on a computer software (GeneWorks; IntelliGenetics, CA), with comparison to non-redundant data bases by the BLAST program on network servers (Altschul et al., 1990).

RNA Gel Blot Analysis

RNA blot analyses were performed as described in Chapter I. RNA blot containing 40 μg RNA per lane was subjected to hybridization with ^{32}P-labeled cDNA probe.

RESULTS

A7 Encodes a Highly Charged Protein, KED

A nearly full-length cDNA of A7 was obtained and its putative amino acid sequence was determined (Figure 5A, B). The deduced polypeptide was rich in lysine (K; 34.7%), glutamic acid (E; 24.95%) and aspartic acid (D; 12.48%), which constituted up to 70% of total amino acids. The A7 gene was designated as *KED*. This amino acid composition makes the *KED* gene product a highly charged protein. A hydropathy plot shows that the KED polypeptide is extremely hydrophilic and is divided into two regions: a highly hydrophilic N-terminal region (amino acids 1-372, pI = 5.40) and a relatively hydrophobic C-terminal region

(amino acids 373-513, pI = 9.33) (Figure 5C). Another distinct feature of the KED polypeptide is that acidic amino acids form small clusters, and so do basic amino acids. These clusters alternate (Figure 5D).

Southern hybridization indicated that one copy, or at most two copies, of *KED* is present in the genome of *N. tabacum* (data not shown). The *KED* transcripts were rapidly accumulated in wounded leaves of *Nicotiana sylvestris*, which is one of the ancestral species of *N. tabacum* (data not shown). This suggests that at least one copy of *KED* originated from *N. sylvestris*.

(A)

```
CCTTGGAGAA ATTAAAGGCA CATTGACGTG CGGACAATTT TAGATAGGAA GGCAAAACTG      60

TTGTTGTTGC CAGGGGAGAA GTAAAGATGG AAAAGGAAAA GAAGATAGAC ATGGAAGAAA     120
                           M  E  K  E  K  K  I  D  M  E  E  K
AGCACGAAAA GGAATTGAAG GAGAAAGAGA AAAAGGACAA AGTAAAAAAT ACGGGGAGCG     180
  H  E  K  E  L  K  E  K  E  K  K  D  K  V  K  N  T  G  S  E
AAGAGGAGTC GGAGGAAACA GAGGACGAGA AAGATGGTGC AACAAAAAAT GTAAAAGAAA     240
  E  E  S  E  E  T  E  D  E  K  D  G  A  T  K  N  V  K  E  K
AGAAATACAA GAAGGAAAAG AAAGAGAAAA AGGATAAAGA AAAGAAAGAC AAGAGTAAAG     300
  K  Y  K  K  E  K  K  E  K  K  D  K  E  K  K  D  K  S  K  E
AGGAGGAGTC GGAGGAAGAA ACCGAGGAAG AGAAGGATGA TGGAAAAGGG AAGAAGGATA     360
  E  E  S  E  E  E  T  E  E  E  K  D  D  G  K  G  K  K  D  K
AAAAGAAGAA ACATAAGACA GATATGAAAG AGAAAAAGGA TAAAGAGATG AAAGACAAGA     420
  K  K  K  H  K  T  D  M  K  E  K  K  D  K  E  M  K  D  K  S
GCAAACATGA GTCAGAAAAA GAAGACAGCA AAGAAATAGA GGAAGAGAAG GATGACGGAG     480
  K  H  E  S  E  K  E  D  S  K  E  I  E  E  E  K  D  D  G  E
AAGGGGAGAA GAAAGATAAA GAGAAGAAAC TCAAGAAGGG AAAGAAAGAC AGAAAGGAAA     540
  G  E  K  K  D  K  E  K  K  L  K  K  G  K  K  D  R  K  E  K
AAGAGAAGAA AGACAAGAGC ATAGAGGAGT CGAAGGAAGA AAAGGATGAT GATAAAGGGG     600
  E  K  K  D  K  S  I  E  E  S  K  E  E  K  D  D  D  K  G  E
AGAAGAAGGA TAAAGAGCAG AAAGACAAGA AGGAGAAAAA GAACAAGGAA GAGAAAGGCA     660
  K  K  D  K  E  Q  K  D  K  K  E  K  K  N  K  E  E  K  G  K
AGAGCAAAGG GGAATCAGAA GAAGAAACCG AGGAAGAGAA GGATGATGAA AAAGGGAAAA     720
  S  K  G  E  S  E  E  E  T  E  E  E  K  D  D  E  K  G  K  N
ACAAGGAATC GGATGAAGAA GACGAGAGAC AGACAGAGGA AGAGGAGAAT GATGAAAAAG     780
  K  E  S  D  E  E  D  E  R  Q  T  E  E  E  E  N  D  E  K  G
GGGTGAAGAA GGATAAGGAG AAGAAAAACA AGGAAAAGAA AGAGAAAAAG GACAACGAAA     840
  V  K  K  D  K  E  K  K  N  K  E  K  K  E  K  K  D  N  E  K
AGAAAGATAA GAGCAAAGAG GAAACGGAGG AAGAGAAGGA TGATGAAAAA GGAGAGAAGA     900
  K  D  K  S  K  E  E  T  E  E  E  K  D  D  E  K  G  E  K  K
AGGATAAAGA GAAGAAATGC AAGAAGAATA AGAAAGAGAA AAAGGATAAA GAAACGAAAG     960
  D  K  E  K  K  C  K  K  N  K  K  E  K  K  D  K  E  T  K  D
ACAAGAGCAA AGAGGTGTCG GATGAAGAGG AAGAGAAAGA TGACGAAGAA GGGGAGAAGA    1020
  K  S  K  E  V  S  D  E  E  E  E  K  D  D  E  E  G  E  K  K
AGGATAAAAA AAAGAAACAC AATAAGGATA AGAAAGAAAC AAAGGATAAA GAAAAGAAAT    1080
  D  K  K  K  K  H  N  K  D  K  K  E  T  K  D  K  E  K  K  Y
ACAAGAGCAA AGAGGAGTCA GAAGAAGAAG ATAAGAAAGA AACGGAGGAA GAGAAGGATG    1140
  K  S  K  E  E  S  E  E  E  D  K  K  E  T  E  E  E  K  D  D
ATGATGAAGA GGGTCAGAAG AAGGAAAAAG AGAAGAAAAA CAAGAAGGAT AAAAAAGAGA    1200
  D  E  E  G  Q  K  K  E  K  E  K  K  N  K  K  D  K  K  E  K
AAAAGGATAA AGAAAAGAAA GTCAAGAGCA AAGAGGAGTC AGATGAAGAA GACAAGCAAG    1260
  K  D  K  E  K  K  V  K  S  K  E  E  S  D  E  E  D  K  Q  D
ACAAGGTGAA TGAGGTTGAA GTCGCCACAA GAGAGATAAA AATTGAGGAT GACAAGAAAA    1320
  K  V  N  E  V  E  V  A  T  R  E  I  K  I  E  D  D  K  K  I
TATCGGACGG TGAAGCAGAC GAGAAAGGTA AAAGGAAGGA GAAAGGAAAA GACAGTAAAG    1380
  S  D  G  E  A  D  E  K  G  K  R  K  E  K  G  K  D  S  K  D
ATGAGAAACA AAAGGATGCA AAAAAGGACA AAGCTGAGAA GACGAGAAAA CTTGAGGACA    1440
  E  K  Q  K  D  A  K  K  D  K  A  E  K  T  R  K  L  E  D  K
AGTATAAAAG CAACGGTAAG TTGAAATCCA AGTTGGAGAA GATCAATGCC AAACTGGAAG    1500
  Y  K  S  N  G  K  (L) K  S  K  L  E  K  (I) N  A  K  L  E  A
CTCTTCAGCA GAAAAAAGCG GACATCATGA AGACAATAAA AGAAGCCGAG GATAAAAACT    1560
 (L) Q  Q  K  K  A  D (I) M  K  T  I  K  E (A) E  D  K  N  L
TAGCCGTGGT TGAGAGTCCT AAAGAGGCAG ACCTGAAGGC ACACGATGGA GTAATGACTG    1620
  A (V) V  E  S  P  K  E  A  D  L  K  A  H  D  G  V  M  T  E
AACAGTGAGT AGTATATTTT TCATAATAAT GTTAGATGTT CTTATACTGT CGTTGTGTAT    1680
  Q  *
ACCTTTCCCT TCCACTGTCA TTTAGAAATG TATTATCTTT GTCTTAATTA AGTTAAAGTA    1740

ATAGAGATGA TATACTTGCA TTTAAAAAAA AAAAAAAA                            1778
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(B)

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MEKEKKIDMEEKHEKELKEKEKKDKVKNTGSEEESEETEDEKDGATKNVK      50
EKKYKKEKKEKKDKEKKDKSKEEESEEETEEEKDDGKGKKDKKKKHKTDM     100
KEKKDKEMKDKSKHESEKEDSKEIEEEKDDGEGEKKDKEKKLKKGKKDRK     150
EKEKKDKSIEESKEEKDDDKGEKKDKEQKDKKEKKNKEEKGKSKGESEEE     200
TEEEKDDEKGKNKESDEEDERQTEEEENDEKGVKKDKEKKNKEKKEKKDN     250
EKKDKSKEETEEEKDDEKGEKKDKEKKCKKNKKEKKDKETKDKSKEVSDE     300
EEEKDDEEGEKKDKKKKHNKDKKETKDKEKKYKSKEESEEEDKKETEEEK     350
DDDEEGQKKEKEKKNKKDKKEKKDKEKKVKSKEESDEEDKQDKVNEVEVA     400
TREIKIEDDKKISDGEADEKGKRKEKGKDSKDEKQKDAKKDKAEKTRKLE     450
DKYKSNGKLKSKLEKINAKLEALQQKKADIMKTIKEAEDKNLAVVESPKE     500
ADLKAHDGVMTEQ                                           513
```
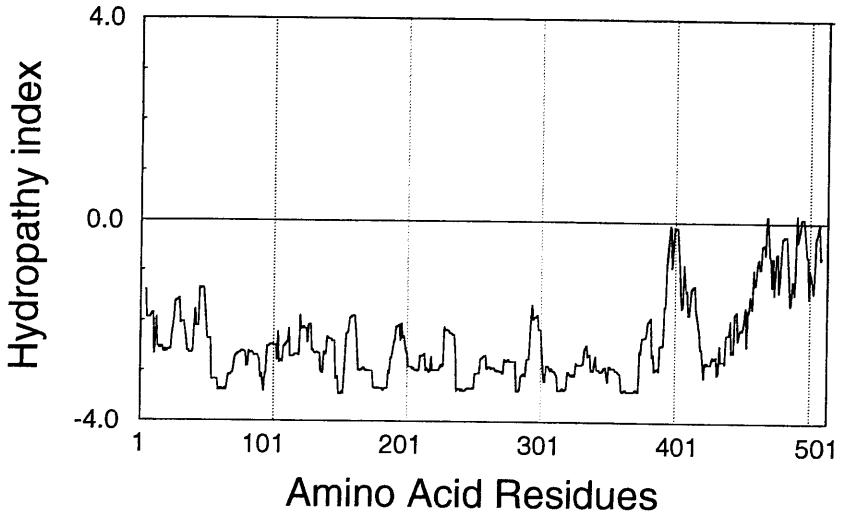

(C)



(D)

Figure 5. Sequences and structural features of the *KED* gene product.

(A) DNA sequence of the *KED* cDNA along with the deduced amino acid sequence. The nucleic acids are presented on the top line and the derived one-letter amino acid sequence is shown below. The stop codon is indicated by an astarisk. The circles indicate hydrophobic residues in the putative leucine zipper motif. The accession number is AB009883. (B) Amino acid sequence of the *KED* gene product. The circles indicate hydrophobic residues in the putative leucine zipper motif (red). Lysine, glutamic acid, and aspartic acid residues are colored as green, purple, and blue, respectively. (C) Hydropathy plot of the KED polypeptide. Hydropathy analysis was performed using a window of nine amino acids (Kyte and Doolittle, 1982). (D) Distribution of acidic and basic amino acid residues in the KED polypeptide. Acidic (Asp and Glu) and basic (Lys and Arg) residues are indicated by vertical bars above and below the center box, respectively. The relatively hydrophobic C-terminal region (amino acids 373-513) is indicated by a light gray box. The putative leucine zipper motif is indicated by a dark gray box.

Immediate and Systemic Response of *KED* to Wounding

In response to wounding, several genes have been shown to accumulate their transcripts not only locally but also systemically. To examine whether or not *KED* is systemically induced, RNA was extracted simultaneously both from wounded leaves and from adjacent healthy unwounded leaves, and RNA blot hybridization was performed (Figure 6). The results showed that *KED* transcripts accumulated in the upper unwounded leaves in response to wounding. The accumulation pattern of *KED* transcripts was similar for both the local and systemic responses, although the transcript level was lower in the systemic response.

To investigate the protein level and cellular localization of *KED* gene products, the antibody against KED protein was planned to prepare by expressing the gene in *E. coli*. However, *E. coli* cells carrying the plasmids for over-expression of KED failed to grow, indicating KED is toxic for bacteria.

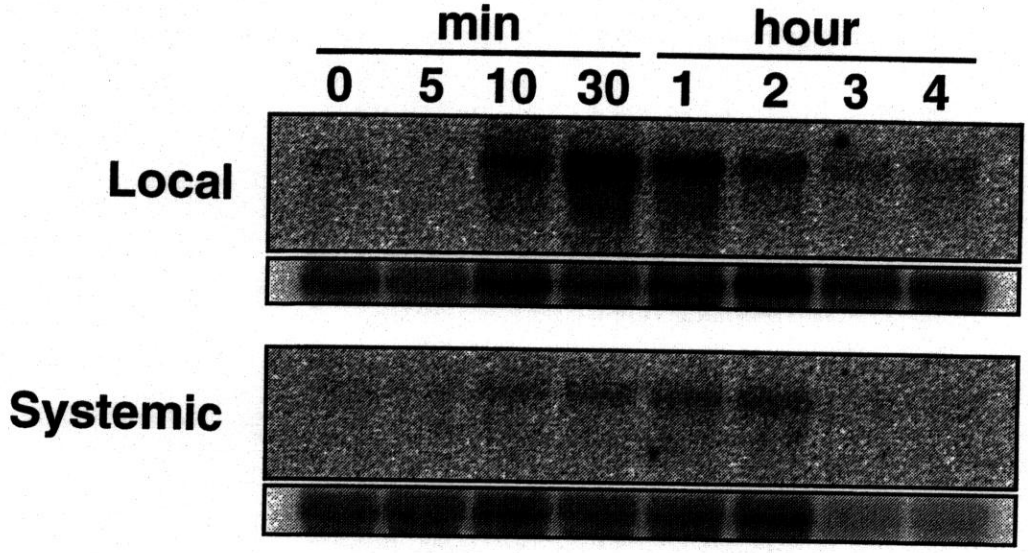


Figure 6. Local and systemic accumulation of *KED* transcripts after wounding.
Healthy leaves were wounded by incising across the midrib with a pair of scissors. The wounded ('Local') and unwounded upper ('Systemic') leaves were harvested at the indicated time intervals. An RNA blot containing 40 μg of total RNA per lane was subjected to hybridization with a ^{32}P-labeled *KED* cDNA probe.

DISCUSSION

Analysis of the full-length cDNA of A7 revealed that it encodes a highly charged protein. The A7 gene was designated as *KED*, since the amino acids K, E and D comprise up to 70% of its gene product. There are none of the clusters of hydrophobic amino acids that are necessary for protein folding, suggesting that the KED polypeptide is unable to form a firm tertiary structure. Although a search of protein sequence data bases revealed that no similar proteins have been reported so far, we identified several putative bipartite nuclear-targeting signal (NTS) sequences (Robbins et al., 1991) and a putative leucine zipper motif located in the C-terminal region. Dehydrins, known as late embryogenesis abundant (LEA) D11 family proteins, are extremely hydrophilic with abundance of charged and polar amino acid residues

(Close, T.J., 1997). They are believed to ameliorate the effects of drought and low-temperature stress by regulation of osmotic balance as compatible solutes and by stabilization of membrane and enzymes (Close, T.J., 1997). Many dehydrins contain putative bipartite NTS sequence and repeated K-rich domains which form amphipathic α-helix. In view of these similarities between KED and dehydrins, it is conceivable that KED stabilizes cellular components, which are damaged by wound stress, through electrostatic and hydrophobic interactions.

II-2 New WRKY Transcription Factor Encoded by C10

MATERIALS AND METHODS

Plant and Treatments

Tobacco plants were grown as described in Chapter I. Wounding was performed by cutting mature leaves across the midrib with a pair of scissors. The wounded leaf and upper unwounded leaf were harvested at an appropriate time point, immediately frozen in liquid nitrogen and stored at -80°C.

Tobacco suspension cultures (*N. tabacum* L. cv. Bright Yellow 2) were maintained by subculture in Murashige-Skoog medium with 30 g/L sucrose, 0.37 g/L KH_2PO_4, 1 mg/L thiamine-hydrochloride and 0.2 mg/L 2,4-dichlorophenoxyacetic acid, at 23°C under continuous dark conditions, and cells of 7th day subculture were employed for transactivation analyses.

cDNA Cloning

The almost full-length cDNA was amplified by RT-PCR with the specific primers (5'-TACACCTGAAGAAGGATTTTAAGC-3' and 5'-GTTGGAAAACATTGAACAATTGTC-3') and cloned into the pGEM-T easy vector (Promega), to give pGEM-C10P. The nucleotide sequence was determined by the dideoxy chain termination method (ABI PRISM Dye Terminator). Editing of DNA sequences and amino acid sequences were carried out on a

computer (GeneWorks; IntelliGenetics, CA), with comparison to non-redundant data bases by the BLAST program on network servers (Altschul et al., 1990).

Plasmid Construction

pGEM-C10P was digested with *Eco*RI and the *WIZZ* cDNA fragment was subcloned into the *Eco*RI site of the pGEX-4T-1 expression vector (Pharmacia) to create pGEX-WIZZ, encoding the GST-WIZZ protein. *E. coli* DH5α cells carrying pGEX-4T-1 or pGEX-WIZZ were cultured in LB broth and treated with 1 mM IPTG for GST or GST-WIZZ production. The harvested cells were disrupted by sonication, and centrifuged. Clear lysates were used for gel shift assays.

The *WIZZ* ORF, sandwiched with *Xba*I (upstream of a start codon) and *Bam*HI (just before a stop codon) sites, was created by PCR amplification and verified by direct sequencing. This *Xba*I-*Bam*HI fragment was inserted into *Xba*I and *Bam*HI sites of the pGFP-2 vector (provided by N. -H. Chua and P. Spielhofer), resulting in pWIZZ-GFP2, encoding WIZZ fused to the N terminus of GFP.

The promoter-less reporter plasmid (PL-LUC) was created by total deletion of the cauliflower mosaic virus (CaMV) 35S promoter by digestion with *Hin*dIII of the 221-luc+ vector (provided by K. Hiratsuka) consisting of the 35S promoter, the *luciferase* (*LUC*) gene, and the *nos* terminator. To generate the -46-LUC plasmid, a 35S minimal promoter, truncated to position -46, was amplified by PCR with incorporation of *Hin*dIII and *Bgl*II sites at the 5' end and an *Nco*I site at the 3' end, respectively. The resulting *Hin*dIII-*Nco*I fragment was inserted into *Hin*dIII and *Nco*I sites of 221-luc+. Three copies of the BS65 sequence (de Pater

et al., 1996) were placed into *Hin*dIII and *Bgl*II sites of -46-LUC to create the 3BS-46-LUC plasmid. Two effector plasmids, 35S-WIZZ and 35S-anti-WIZZ, were constructed as follows; the *Not*I fragment derived from pGEM-WIZZ (see above) was cloned into the *Not*I site of pBlueScript II SK (+) (Stratagene) in both orientations. Then the respective *Xba*I-*Sac*I fragments were subcloned into *Xba*I and *Sac*I sites of the pBI221 vector (Clontech).

Gel Mobility Shift Assay

Gel shift assays were performed essentially as described earlier (Kusano et al., 1995). The IMU sequence is the mutated derivative of a motif I, which contains a G-box-related core sequence (Salinas et al., 1992). The BS65 sequence is the optimal binding site of the *Arabidopsis* WRKY protein ZAP1 (de Pater et al., 1996). The AS1 sequence is the *as-1* element found in the CaMV 35S promoter (Lam et al., 1989). Monomeric double-strand probes (described in Figure 11A) were labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase. The labeled DNA probe (100 fmol) was mixed with *E. coli* crude extract (3 μg protein each) and competitors or EDTA as appropriate in a 15 μl of reaction solution. After 15-min incubation at 30°C, the sample was subjected to electrophoresis.

Particle Bombardment

BY-2 cells were spread onto MS agar medium and the plates were kept in clean bench to evaporate the liquid medium. In addition, a 3 cm square onion scaly leaf fragment was placed on wet paper in a Petri dish. Particle bombardment was performed according to the manufacturer's instructions (PDS-1000, Bio-Rad), with 1.0 μm diameter gold particles coated

with plasmids according to the manufacturer's protocol. For transactivation analysis, an appropriate set of a reporter plasmid (0.67 μg) and an effector plasmid (0.67 μg) was bombarded with the reference pRTL2-GUS vector (provided by J. Carrington) (0.33 μg) carrying two copies of the 35S promoter and a translation leader enhancer sequence (Restrepo et al., 1990). The plated cells or the onion scaly leaf were placed under the stopping screen at a distance of 6 cm or 9 cm and bombarded twice per sample in a vacuum of 28 inches of mercury using a helium pressure of 1100 psi to accelerate the macrocarrier. Bombarded cells and the leaf were kept in the dark for 16 h at 23°C before analysis.

Transactivation Assay

Luciferase and GUS activities were measured using a luciferase assay system (PicaGene; Toyo Ink, Tokyo) and a GUS assay system (AURORA GUS; ICN Pharmaceuticals, Inc., CA) with a luminometer (Lumat LB9507; EG & G Berthold, Germany).

RESULTS

C10 Encodes a WRKY Transcription Factor, WIZZ

The nearly full-length cDNA of C10, obtained by 5'-RACE and following RT-PCR, was found to be 1,251 bp in size and to contain an open reading frame encoding a protein with 356 amino acids in length with a predicted relative molecular mass of 39,082 Da (Figure 7). Within the 5' untranslated region, two stop codons in frame exist, suggesting that it

contains the entire coding region. The inferred amino acid sequence has two characteristics; it contains a conserved domain, the so-called WRKY domain after (N)-**WRKY**GQK-(C) in the parsley WRKYs region (Rushton et al., 1996), in its central region and a heptad leucine repeat in the amino-ternimal region (Figure 8A). Thus the gene was designated as *WIZZ* (*w*ound *i*nduced leucine-*z*ipper and *z*inc finger protein). The WRKY domain is shared by SPF1 from sweet potato (Ishiguro and Nakamura 1994), ABFs from oat (Rushton et al., 1995), WRKYs from parsley (Rushton et al., 1996), ZAP1 from *Arabidopsis* (de Pater et al., 1996), and tWRKYs from tobacco (Wang et al., 1998). These WRKY family proteins are classified into two groups, a single domain group and a two domain one. WIZZ, ABF2 and WRKY3 proteins belong to the former, whereas SPF1, ABF1, ZAP1, WRKY1, WRKY2, tWRKY1 and tWRKY2 are in the latter (Figure 8A). The WRKY domain contains a zinc-finger motif consisting of two cysteines and two histidines. An amino acid alignment of WRKY domains among WIZZ, ABF2 and WRKY3 is presented in Figure 8B. The ABF2 but not WRKY3 also has a leucine-zipper motif in the distal region close to the amino-terminus. As shown in the schematic illustration, WIZZ resembles ABF2 in structure (Figure 8A).

```
ATTAATTACA CCTGAAGAAG GATTTTAAGC TTTTTGGATT TGTTGGAATT TTATTAAGAA      60

GAAATTAAAG CAATGGAATT CACAAGTTTG GTTGATACTT CCTTGGATTT GAGTTTTAGA     120
            M  E  F  T  S  L  V  D  T  S  L  D  L  S  F  R

CCTCTTCCAG TTCTTGATAA AGTGCTGAAA CAAGAAGTTC AGAGTAATTT CACTGGATTG     180
P  L  P  V  L  D  K  V  L  K  Q  E  V  Q  S  N  F  T  G  L

AGCAGAGACA ATATGCTGGT GAAAGATGAG GCAGGTGATT TGTTGGAGGA ACTGAACAGA     240
S  R  D  N  M  L  V  K  D  E  A  G  D  L  L  E  E  L  N  R

GTGAGCAGTG AAAACAAGAA ACTAACAGAG ATGCTCACAG TGGTGTGTGA AAATTACAAT     300
V  S  S  E  N  K  K  L  T  E  M  L  T  V  V  C  E  N  Y  N

GCATTAAGAA ACCAACTAAT GGAGTATATG AACAACCAGA ATAATGGTGT AGTAGATGAT     360
A  L  R  N  Q  L  M  E  Y  M  N  N  Q  N  N  G  V  V  D  D

AGTGCTGGAT CAAGGAAAAG AAAAGCTGAA AATATCTCCA ATCCCAACAA CAACAACAAC     420
S  A  G  S  R  K  R  K  A  E  N  I  S  N  P  N  N  N  N  N

AACAAAAACA ACAACTTGGA TATTGTTTGT GGACGTTTAT CAGAAAGCAG TTCAAGTGAT     480
N  K  N  N  N  L  D  I  V  C  G  R  L  S  E  S  S  S  S  D

GAAGAGTCTT GTTGCAAGAA ACCTAGAGAA GAGCACATAA AAACTAAGGT TTCTGTCGTT     540
E  E  S  C  C  K  K  P  R  E  E  H  I  K  T  K  V  S  V  V

TCTATGAGGA CAGAAGCATC TGATACCTCT CTTATTGTAA AGGATGGTTA TCAATGGAGG     600
S  M  R  T  E  A  S  D  T  S  L  I  V  K  D  G  Y  Q  W  R

AAATATGGTC AGAAAGTAAC TAGAGACAAT CCTTCTCCAA GAGCTTACTT CAGGTGCTCT     660
K  Y  G  Q  K  V  T  R  D  N  P  S  P  R  A  Y  F  R  C  S

TTTGCTCCTG GCTGCCCCGT CAAGAAAAAG GTGCAAAGAA GCATAGAAGA TCAGTCAGTT     720
F  A  P  G  C  P  V  K  K  K  V  Q  R  S  I  E  D  Q  S  V

GTGGTGGCAA CATATGAAGG AGAGCATAAC CATCCAGTAA ACCCTTCAAA ACCAGAGGCT     780
V  V  A  T  Y  E  G  E  H  N  H  P  V  N  P  S  K  P  E  A

GCTGCTGGTA CTGCTACTTC CACCGGCAGC CGTTTAAATG TGAGAACTAT TGGGGGTACT     840
A  A  G  T  A  T  S  T  G  S  R  L  N  V  R  T  I  G  G  T

ACAGCTTCAG TCCCTTGCTC TACCACTCTC AATTCATCAG GACCAACCAT TACTCTCGAT     900
T  A  S  V  P  C  S  T  T  L  N  S  S  G  P  T  I  T  L  D

CTTACTGAAC CTACAACAGT AGCAAAAGGC GATATCATGA AGATGAGTAG CAGTATTAGT     960
L  T  E  P  T  T  V  A  K  G  D  I  M  K  M  S  S  S  I  S

CCTACAGGTG GCAGTAGCCA AAGAACAACA GAAGGTGATC ACTATAGTAG GCCAGAGTTT    1020
P  T  G  G  S  S  Q  R  T  T  E  G  D  H  Y  S  R  P  E  F

CAACAGTTCT TGATAGAGCA AATGGCTTCT TCATTGACTA AAGATCCAAG TTTCAAAGCA    1080
Q  Q  F  L  I  E  Q  M  A  S  S  L  T  K  D  P  S  F  K  A

GCACTTGCTG CCGCCATTTC AGGAAAAATT CTCCAACATA ATAATCAAAC AAGTAGATGG    1140
A  L  A  A  A  I  S  G  K  I  L  Q  H  N  N  Q  T  S  R  W

TAAACAAAAG TCCTGCACAC CAGTCAATTT CTATTTATGG ATAGGCTAGA CAATTGTTCA    1200
*

ATGTTTTCCA ACATAGACTC ATTTGGAAAG TTCAGCAAAA AAAAAAAAAA A             1251
```

Figure 7. DNA sequence of the WIZZ cDNA along with the deduced amino acid sequence.
The nucleic acids are presented on the top line and the derived one-letter amino acid sequence is shown below. The stop codon is indicated by an astarisk. The hydrophobic residues in the putative leucine zipper motif are double-underlined. The WRKY domain is shown in Italics. The two cysteines and two histidines in the zinc finger motif are circled. The original C10 clone obtained by FDD screening is underlined. The accession number of WIZZ cDNA is AB028022.

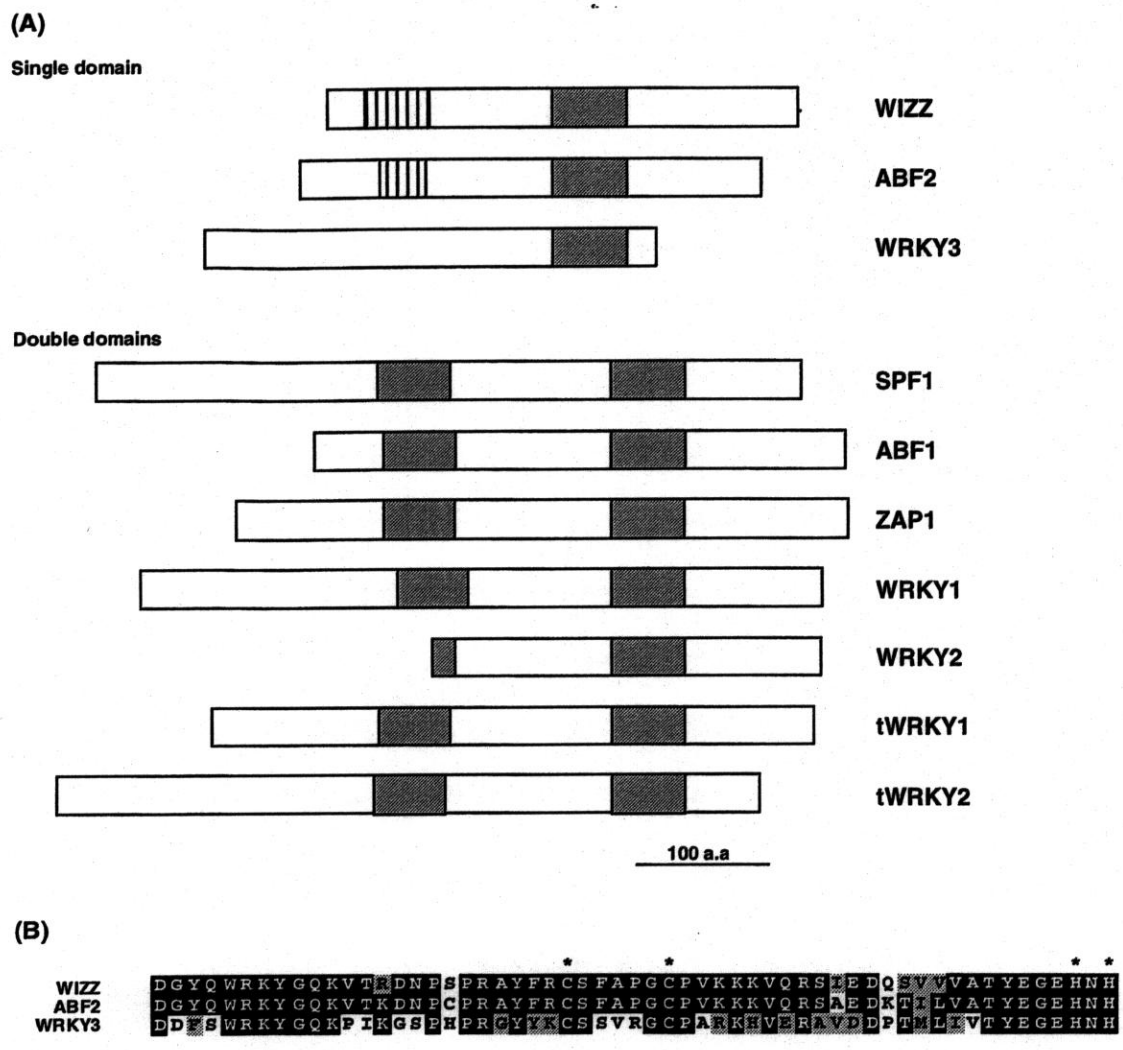


Figure 8. Structural features of WIZZ.
(A) Schematic presentation of WIZZ and other WRKY family proteins. The WRKY domains are shaded and the leucine-zipper regions are striped. The bar represents a length of one hundred residues. (B) Alignment of WRKY domains from WIZZ, ABF2 and WRKY3. Identical and similar residues are highlighted in black and gray, respectively. The two cysteines and two histidines for the putative zinc finger are indicated by stars.

Rapid and Systemic Accumulation of *WIZZ* Transcripts upon Wounding

Wound-responsive transcripts are often accumulated not only locally but also systemically upon wounding (Seo et al., 1995). To determine whether this is also the case with the *WIZZ* gene, RNA blot analysis was performed using total RNAs prepared from

wounded and unwounded leaves (Figure 9). When the full-length cDNA of *WIZZ* was used as probe, at least 3 differently sized transcripts were detected. Judging from the molecular size, we consider that the middle-sized transcripts correspond to *WIZZ* cDNA. All of them demonstrated systemic in addition to local responses upon wounding.

To investigate the protein level of *WIZZ* gene products, an antibody against mixed synthetic peptides (N107 to L122 and I347 to W356 of WIZZ) was produced in rabbits. However, immunoblot analyses of proteins from wounded leaves showed no specific signals, suggesting either a lack of specificity of antibodies or very low level of WIZZ protein in wounded leaves (data not shown).

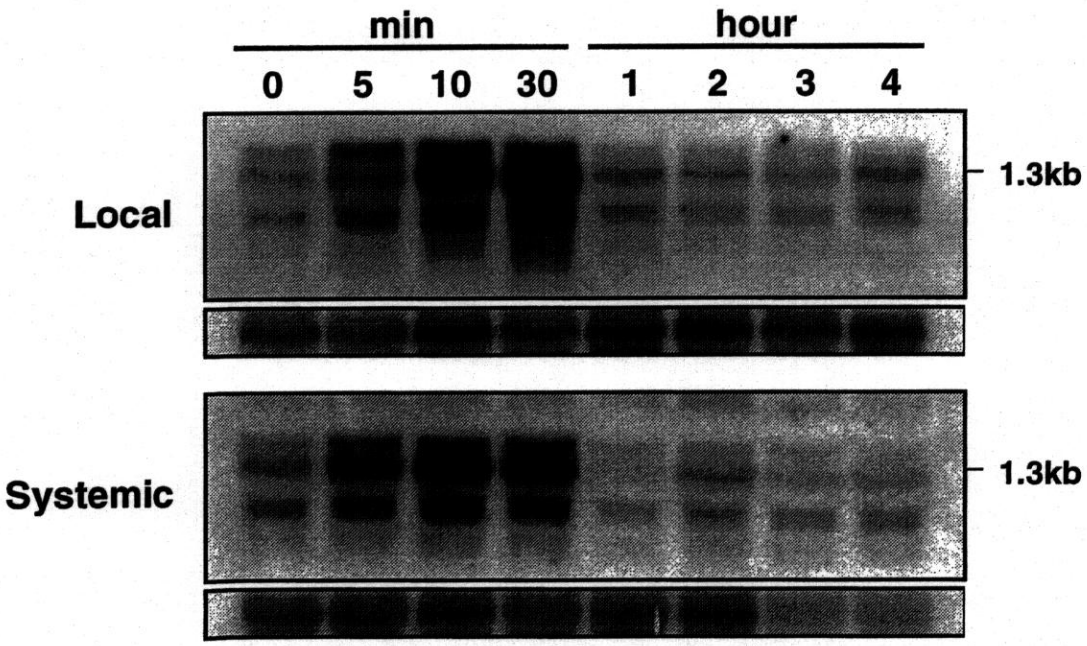


Figure 9. Local and systemic induction of *WIZZ* expression by wounding.
Healthy leaves were wounded by cutting. The wounded (Local) and upper unwounded (Systemic) leaves were harvested at the indicated time points. The blot, containing 40 μg of total RNA per lane, was subjected to hybridization with the full-length *WIZZ* cDNA (upper panel) or actin probe (lower panel).

Nuclear Localization of the WIZZ Protein

PSORT analysis (Nakai and Kanehisa 1992) indicated a high likelihood of nuclear localization of the WIZZ protein. To confirm this, we constructed a cauliflower mosaic virus (CaMV) 35S::WIZZ-GFP gene fusion product that could be used in transient assays. After biolistic bombardment of onion epidermis with a CaMV 35S::GFP control construct (pGFP-2), GFP signals were observed in both the cytoplasm and the nucleus (Haseloff et al., 1997) (Figure 10A). In contrast, the WIZZ-GFP fusion protein was found exclusively in the nucleus, indicating that WIZZ is a nuclear factor (Figure 10B).

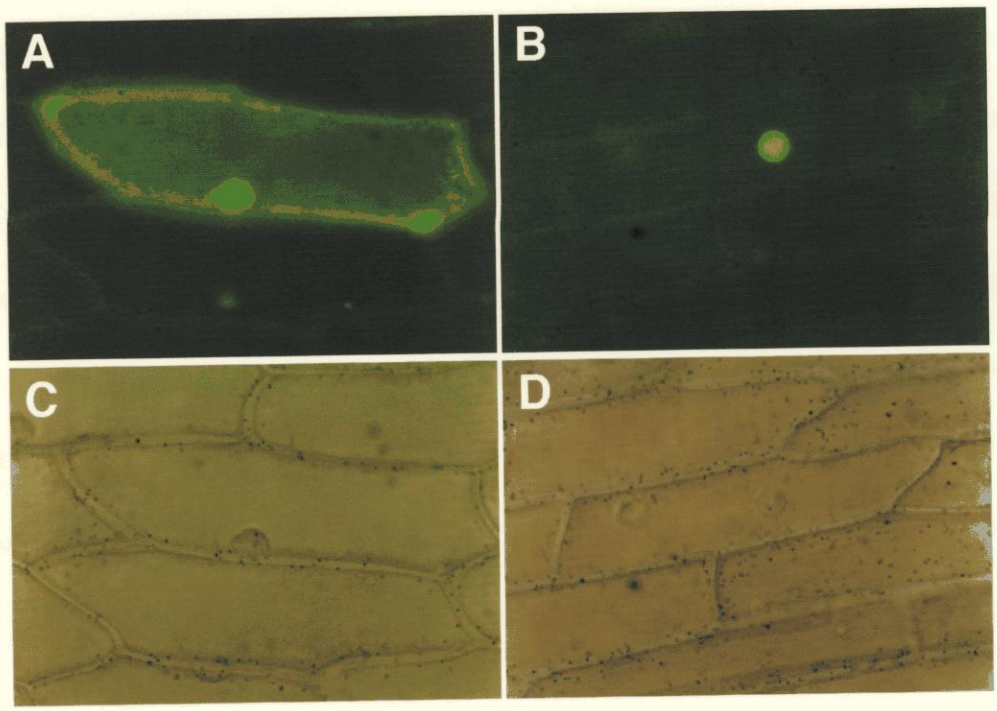


Figure 10. Nuclear localization of WIZZ-GFP fusion protein in onion cells.
(A) and (C) Onion epidermis cells transfected with GFP-expressing plasmids. (B) and (D) Onion epidermis cells transfected with WIZZ-GFP fusion protein-expressing plasmids. Green fluorescence under UV light excitation [(A) and (B)] or corresponding views by differential interference contrast [(C) and (D)] are shown in the same magnification.

DNA Binding Specificity of WIZZ

Since WRKY proteins have been reported to bind DNA, we investigated whether WIZZ does so by a gel mobility shift assay using an *E. coli* crude extract containing GST-fused WIZZ. As used in the previous report on ZAP1 (de Pater et al., 1996), three probes, BS65, IMU and AS1, containing two TGAC sites with variable neighboring nucleotides, were primarily tested (Figure 11A). The GST-WIZZ fusion protein efficiently bound to the BS65 and the IMU probes, but not to AS1. Binding to BS65 and IMU was dramatically reduced by addition of EDTA or unlabeled corresponding competitor probes. When both TGAC-core sequences in BS65 were mutated, the fusion proteins lost the binding ability, indicating the core TGAC to be important (Figure 11B). Three probes tested had two TGAC-core sequences, but differed in interspace and flanking regions. To clarify the DNA binding specificity of WIZZ, further gel mobility shift assays were performed using modified probes. The interspace between two core TGAC sites was varied (BS65s4-BS65s8). The results showed that the GST-WIZZ protein preferred the BS65s5, BS65s6 and BS65s8 sequences than the BS65s4 and BS65s7. When the two TGAC motifs, CTGACG and ATGACG, of the AS1 probe were changed to TTGACC and TTGACT, respectively, to yield the AS1m probe, WIZZ protein gains the binding activity to the AS1m probe (Figure 11C). To examine whether or not both TGAC motifs in the BS65 probe are required for WIZZ binding, each core site was appropriately mutated (to BS65tm and BS65mt). Signal intensities for each binding complex were considerably decreased in comparison with the intact BS65 case (Figure 11D).

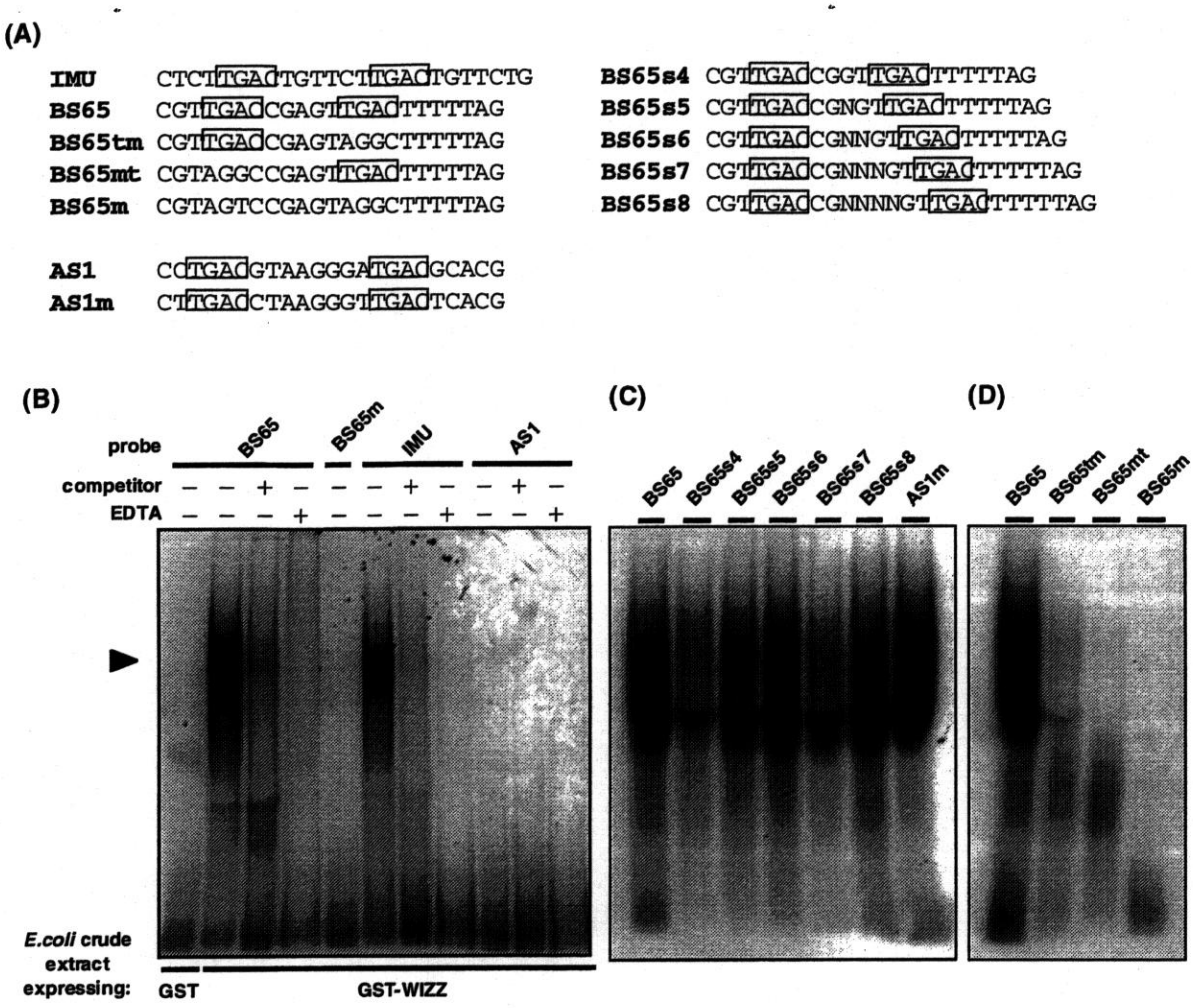
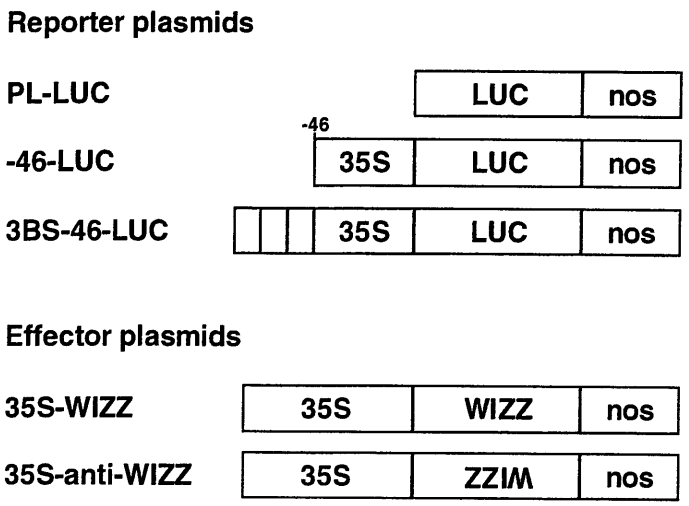


Figure 11. Gel mobility shift assay results.
(A) Nucleotide sequences of DNA probes with TGAC motifs boxed. Modifications from the BS65 and the AS1 probes are underlined. N indicates a mixture of four nucleotides (A, C, G, or T). (B) Effects of a competitor and a chelator on DNA binding activity of WIZZ. Gel shift assays were performed with (+) or without (-) unlabeled competitor BS65, IMU, or AS1 in a 100-fold molar excess or a final concentration of 50 mM EDTA. DNA binding complexes of WIZZ are indicated by the arrowhead. (C) Effects of modified probes on DNA binding activity of WIZZ. (D) Effects of the number of TGAC-core sites in the BS65 probe.

Lack of Transactivation Activity of WIZZ

To test the transactivation activity of WIZZ, a fusion construct of the GAL4 DNA binding domain and WIZZ in-frame was created and introduced into yeast cells carrying two reporter plasmids consisting of the GAL4 binding sequence upstream of lacZ and HIS3 genes. No transactivation activity of WIZZ was detected in this yeast system (data not shown). Transactivation activity of WIZZ was also addressed using tobacco BY-2 cells as follows; three reporter plasmids (PL-LUC, -46-LUC and 3BS-46-LUC) and two effector plasmids (35S-WIZZ and 35S-anti-WIZZ) were constructed and transfected into tobacco suspension culture cells (Figure 12). Co-transfection of the 3BS-46-LUC reporter with the WIZZ binding sites and the 35S-WIZZ effector plasmid did not show WIZZ to activate gene expression. Similarly, co-transfection with the 35S-anti-WIZZ effector plasmid did not affect the reporter activity in the presence or the absence of the WIZZ binding sites, indicating that WIZZ alone can not activate gene expression.

(A)



(B)

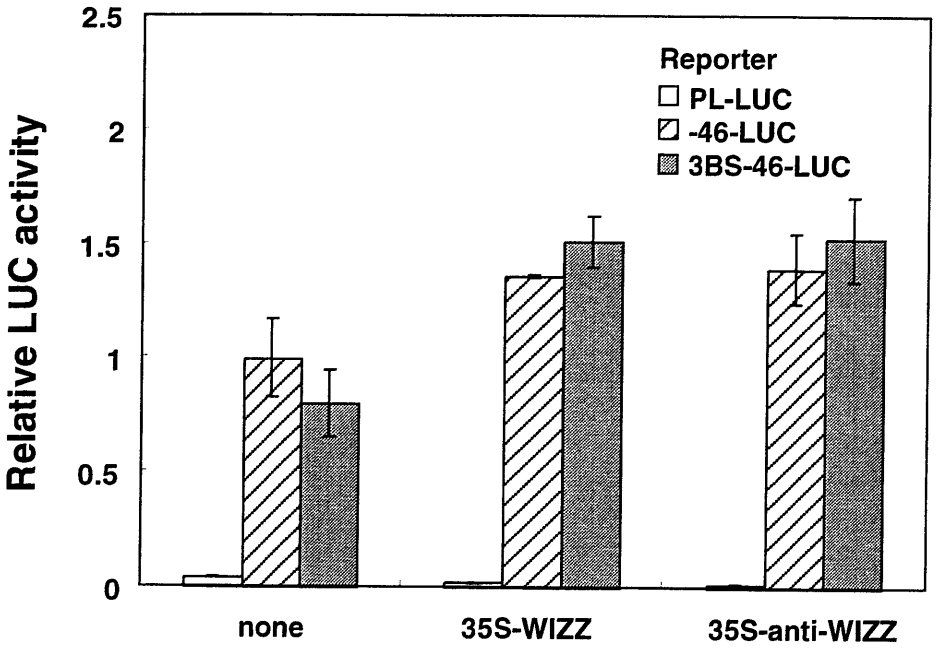


Figure 12. Transactivation analysis of WIZZ in BY-2 cells.
(A) Schematic representation of reporter and effector plasmids. (B) Transcriptional activity. The reporter and effector plasmids were bombarded into BY-2 cells with the reference vector to monitor the transformation efficiency. Reporter activity is normalized to the reference activity. The reporter activity with the −46-LUC plasmid alone is set as 1. Three independent experiments were carried out. Vertical bars represent ±SE (n=3).

DISCUSSION

The present FDD screening of immediate early wound-responsive genes resulted in isolation of a gene encoding a new WRKY protein, designated as WIZZ. Several genes in the WRKY protein family have recently been identified in potato (Ishiguro and Nakamura 1994), wild oat (Rushton et al., 1995), parsley (Rushton et al., 1996), *Arabidopsis* (de Pater et al., 1996), and tobacco (Wang et al., 1998). Furthermore, dozens of similar genes have been found in *Arabidopsis* genome. In the tobacco plants, at least seven *WRKY* genes are present including *WIZZ*, five earlier reported homologues (Wang et al., 1998; accession AB022693, AB020590, AB020023), and another member responding to TMV-infection that we have recently isolated (unpublished data).

The WRKY protein family is categorized into two groups based on the number of WRKY domains. The WIZZ protein has a single WRKY domain as do ABF2 from wild oat and WRKY3 from parsley, and a leucine zipper motif in the amino terminal region like ABF2. However, it does not contain the PVQ repeats found in WRKY3. The conserved WRKY domain of WIZZ is closer to that of ABF2 than WRKY3, suggesting that the WRKY sub-family with a single domain could be subclassified on the basis of characteristic motifs, a leucine zipper or PVQ repeats.

RNA blot analysis with probes containing the conserved WRKY domain showed that at least three kinds of transcripts were locally and systemically induced by wounding. The transcripts of large size were not detected by the C10 probe, which lacked the conserved sequence encoding the WRKY domain, suggesting that they were derived from the other

member of the WRKY family. The small-sized transcripts may correspond to derivatives of the *WIZZ* transcript by degradation or alternative splicing. Their patterns of accumulation were very similar, indicating control by a common regulatory system. In parsley cells, two *WRKY* transcripts were rapidly induced by elicitor treatment (Rushton et al., 1996). Among them, the *WRKY1* gene was recently reported to be expressed in response not only to elicitors but also to mechanical stress (Gus-Mayer et al., 1998). Further study should shed light on the control of gene expression during the early stage of various defense responses, especially systemic responses to wounding.

The gel mobility shift assays showed that WIZZ binds to both BS65 and IMU probes like *Arabidopsis* ZAP1, and that such binding is abolished by metal-ion chelator EDTA. This indicates that WIZZ specifically binds to DNA depending on bivalent cations, probably of zinc. The probes employed contain two common TGAC core sequences, modifications of which completely inhibited binding activity. Also the number of nucleotides between two core TGAC motifs greatly affected binding. For example, 5, 6 and 8 spacer nucleotides confer equally high binding, whereas 4 and 7 did not. However, WIZZ did not bind AS1 probe, which also contains two TGAC sites with 8 nucleotide spacer, suggesting the flanking region and/or the spacer length of the two TGAC sites to be important in recognition by WIZZ. Indeed, WIZZ recovered binding activity to the AS1m probe with modified flanking nucleotides of the TGAC sites in the AS1 probe. These observations suggest that WIZZ binds specifically to the sequence containing two TTGAC(T/C) motifs with an appropriate spacing at least. This assumption well explains the binding specificity of the WRKY family proteins, despite their common TGAC recognition sequence. Although WIZZ could bind to probes with

a single TGAC site (BS65tm and BS65mt), the binding activity was synergistically increased by further TGAC sites nearby. This is consistent with observations that, by the mutational analysis of parsley *WRKY1* promoter, multiple W-Box elements containing TTGAC(C/T) motif generated a synergistic effect on gene expression (Eulgem et al., 1999). This suggests that protein-protein interaction, probably homologous dimerization through the leucine zipper motif, may be critical for binding. Recently, a number of studies have pointed to the importance of protein-protein interactions for the WRKY family. In tobacco plants, DNA binding activity induced by TMV-infection (TDBA12) was abolished with a protein dissociating agent, sodium deoxycholate (Yang et al., 1999). The binding activity of potato PBF-1, which is the DNA binding factor for the elicitor responsive element of *PR-10a* promoter, was induced by elicitor and wound treatment and also proved sensitive to sodium deoxycholate (Després et al., 1995). Since this element of the promoter contains two TGAC sites, the PBF-1 is assumed to be a member of the WRKY family. Moreover, DNA sequences bound by WRKY protein family members contain multiple TGAC core sites. For example, BS65, the optimal binding sequence for ZAP1, contains two TGAC sites (de Pater et al., 1996), and Box 2, a conserved element in the promoter of α-amylase genes, contains three TGAC sites recognized by ABFs (Rushton et al., 1995).

Among putative WRKY protein family members, *Arabidopsis* ZAP1 and parsley WRKY1 are the case so far, in which transcriptional activation was demonstrated in yeast and plant cells (de Pater et al., 1996; Eulgem et al., 1999). In the present study, WIZZ did not transcriptionally activate reporter genes, consistent with a lack of putative activation domains, such as a proline-rich domain. This suggests that a co-activator, which may heterologously

interact with WIZZ, is necessary for transcriptional activation. We could not, however, eliminate the possibility that WIZZ might be activated by post-translational modification, since both TDBA12 and binding activity of PBF-1 were affected by alkaline phosphatase treatment, suggesting involvement of protein phosphorylation.

TGAC core motif was originally identified as an elicitor-responsive element in parsley *PR1-1*, a PR-10 class gene (Meier et al., 1991). Later this sequence was also found in the elicitor-responsive element of the maize PRms gene (Raventos et al., 1995) and in the tobacco basic class I chitinase gene (Fukuda and Shinshi 1994; Fukuda, Y., 1997), so that it may be conserved in both dicot and monocot plants. Further analyses have revealed that this sequence is located in the promoter regions of various wound-response associated genes encoding tobacco osmotin (Nelson et al., 1992), chitinase (van Buuren et al., 1992; Ohme-Takagi et al., 1998), basic β-1,3-glucanases (Linthorst et al., 1990), PI-II (Balandin et al., 1995) and basic PR-1 (Eyal et al., 1992). Moreover, elicitor- and wound-responsive regions of the tobacco osmotin gene contain two TTGACC sequences (Raghothama et al., 1993), and DNA binding activity was also found to be induced by TMV-infection (Wang et al., 1998; Yang et al., 1999). Therefore, such a broad but defined distribution of the TGAC sequence suggests that it is a common *cis*-acting element and that WRKY family members act as trans factors during general defense responses. In fact, transcript accumulation of parsley WRKY1 and DNA binding activity of potato PBF-1 were induced not only by an elicitor treatment but also by mechanical stimulation (Després et al., 1995; Gus-Mayer et al., 1998). Understanding complex interactions of the WRKY family should facilitate elucidation of the general mechanisms underlying activation of defense-related gene expression.

Chapter III

Promoter Analyses of *KED* and *WIZZ*

In Chapter II, I described that WIZZ protein plays an important role for regulation of defense-related genes' expression. On the other hand, *WIZZ* itself and *KED* are also expected to be regulated at transcriptional level since no transcript accumulation is detected in healthy tobacco leaves. This raises several questions as to the nature of immediate-early wound-responsive element(s) and of *trans*-acting factor(s), and as to whether the regulatory system are common or not. To address these questions, deletion analyses of both *KED* and *WIZZ* promoters were performed in transiently transformed tobacco leaves.

MATERIALS AND METHODS

Plant and Treatments

Tobacco plants were grown as described in Chapter I. Wounding was performed by cutting mature leaves into pieces with a pair of scissors. The resulting leaf fragments were floated on water. The samples were harvested at an appropriate time point, immediately frozen in liquid nitrogen and stored at -80°C.

Promoter Cloning

The promoter regions of the *KED* gene and the *WIZZ* gene were isolated by TAIL-PCR (Yagi,

M., 1999). Three nested primers of each gene were synthesized. A set for the *KED* gene consisted of 5'-GCACCATCTTTCTCGTCCTCTGTTTCCTCCG-3' (ked-LA4), 5'-GTTTCCTCCGACTCCTCTTCGCTCCCCG-3' (ked-LA3), and 5'-TCTTTTCCTTTTCCATCTTTACTTCTCCCCTGGC-3' (ked-LA1). A set for the *WIZZ* gene consisted of 5'-CTTTCACCAGCATATTGTCTCTGCTCAATCCAGTG-3' (wizz-LA1), 5'-GAATCCAAAAATCTCCACATACCAGCAC-3' (wizz-LA3), and 5'-AAGGAAGTATCAACCAAACTTGTGAATTCCATTGC-3' (wizz-LA2). These primers correspond to the sequences at the 5' end of the *KED* cDNA and the *WIZZ* cDNA respectively. Arbitrary degenerate primer was 5'-NGTCGASWGANAWGAA-3' (TP1). After three round PCR, TAIL-PCR products were cloned and the nucleotide sequences were determined.

The *WIZZ* promoter region was amplified by RT-PCR with 5'-ATGGCTGGGGGGCAGCCAACTTTGACACC-3' (wizz-5'GSP2) and wizz-LA1 primer. The resulting products were cloned into the pGEM-T easy vector (Promega), to give pGEM-WP13.

The nucleotide sequences were determined by the dideoxy chain termination method (ABI PRISM Dye Terminator). Editing of DNA sequences was carried out on a computer (GeneWorks; IntelliGenetics, CA). Similar sequences and functional elements were searched by the BLAST program (Altschul et al., 1990) and by the PLACE database (Higo et al., 1999) on network servers respectively.

Plasmid Construction

A series of the *KED* promoter deletions, sandwiched with *Pst*I (5' end of the *KED* promoter

region) and *Nco*I (at a start codon) sites, were created by PCR amplification with three sets of primers. These products were cloned into pGEM-T easy vector or pBluescript vector and verified by sequencing, resulting in pBS-KP-A11, pBS-KP-B3, and pGEM-KP-C1.

The 5' untranslated region of the *WIZZ* cDNA between *Hin*dIII site and a start codon was amplified by PCR with incorporation of tandemly located *Nco*I (at a start codon) and *Bam*HI sites at the 3' end. The resulting *Hin*dIII-*Bam*HI fragment was inserted into *Hin*dIII and *Bam*HI sites of pUC18 vector, to give pUC-NB21. The *Apa*I-*Hin*dIII fragment of deleted pGEM-WP13 and *Hin*dIII-*Kpn*I fragment of the pUC-NB21 were inserted into *Apa*I and *Kpn*I sites of the pBlueScript II SK (+) (Stratagene), resulting in pBS-WPNB10. The *WIZZ* promoter deletions were created by enzymatic digestion of pBS-WPNB10 with Exonuclease III, resulting in pBS-WP-A to -H.

The *Pst*I-*Nco*I fragments of pBS-KP, pGEM-KP and pBS-WP were inserted into *Pst*I and *Nco*I sites of 221-luc+ vector, resulting in pKP-LUC-A to -C with promoter sizes of 703, 373, and 182 bp, and pWP-LUC-C to -H with promoter sizes of 1494, 1281, 1115, 926, 273, and 177 bp.

Particle Bombardment

A 2.5 cm square tobacco leaf fragment was placed on wet paper in a Petri dish. Particle bombardment was performed according to the manufacturer's instructions (PDS-1000, Bio-Rad), with 1.0 μm diameter gold particles coated with plasmids according to the manufacturer's protocol. For transient reporter assays, an appropriate reporter plasmid (1.11 μg) was bombarded with the reference pRTL2-GUS vector (0.56 μg) (Restrepo et al., 1990).

The leaf fragments were placed under the stopping screen at a distance of 6 cm and bombarded twice per sample in a vacuum of 28 inches of mercury using a helium pressure of 1100 psi to accelerate the macrocarrier. Bombarded leaf fragments were wounded by cutting immediately after bombardment and kept under light at room temperature for appropriate time intervals. The treatments were performed within 30 min.

Transient Reporter Assay

Luciferase and GUS activities were measured using a luciferase assay system (PicaGene; Toyo Ink, Tokyo) and a GUS assay system (AURORA GUS; ICN Pharmaceuticals, Inc., CA) with a luminometer (Lumat LB9507; EG & G Berthold, Germany).

RESULTS

Sequence Analyses of *KED* and *WIZZ* promoter

The *KED* and *WIZZ* promoters were isolated by TAIL-PCR (Yagi, M., 1999). Primer extension analysis was performed, but the transcriptional start point of the *WIZZ* promoter could not be determined. Accordingly, positions corresponding to 5' end of each cDNA were assigned provisionally as the transcriptional start point.

In the promoter sequences of *KED* (718 bp) and *WIZZ* (1997 bp), five consensus sequences ranging from 12 to 9 bp long were found and designated as W1 to W5 respectively. Among them, W1 and W2 contained binding sites of Dof and bZIP type transcription factors,

respectively, while the other three sequences did not contain any known motifs. Drought and ABA-related elements were found in both promoters. However, defense-related W-boxes and -624 boxes were found only in the *WIZZ* promoter sequence (Rushton et al., 1996; Lebel et al., 1998) (Figures 13, 14, Table 3). In addition, the *WIZZ* promoter was found to contain a region with exceptionally high degree of similarity to regions of the polygalacturonase and chitinase genes from tobacco. This contained several inverted repeats and direct repeats, and was shown to be a putative remnant of transposable elements (Figure 15).

Table 3. Conserved sequence motifs in the region of the *KED* and *WIZZ* promoter

Motif	Consensus sequence	Sequence and position in the *KED* promoter	Sequence and position in the *WIZZ* promoter	
W1	AAAAAGAAAGAA	AAAAAGAAAGAA (-236)	AAAAAGAAAGAA (-124)	Binding site for Dof
W2, A-Box	AAATACGTAGT	AAATACGTAGT (-200)	AAATACGTAGT (-488)	Binding site for bZIP
W3	TAATTTATATC	TAATTTATATC (-441)	GATATAAATTA (-977)	
W4	TGATTCATAT	TGATTCATAT (-394)	TGATTCATAT (-1126)	
W5	TTATATCGT	TTATATCGT (-437)	TTATATCGT (-969)	
W-Box	TTGACC or TTGACT		TTGACT (-1913), TTGACT (-633), GGTCAA (-394), TTGACC (-268), TTGACT (-262), AGTCAA (-245), GGTCAA (-72)	Binding site for WRKY protein
Myb	TAACTG	CAGTTA (-655)	TAACTG (-1305)	Binding site for ATMYB2
Myc	CACATG	CATGTG (-398), CACATG (-56)	CATGTG (-1884), CACATG (-1411), CATGTG (-1286)	Binding site for MYC (rd22BP1)
ARF	TGTCTC	GAGACA (-330)		Binding site for ARF (auxin response factor)
SURE	AATAGAAAA		AATAGAAAA (-1461)	Sucrose Responsive Element
-624 Box	ATATTCTT		AAGAATAT (-1086), ATATTCT (-918)	
LTRE	CCGAAA, CCGAC		CCGAAA (-733), CCGAC (-228)	

(A)

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TAGTCGAGAG AGAAGAAAAC TGTAGTGAAG TGAGGCTTAT AAAGAACAAG AAATGCTGAA   -659
AATCAGTTAC AAGCTGAAAC AATAATGTGG CATGGGATAT AGAGGCGCAG GAAAAGGATA   -599
TAAGAAAATG TTTCTTTGAA TGATATTTTT GGAATCAGGT TTACATAATA CTGATGGTTC   -539
TTTTCTTCTC TGGGTATGTG TAAGTTACAG CTAGTTAATC CATATTGCCT CTTTACCATA   -479
TTTTCATATG GTCGACTTCC CTTTGATATT TAGCTTTTAA TTTATATCGT GTTACAACTG   -419
ATAATGACTC TGTTGCTTAC CATGTGATTC ATATGGTAAT ACCAGAACAA AACTGCCAAT   -359
GAATCTGCTG AACAAAACTG CCTTGTGAGA GACAACTTTG TGGTTCATTA GGTTCTAATT   -299
GCCTAGTGCC TACTCGACGT TAATCCGGAT TAGTTGGGCG TGGTTTAGAT ACCGGATGTC   -239
TAAAAAAGAA AGAAAAAACT GCCTAGTGCC AGTCGACCAA ATACGTAGTC ATTTAGTTAT   -179
CCTTGTTCTT TGCAACCTGT GAAAACATTA TTTGTCCTTC ATTGGTTTGA GAAAAATCTT   -119
AAGTTGTTTG CCAGTCTTTT TACCATTATT GTTATTTTCT TGTCTACCTA GTGCTTTCAT    -59
TACACATGAG AATTATAGAC TAATTGTTTT CTTAATTTTT TCATCTCCTT CTCAGATACC      2
                                                              cc
TTGGAGAAAT TAAAGGCACA TTGACGTGCG GACAATTTTA GATAGGAAGG CAAAACTGTT     62
uuggagaaau uaaaggcaca uugacgugcg gacaauuuua gauaggaagg caaaacuguu
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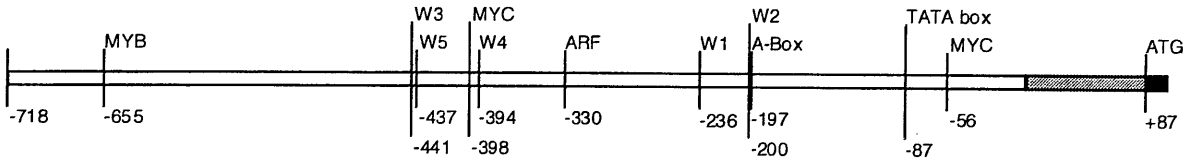
                        MetGlu LysGluLysI
```

(B)



(C)

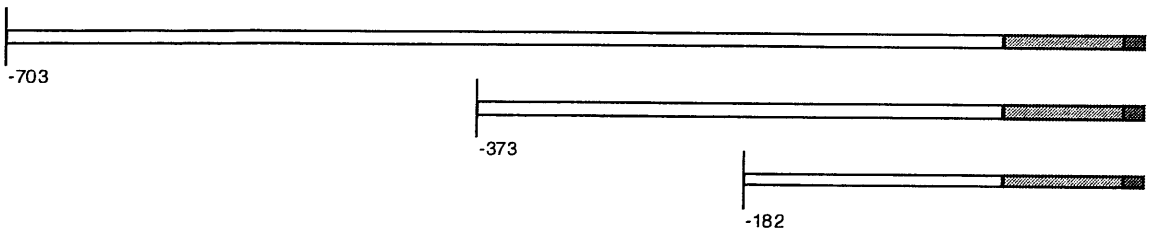
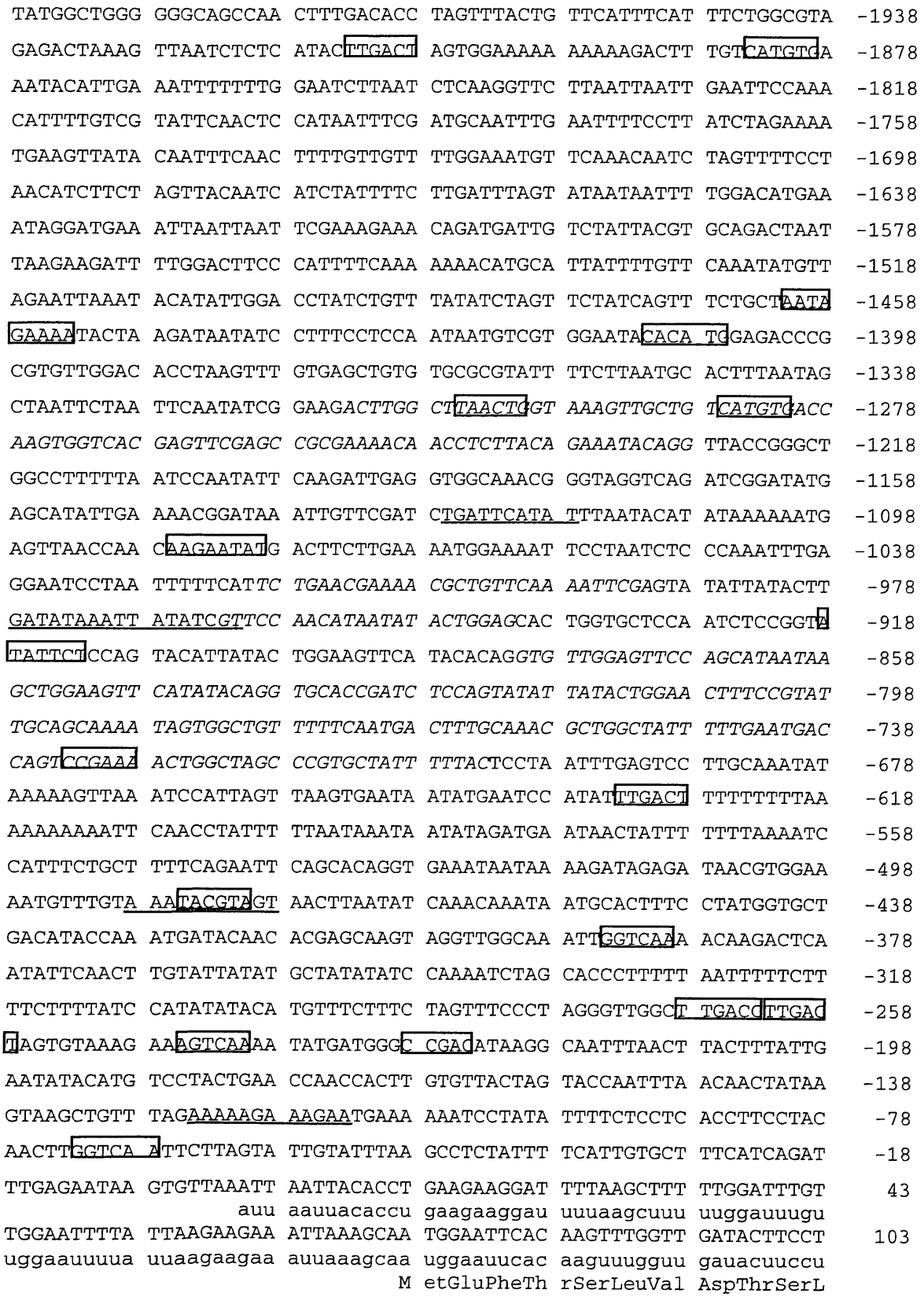


Figure 13. *KED* promoter.
(A) DNA sequence of the *KED* promoter region. The genomic DNA sequence is presented on the top line and the mRNA sequence and the derived three-letter amino acid sequence are shown below. Consensus sequences in the *KED* and *WIZZ* promoter region are underlined. The boxes indicate proposed functional elements. (B) Schematic overview of putative *cis*-elements and consensus sequences in the *KED* promoter. (C) Constructs of deletion mutants for transient assays. A light gray box indicates the 5' untranslated region (1-86). A black box and a dark gray box indicate the coding region of KED and LUC, respectively.

(A)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CATTTTGTCG TATTCAACTC CATAATTTCG ATGCAATTTG AATTTTCCTT ATCTAGAAAA -1758
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GACATACCAA ATGATACAAC ACGAGCAAGT AGGTTGGCAA ATTGGTCAAA ACAAGACTCA -378
ATATTCAACT TGTATTATAT GCTATATATC CAAAATCTAG CACCCTTTTT AATTTTTCTT -318
TTCTTTTATC CATATATACA TGTTTCTTTC TAGTTTCCCT AGGGTTGGCT TGACCTTGAC -258
TTAGTGTAAAG AAAGTCAAAA TATGATGGGC CGACATAAGG CAATTTAACT TACTTTATTG -198
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GTAAGCTGTT TAGAAAAAGA AAGAATGAAA AAATCCTATA TTTTCTCCTC ACCTTCCTAC -78
AACTTGGTCA ATTCTTAGTA TTGTATTTAA GCCTCTATTT TCATTGTGCT TTCATCAGAT -18
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auu aauuacaccu gaagaaggau uuuaagcuuu uuggauuugu
TGGAATTTTA TTAAGAAGAA ATTAAAGCAA TGGAATTCAC AAGTTTGGTT GATACTTCCT 103
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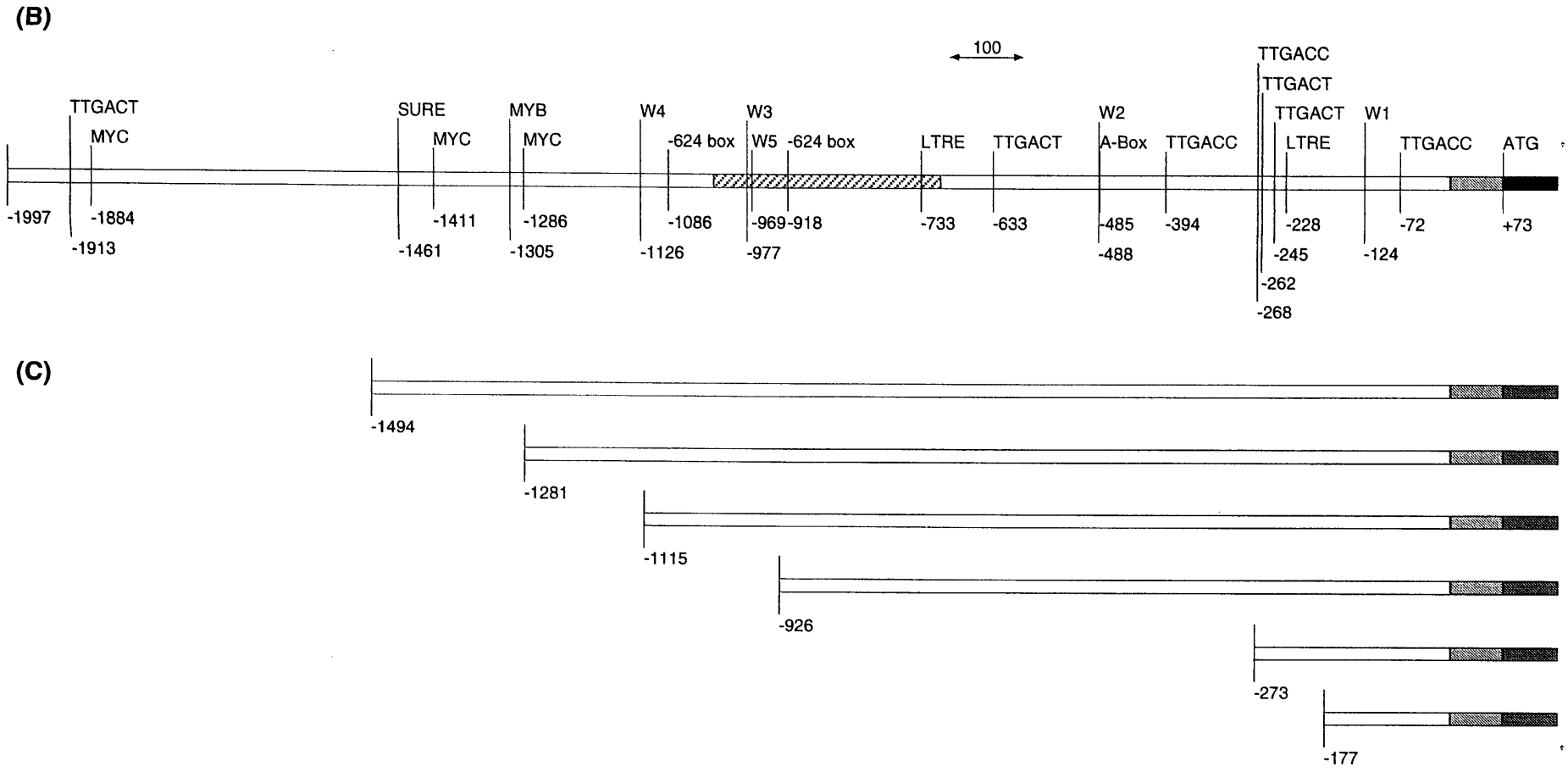
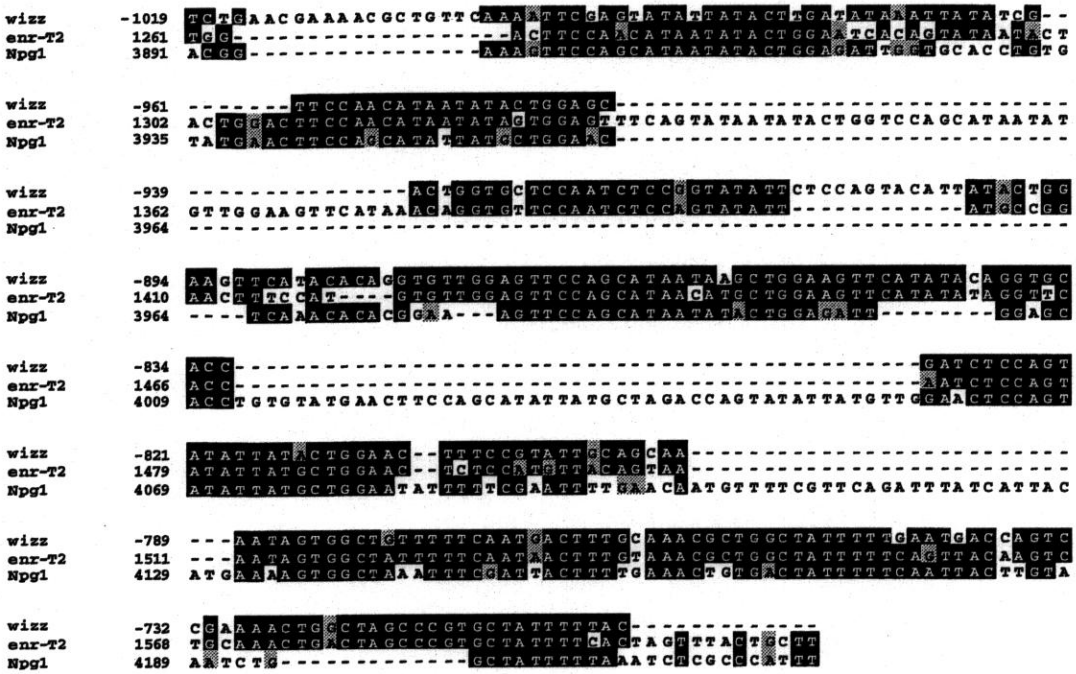


Figure 14. *WIZZ* promoter.

(A) DNA sequence of the *WIZZ* promoter region. The genomic DNA sequence is presented on the top line and the mRNA sequence and the derived three-letter amino acid sequence are shown below. Consensus sequences in the *KED* and *WIZZ* promoter region are underlined. The boxes indicate proposed functional elements. The italic sequences indicate homologous regions to transposable element. (B) Schematic overview over putative *cis*-elements and consensus sequences of the *WIZZ* promoter. (C) Constructs of deletion mutants for transient assays. A light gray box indicates the 5' untranslated region (1-72). A black box and a dark gray box indicate the coding region of WIZZ and LUC, respectively. Striped boxes indicate homologous regions to defective transposable element.

(A)



(B)

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TCTGAACGAA AACGCTGTTC AAAATTCGAG TATATTATAC TTGATATAAA TTATATCGTT   -960
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CCAACATAAT ATACTGGAGC ACTGGTGCTC CAATCTCCGG TATATTCTCC AGTACATTAT   -900
GGTTGTATTA TATGACCTCG TGACCACGAG GTTAGAGGCC ATATAAGAGG TCATGTAATA

ACTGGAAGTT CATACACAGG TGTTGGAGTT CCAGCATAAT AAGCTGGAAG TTCATATACA   -840
TGACCTTCAA GTATGTGTCC ACAACCTCAA GGTCGTATTA TTCGACCTTC AAGTATATGT

GGTGCACCGA TCTCCAGTAT ATTATACTGG AACTTTCCGT ATTGCAGCAA AATAGTGGCT   -780
CCACGTGGCT AGAGGTCATA TAATATGACC TTGAAAGGCA TAACGTCGTT TTATCACCGA

GTTTTTCAAT GACTTTGCAA ACGCTGGCTA TTTTTGAATG ACCAGTCCGA AAACTGGCTA   -720
CAAAAAGTTA CTGAAACGTT TGCGACCGAT AAAAACTTAC TGGTCAGGCT TTTGACCGAT

GCCCGTGCTA TTTTTAC                                                  -703
CGGGCACGAT AAAAATG
```

Figure 15. *WIZZ* promoter region similar to the defective transposable element.
(A) Multiple alignment of partial sequences of tobacco enoyl-ACP reductase (enr-T2) and polygalacturonide gene (Npg1) showing similarity to the *WIZZ* promoter. (B) Inverted and direct repeats in the *WIZZ* promoter region. Five inverted repeats are indicated by arrow. Two direct repeats are indicated by different broken arrows, respectively. Several putative elements are boxed or underlined.

Promoter Analyses by Deletion Mutants of *KED* and *WIZZ*

It has previously been reported form transient reporter assays that wound-induced *WIZZ* transcript accumulation is regulated at the transcriptional level (Yagi, M., 1999). To confirm and extend the findings, further transient reporter assays were performed with *KED* and *WIZZ* promoter-LUC constructs and two or five deletion constructs, bombarded into tobacco leaves (Figures 12C, 13C).

Time course analyses with the longest constructs of each promoter showed contrasting results. Reporter activities from the 703-bp *KED* promoter-LUC could not be detected 3 to 6 hr after bombardment. On the other hand, those from the 1494-bp *WIZZ* promoter-LUC were shown to increase transiently reaching a maximal level at 4 to 5 hr after bombardment (Figure 16). Transient reporter activation reaching a maximal level 4 hr after wounding was also observed in leaves bombarded with a 926-bp *WIZZ* promoter-LUC construct followed by overnight incubation (data not shown).

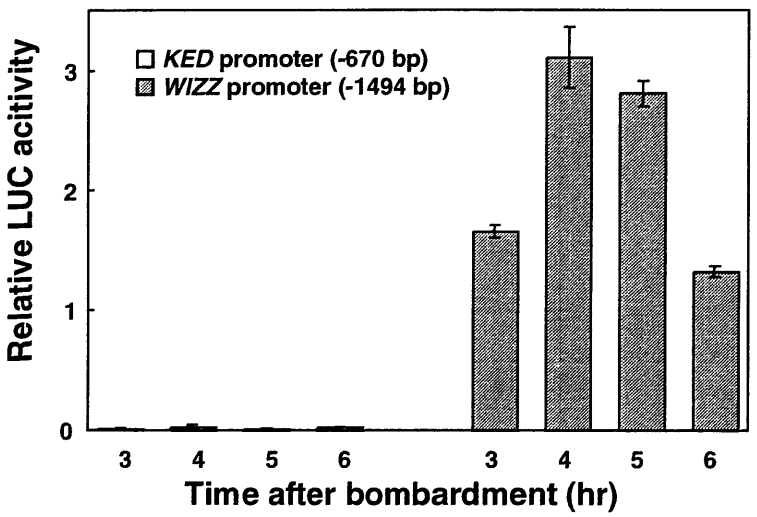

Figure 16. Time course analyses of *KED* and *WIZZ* promoter.
Tobacco leaf fragments were bombarded either 703-bp *KED* promoter-LUC or 1494-bp *WIZZ* promoter-LUC construct with a reference vector to monitor the transformation efficiency. Immediately after bombardment, the leaves were wounded by cutting. Several independent experiments were carried out. Vertical bars represent ±SE (n=3 to 6).

To determine regulatory sequences that may control wound-induced *WIZZ* transcription, six deletion constructs were introduced into tobacco leaves, and reporter activities were measured 5 hr after wounding (Figure 17). The reporter activities of 1494-bp through 177-bp *WIZZ* promoter-LUC constructs were reduced proportionally to their size. Similar results were obtained when these constructs were introduced into tobacco BY-2 cells after incubation for 16 hr (data not shown). Three deletion constructs of *KED* promoter-LUC were tested, but no activities were detected 5 hr after wounding (Figure 17).

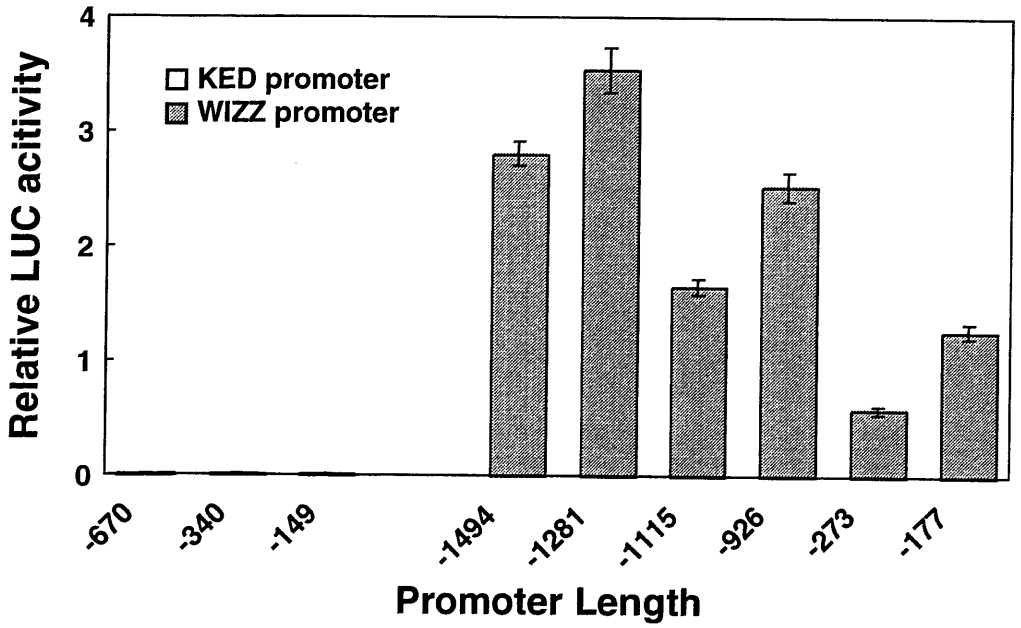

Figure 17. Deletion analyses of *KED* and *WIZZ* promoter.
Tobacco leaf fragments were bombarded either three *KED* promoter-LUC deletion constructs or six *WIZZ* promoter-LUC deletion construct with a reference vector to monitor the transformation efficiency. Immediately after bombardment, the leaves were wounded by cutting. Samples were harvested 5 hr after wounding. Several independent experiments were carried out. Vertical bars represent ±SE (n=3 to 6).

DISCUSSION

To date, a considerable number of wound-responsive genes and their promoters have been identified and characterized. By means of transient reporter assay and transgenic plant approaches, several elements in the promoters were found to be critical for wound-responses. Transcripts of most of these genes accumulated several hours to days after wounding and promoter analysis resulted in identification of secondary signals, including, for example, jasmonic acid, rather than wound-responsive elements themselves. In this sense, because of their quick response, *WIZZ* and *KED* serve as better model systems for elucidation of the mechanisms underlying wound responses.

Transient reporter assays of the *KED* promoter revealed that this region was not sufficient for the wound response. In contrast, transient reporter activation was observed with the *WIZZ* promoter, suggesting inclusion of wound responsive element(s). To elucidate the *cis*-acting elements important for wound-induced transcriptional activation of *WIZZ*, deletion analysis of the *WIZZ* promoter was carried out. However, the results showed no drastic reduction in wound responsiveness. When different sized *WIZZ* promoter regions of 1281-, 926-, and 177-bp were tested, reporter activity was greater with the shorter promoter size, indicating that *WIZZ* activation upon wounding is regulated by complicated mechanisms, with the essential elements residing within the 177-bp promoter region.

Sequence analyses of both promoters showed several consensus sequences (termed W1 to W5) and putative functional elements. The longest consensus sequence W1 contains two AAAG sequences, to which Dof-type transcription factors could bind. These are unique

to plants and are reported to be involved in expression of photosynthetic and seed-specific genes (Vicente-Carbajosa et al., 1997; Yanagisawa and Sheen, 1998). In addition, Dof transcription factors were recently reported to be induced in response to wound- and elicitor-treatment and to act as transcriptional activators in pea (Seki et al., 1999). The W2 sequence contains an ACGT motif, possibly recognized by bZIP-type transcription factors, which have been found to play critical roles in defense signaling (Zhang et al., 1999). The other sequences, W3, W4, and W5, with no similarity to any other known elements, remain to be explored.

Both promoter regions contain binding sites for myb- and myc-type transcription factors, reported to contribute to *Arabidopsis rd22* gene expression upon ABA- and drought-response (Abe et al., 1997; Urao et al., 1993). Two low-temperature responsive elements (LTRE) were found only in the *WIZZ* promoter. The presence of these motifs may suggest that *KED* and *WIZZ* are induced not only by wounding but also by water-stress caused by drought or low-temperature. This assumption is supported by an observation that KED is structurally similar to dehydrins, because of its biased amino acid composition, making them hydrophilic polypeptide.

W-boxes, which are fungal elicitor-responsive elements of the parsley PR-10 class gene and the recognized sequences by WIZZ, were found only in the *WIZZ* promoter. At present, the W-box with the TGAC core sequence is the only common element found in a large subset of pathogen-responsive gene promoters recognized by a WRKY protein family (Rushton and Somssich, 1998; discussion in Chapter II-2). Multiple TGAC containing sequences are frequently recognized by WRKY proteins, for example, BS65 by ZAP1 (de Pater et al., 1996), and Box 2 by ABFs (Rushton et al., 1995). Parsley WRKY1 binds to three

W-boxes in its own promoter, and this is necessary and sufficient for its gene activation, suggesting autoregulation (Eulgem et al., 1999). Three W-boxes were also found to be located nearby at positions -268 to -240 in the *WIZZ* promoter, indicating a autoregulatory loop like the case of WRKY1.

The *WIZZ* promoter contains a region homologous to a putative defective transposable element with several inverted and direct repeats. Similar regions were previously found in the third intron of the pollen-specific polygalacturonase gene, in the upstream region of the endochitinase gene from tobacco (Tebbutt and Lonsdale, 1993), and in other tobacco genes including those for enoyl-ACP reductase (accession number Y13861), the ubiquitin extension protein (AJ223329), and the ATPase β subunit (U96498). Transposable elements are believed to have contributed to genome evolution by changing structures and by providing *cis*-regulatory elements leading to changes in expression patterns of genes. For example, in the promoter of the asparagus defense gene *AoPR1*, several regions have been found to be similar to the sequence of the long terminal repeat promoter of tobacco retrotransposon *Tto1*, which is transcriptionally activated by jasmonic acid treatment or wounding (Takeda et al., 1998). Thus, the presence of a defective transposable element in *WIZZ* leads to the speculation that its wound-responsiveness may have resulted from genetic modification by transposable elements.

Chapter IV

Signal Transduction in Early Wound Response

The *KED* and *WIZZ* transcripts are rapidly increased and transiently accumulated on wounding. These responses occur not only locally but also systemically. Of particular interest is that systemic response occurs almost simultaneously with local response. This implies that the wound signal is immediately transmitted from the injured site to the whole plant. The similarity of expression patterns of these genes suggests a common regulatory system. Consequently, the question arises as to what are the signals and components leading to the activation of immediate-early wound-responsive genes. To address this question, I tested the effect of several chemicals, which are known to affect expression of wound-responsive genes, on expression of four rapid and transient wound-responsive genes (*KED*, *WIZZ*, *WIPK*, and *PHI-1*).

MATERIALS AND METHODS

Plant and Treatments

Tobacco plants were grown as described in Chapter I. Plants were wounded and/or treated by chemicals as described in the legend of Figure 18. The samples were harvested at an appropriate time point, immediately frozen in liquid nitrogen and stored at -80 °C.

The compounds tested were diluted to their final concentration from 100- or 1000-

fold concentrated stock solutions prepared as follows: α-linolenic acid (LNA) in water with 0.1% Triton, propionic acid (PA) (Na-salt) and 1,2-*bis*-(*o*-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid (BAPTA) in water, cycloheximide (CHX) and staurosporine (STAU) in DMSO (from Sigma), methyl jasmonate (MeJA) in N,N dimethyl-formamide, abscisic acid (ABA) in ethanol (from Wako Pure Chemicals Industries, Ltd.), A23187 in DMSO (from RBI) and okadaic acid in DMSO (from Life Technologies, Inc.).

RNA Blot Analysis

RNA blot analyses were performed as described in Chapter I. RNA blot containing 20 μg total RNA per lane was subjected to hybridization with ^{32}P-labeled cDNA probe.

RESULTS

Repeated Accumulation of *KED* and *WIZZ* Transcripts on Second Wounding

Pharmacological approaches have proved to be useful for characterization of components of signal transduction pathways in biological phenomena. To apply the same strategy to early wound signaling pathway in tobacco plants, a simple two-step system using leaf fragments with pre-incubation in a solution containing test chemical(s) was established. To investigate the effects of incubation on the early wound response, healthy tobacco leaves were cut into pieces and floated on a buffer solution (first step). Both *KED* and *WIZZ* transcripts were rapidly induced within 1 h and disappeared 2 h later. When these leaf pieces

were cut again 4 h after the first wounding (second step), both transcripts again accumulated (Figure 18A). The same experimental system was then adopted for further analyses (Figure 18B).

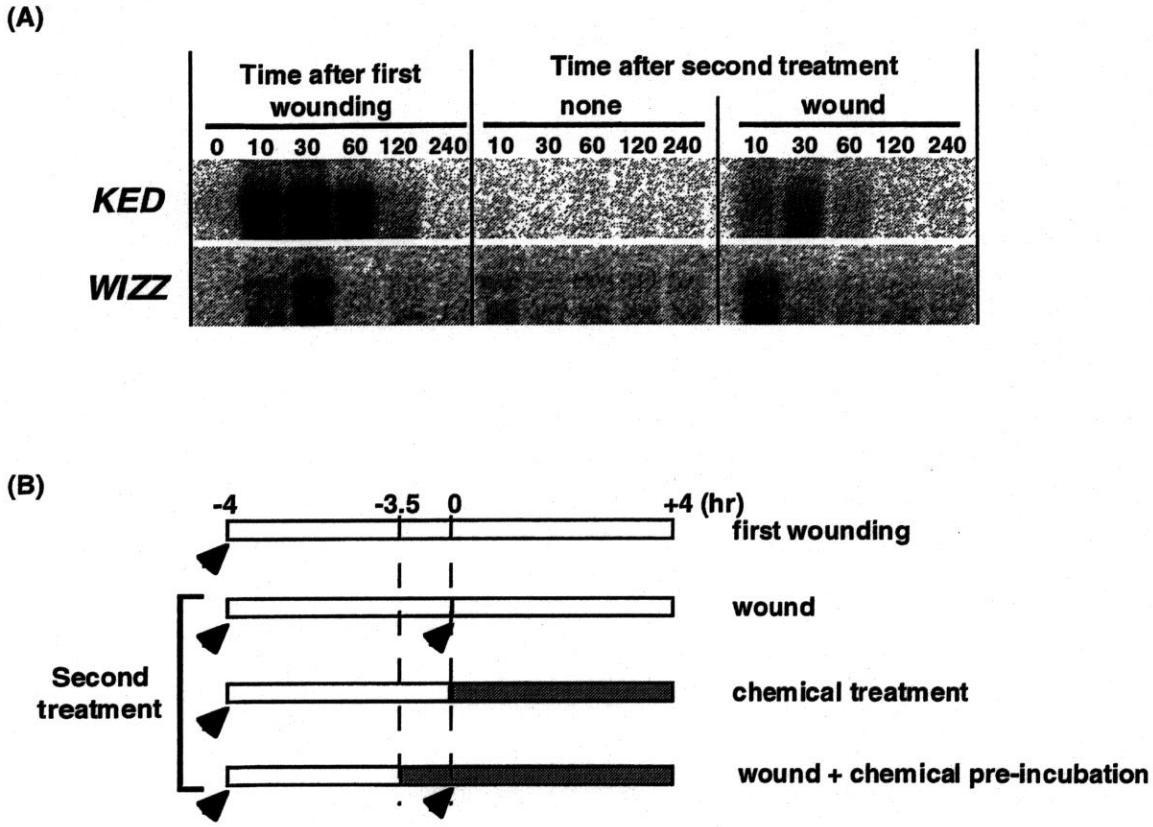

Figure 18. RNA blot analysis of immediate-early gene expression upon wounding.
(A) Effect of second wound treatment on *KED* and *WIZZ* expressions. (B) Schematic illustration of experimental system for wound induction and chemical treatments. Well-expanded leaves of wild-type tobacco plants were wounded by cutting into pieces with a pair of scissors, and quickly floated on a buffer solution (0.05% MES-KOH pH5.7). After 4-hr incubation, second treatment was performed by three ways as follows. The floated leaves were (i) wounded again (wound), (ii) transferred to a buffer solution containing chemical(s) (chemical treatment) or (iii) pre-incubated for 0.5 hr and wounded again (wound + chemical pre-incubation).

Response to MeJA, LNA, and ABA

Octadecanoids and abscisic acid (ABA) have been shown to be involved in induction of several wound-responsive genes. To investigate whether or not *KED* and *WIZZ* transcript accumulations are induced by jasmonic acid methyl ester (MeJA), linolenic acid (LNA) or ABA, RNA blot analysis was performed (Figure 19). Instead of the second wounding, leaf pieces were transferred to a buffer solution containing MeJA, LNA, or ABA 4 h after the first wounding. Essentially, no accumulation of *KED* and *WIZZ* transcripts was observed.

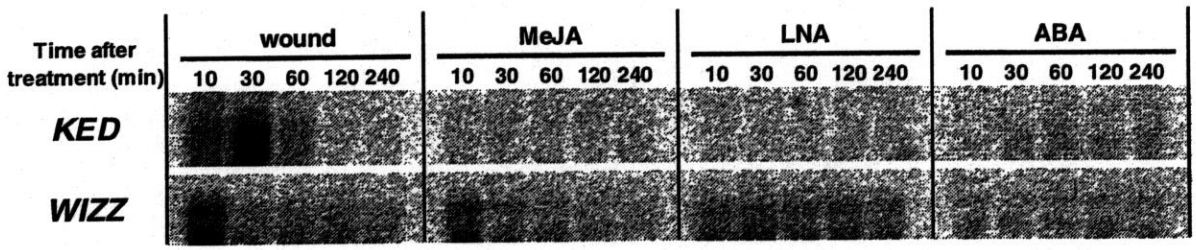

Figure 19. Response to MeJA, LNA and ABA.
Effect of chemical treatment on *KED* and *WIZZ* transcript accumulation. After 4 hr from the first wounding, leaf pieces were transferred to the buffer solution containing 50 μM methyl jasmonate (MeJA), 50 μM linolenic acid (LNA), or 100 μM abscisic acid (ABA).

Effects of Inhibitors of Protein Synthesis, Protein Kinases and Protein Phosphatases

To investigate whether or not *de novo* protein synthesis and protein phosphorylation/dephosphorylation are involved in *KED* and *WIZZ* transcript accumulation by wounding, three kind of inhibitors, cycloheximide (CHX), staurosporine (STAU) and okadaic acid (OKA), were used for pre-incubation. High-levels of transcript accumulation of both *KED* and *WIZZ* for up to 4 h were induced by CHX treatment alone (Figure 20A). Transcript accumulation of *KED* upon wounding was not affected by either STAU or OKA.

That of *WIZZ* was inhibited and enhanced by STAU and OKA, respectively. Effects of OKA treatment on *PHI-1* and *WIPK* expression were also studied (Figure 20B). Wound-inducibility of *PHI-1* transcript accumulation was not affected like *KED*. That of *WIPK* was enhanced like *WIZZ*. Accumulation of both *PHI-1* and *WIPK* transcripts was also induced by CHX-treatment alone (Figure 21B).

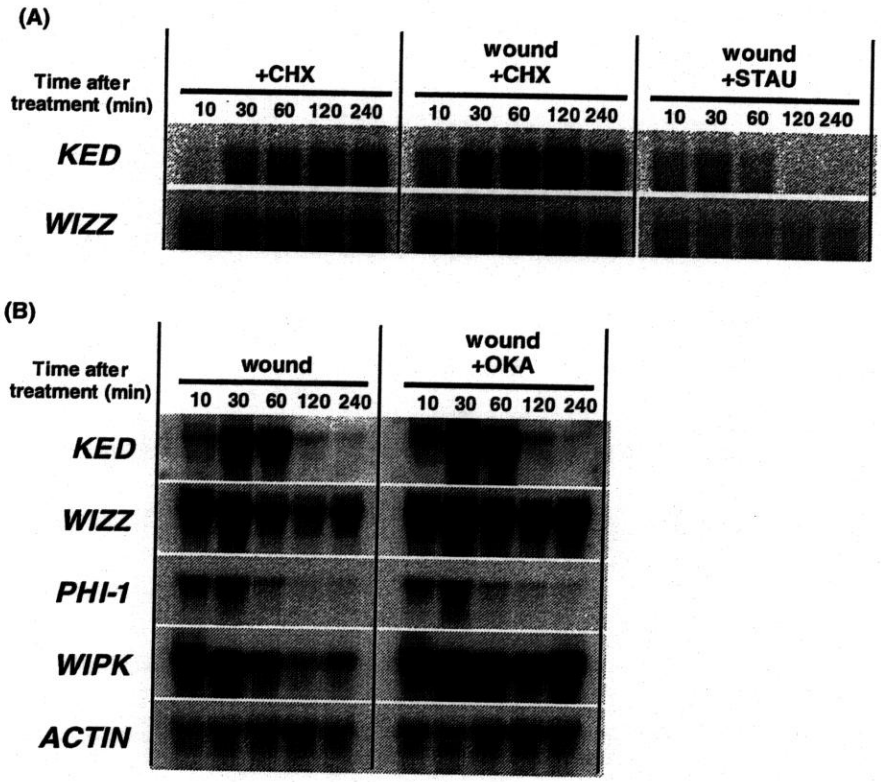

Figure 20. Effects of CHX, STAU, and OKA on immediate-early gene expression upon wounding.
(A) Effects of cycloheximide and staurosporine. (B) Effects of okadaic acid. After 3.5-hr incubation from the first wounding, leaf pieces were transferred to the buffer solution containing 50 μM cycloheximide (CHX), 1 μM staurosporine (STAU) or 0.1 μM okadaic acid (OKA). After further 0.5-hr pre-incubation, the floated leaves were wounded again.

Effects of NADPH-Oxidase Inhibitors

The oxidative burst has been reported to be involved in systemic wound responses in many plants (Orozco-Cardenas and Ryan, 1999), and to be induced by CHX treatment in soybean cell suspension culture (Tenhaken and Rübel, 1998). To investigate whether or not it contributes to rapid transcript accumulation of four genes, diphenylene iodonium chloride (DPI), an inhibitor of NADPH oxidase (Cross and Jones, 1986; Auh and Murphy, 1995), was used for pre-incubation before wounding (Figure 21). The results showed no obvious effect on transcript accumulation induced by wounding or CHX-treatment.

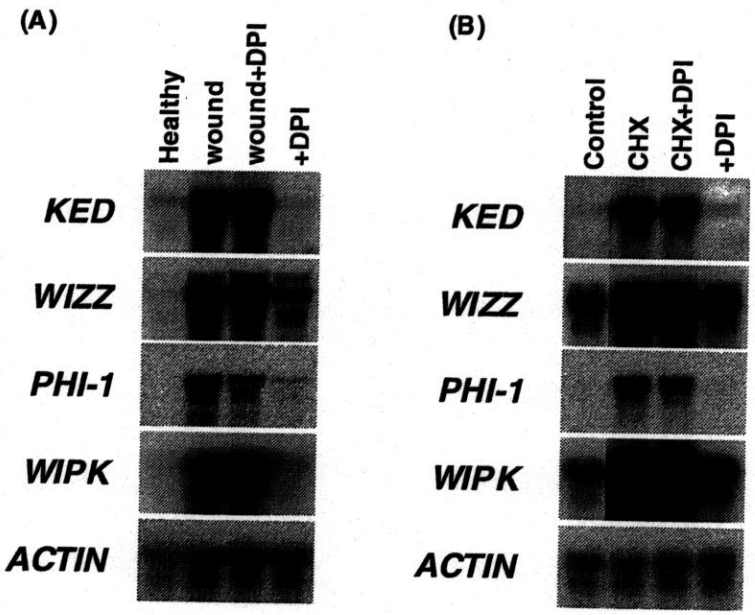

Figure 21. Effects of DPI on immediate-early gene expression upon wounding and CHX-treatment.
(A) Effects of DPI on wound-induction. After 3.5-hr incubation from the first wounding, leaf pieces were transferred to the buffer solution in the presence or absence of 20 μM diphenylene iodonium chloride (DPI). After further 0.5-hr pre-incubation, the floated leaves were treated or untreated with the second wounding, and harvested 0.5 hr thereafter. (B) Effects of DPI on CHX-induction. After 3.5-hr incubation from the first wounding, leaf pieces were transferred to the buffer solution in the presence or absence of 50 μM DPI ("+DPI" or "Control"). After further 0.5-hr pre-incubation, samples were treated or untreated with 50 μM CHX, and harvested 1 hr later.

Calcium Signaling and Immediate-Early Transcript Accumulation upon Wounding

Recent work on systemin, which is an 18-amino acid polypeptide from tomato plants believed to act as a systemic wound signal, showed that it triggers an increase of intracellular calcium in tomato mesophyll cells and rapidly and transiently induces calmodulin (CaM) gene expression (Moyen et al., 1998; Bergey and Ryan, 1999). These findings suggest that intracellular calcium is a part of signal transduction that contributes to the early wound response in tomato plants. To determine the relationship between wound responses and increase of intracellular calcium, the effects of calcium chelator 1,2-*bis*-(*o*-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid (BAPTA), calcium channel blocker La^{3+}, and ionophore A23187 on the immediate-early wound-responsive gene expression were assessed. The transcript patterns and levels were not significantly affected by pre-incubation of BAPTA and La^{3+} (Figure 22A, B). When leaf pieces were treated in combination with ionophore and BAPTA, accumulation of transcripts was not observed (Figure 22C).

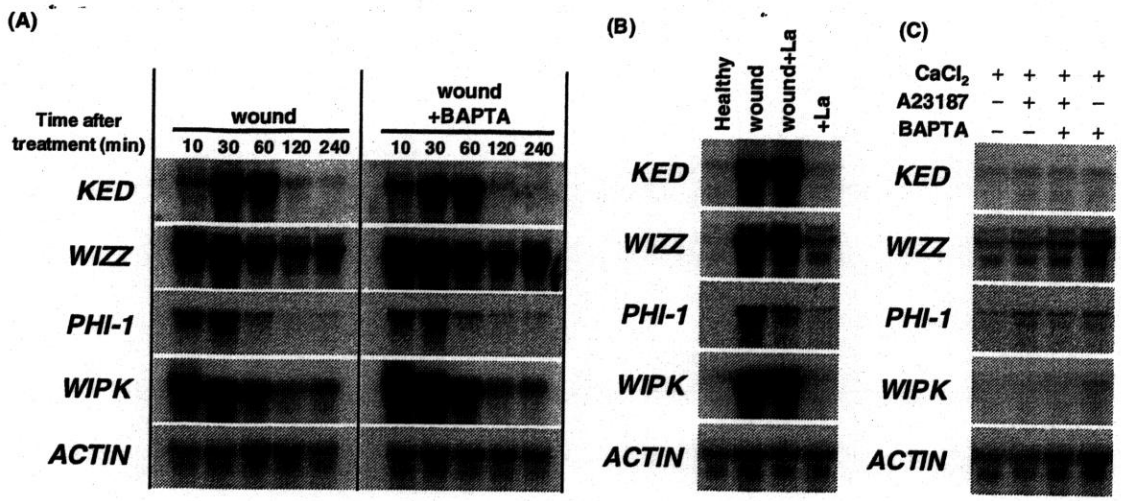

Figure 22. Calcium signaling and immediate-early gene expression upon wounding.
(A) Effects of BAPTA. After 3.5-hr incubation from the first wounding, leaf pieces were transferred to the buffer solution in the presence (+BAPTA) or absence of 5 mM BAPTA. After further 0.5-hr pre-incubation, the floated leaves were wounded again. (B) Effects of La^{3+}. After 3.5-hr incubation from the first wounding, leaf pieces were transferred to the buffer solution in the presence (+La) or absence of 1 mM $LaCl_3$. After further 0.5-hr pre-incubation, the floated leaves were wounded again, and were harvested 0.5 hr after the second wound treatment. Healthy untreated leaves were compared as control (Healthy). (C) Effects of calcium ionophore. After 4-hr incubation from the first wounding, leaf pieces were transferred to the buffer solution in combination with 5 mM $CaCl_2$, 50 μM A23187, and 5 mM BAPTA. The floated leaves were harvested 0.5 hr after the second treatment.

Effects of H^+-ATPase Inhibition and Activation

Regulation of intracellular pH has been proposed to be involved in defense signaling, mainly by modulating plasma membrane H^+-ATPase activity (Roberts and Bowles, 1999; Schaller and Oecking, 1999). The activity of H^+-ATPase is inhibited by vanadate, dicyclohexylcarbodiimide, diethylstylbestrol, and erythrosin B (EB), and is activated by fungal toxin, fusicoccin (FC) (Michelet and Boutry, 1995; Chung et al., 1999). Effects of EB and FC on early wound response were tested with the pre-incubation method. Transcript accumulation with all four marker genes upon wounding was not affected (Figure 23). However, EB alone increased the transcript levels of *KED* and *PHI-1* after 40-min pre-

incubation and those of *WIZZ* and *WIPK* after 2-hr pre-incubation (Figure 23A). Such transcript accumulation for *KED*, *WIZZ*, and *WIPK* with EB treatment was also observed in tobacco BY-2 cells (data not shown). FC alone also increased the transcript levels of all four genes within 40 min. The effect was sustained only for *PHI-1* transcript accumulation (Figure 23B). These results suggest that cellular pH change may play an important role in the immediate-early transcript accumulation.

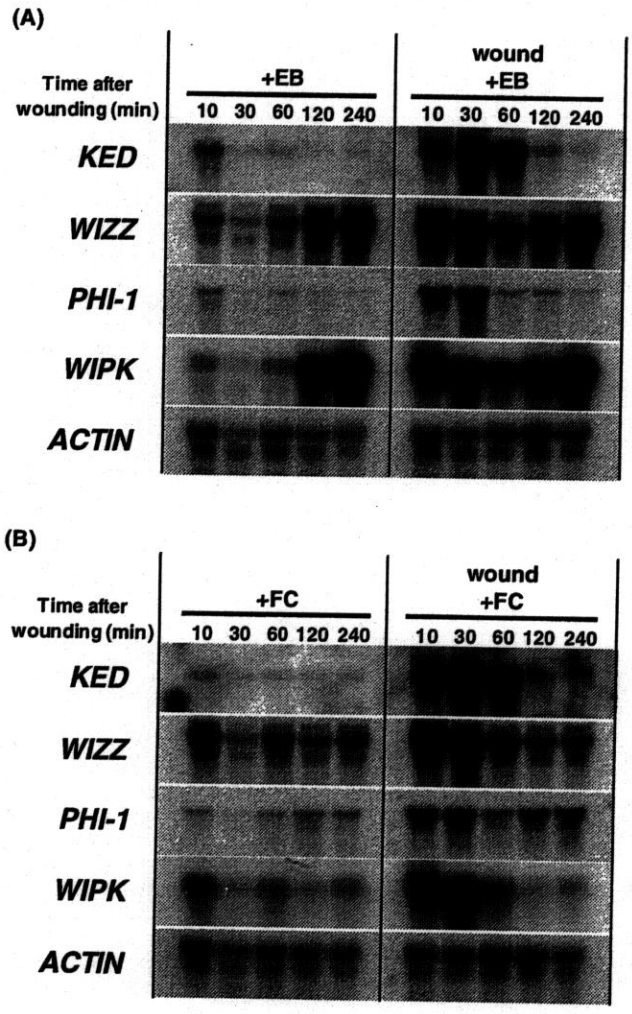

Figure 23. Effects of the inhibitor and activator of H^+-ATPase on immediate-early gene expression upon wounding.
(A) Effects of erythrosin B. (B) Effects of fusicoccin. After 3.5-hr incubation from the first wounding, leaf pieces were transferred to the buffer solution containing 50 μM erythrosin B (EB) or 1 μM fusicoccin (FC). After further 0.5-hr pre-incubation, the floated leaves were wounded again.

Effects of Cytosolic Acidification

To test whether cytosolic pH change itself induces transcript accumulation of immediate-early responsive genes, effects of the weak organic acid, propionic acid (PA), were examined (Figure 24A). Transcripts of *WIZZ*, *WIPK*, and *PHI-1* were accumulated 4h after treatment. To investigate the effects of mechanical stress and rapid change of cytosolic pH, plants were vacuum-treated in the presence or absence of PA (Figure 24B). Although vacuum infiltration itself induced two-step, fast and late, transcript accumulation of all tested genes, PA obviously raised transcript levels of four genes.

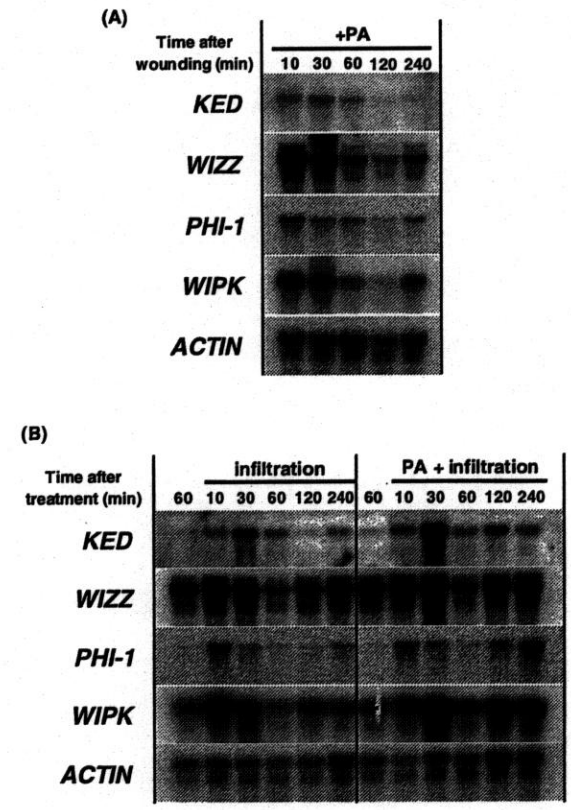

Figure 24. Effects of cytosolic acidification on immediate-early gene expression.
(A) Effects of propionic acid (PA). After 4-hr incubation from the first wounding, leaf pieces were transferred to the buffer solution with 5 mM PA. (B) Effects of mechanical stress and acid loading. After 4-hr incubation from the first wounding, leaf pieces were transferred to the buffer solution in the presence or absence of 10 mM PA, and were subjected to vacuum infiltration. Controls were untreated samples.

DISCUSSION

General Remarks

It is difficult to dissect the molecular processes of early stage wound responses because of the complexity of the multiple responses that occur simultaneously, including leakage of electrolytes, and collapse of intracellular compartmentation. To our knowledge there are no compounds which elicit a wound response in tobacco plants, such as for example, the systemin used in studies of tomato wound responses and elicitors for pathogen responses. This disadvantage makes cell suspension culture systems unsuitable for wound analysis. In order to specify early wound response inducing factor(s), a simple experimental system for tobacco plants adopting pharmacological assays, in which expression of the immediate-early wound-responsive genes could be easily monitored, was therefore established. This chapter describes the results of such experiments, cytosolic pH change and protein phosphorylation being found to play key roles in the early wound response. This conclusion was also supported by further analyses with other compounds such as lactacystin that inhibits proteasome activity, or indomethacin that inhibits activity of phospholipase A_2 and cyclooxygenase (León et al, 1998; Ito et al, 1999).

Octadecanoids and ABA

Accumulation of *KED* and *WIZZ* transcripts was not induced by MeJA or its precursor LNA, or by ABA, so that direct mediation through octadecanoids or ABA is unlikely. In *Arabidopsis*, several wound-responsive genes identified by the differential display

method have been classified into JA-dependent and JA-independent groups (Titarenko et al., 1997). For example, the choline kinase gene is rapidly induced by wounding but not by JA or ABA. In tomato plants, a wound- and pathogen-responsive gene, *Twi1*, is also rapidly induced by wounding but is independent of ethylene and salicylic acid. Neither JA nor systemin induce *Twi1* expression (O'Donnell et al., 1998). These observations suggest that a novel transduction pathway which is distinct from JA- and ABA-pathways may be involved in immediate-early activation upon wounding.

Effects of CHX

To investigate whether or not *de novo* protein synthesis is necessary for immediate-early transcript activation, the effects of the protein synthesis inhibitor, CHX, were examined. The results showed no inhibition of *KED* and *WIZZ* transcript accumulation, clearly indicating that nascent protein synthesis is not required for activation of either gene. However, CHX treatment alone induced and sustained accumulation of transcripts of all four tested genes. Similar phenomena have been observed with many genes, including mammalian oncogenes and early auxin-, salicylic acid- and low temperature-responsive genes (Herschman, 1991; Abel and Theologis, 1996; Horvath and Chua, 1996; Berberich and Kusano, 1997). Accumulation of transcripts for ethylene-responsive transcription factors (ERFs) from tobacco was induced by either wounding or CHX treatment (Suzuki et al., 1998). Three possible mechanisms for this CHX action were proposed: first, direct activation of transcription by CHX; second, indirect activation of transcription because production of short-lived transcriptional repressors was inhibited; and third, enhancement of mRNA stability due

to suppressed RNase production (Koshiba et al., 1995).

Protein Phosphorylation/Protein Dephosphorylation

Protein phosphorylation is one of the earliest responses of tobacco cells upon wounding (Seo et al., 1995; Usami et al., 1995) and two mitogen-activated protein (MAP) kinases, WIPK (wound-induced protein kinase) and SIPK (SA-induced protein kinase), have been shown to be rapidly and transiently activated (Zhang and Klessig, 1998a, Seo et al., 1999). TMV-infection induces both WIPK and SIPK enzymatic activity, and also *WIPK* transcript accumulation (Zhang and Klessig, 1998b). In transgenic tobacco plants overexpressing the tomato *Cf-9* resistance (R) gene, two protein kinases, possibly WIPK and SIPK, were found to be rapidly activated in a gene-for-gene manner within 2 to 5 min after applying pathogen-derived avirulence gene product Avr9 (Romeis et al, 1999). This was inhibited by staurosporine, but not DPI treatment. Furthermore, in the *Cf-9* overexpressing transgenic plants, *WIPK* transcript accumulation was induced by Avr9 treatment. These results indicate that wound signaling and R/Avr-dependent signaling are interlinked at the MAP kinase cascade leading to *WIPK* transcript activation. The effects of okadaic acid or DPI on *WIPK* transcript level upon wounding are in accord with these reports. The pattern of *WIZZ* transcript accumulation is similar to that of *WIPK*, suggesting that both genes are regulated in a common system during the early wound response. Phosphatase inhibition did not affect the transcript levels of two other genes, *KED* and *PHI-1*, indicating that phosphatase activity is not required for down-regulation of these genes to basal level. However, the necessity of protein kinase activity for their wounding-induction remains to be

explained.

Oxidative Burst and Calcium Signaling

To investigate whether or not the oxidative burst and calcium signaling are involved in the immediate-early transcript accumulation, the effects of several reagents were examined. The results showed that transcript accumulation of all genes tested was not mediated by either the oxidative burst produced by NADPH-oxidase or calcium influx through the plasma membrane. In soybean cells, medium alkalinization by CHX treatment was followed by DPI-sensitive H_2O_2 production, indicating that pH change is faster than oxidative burst (Tenhaken and Rübel, 1998). It is known that variation of the concentration, and localization of cytosolic Ca^{2+} is important for differential responses to many stimuli in animal cells (Berridge et al., 1998). In plant cells, cytosolic Ca^{2+} has been reported to be elevated in response to many environmental stimuli such as red light, drought, touch, cold, and elicitors. (Sanders et al., 1999). Involvement of Ca^{2+} from different sources such as intracellular stores in immediate-early transcript accumulation cannot be ruled out.

Cytosolic pH

In animal systems, cytosolic pH plays an important role in cellular homeostasis. For example, cytosolic acidification by growth-factors results in cell proliferation, immediate-early gene expression, and protein phosphorylation (Isfort et al., 1993). In tobacco plants, there is increasing evidence that modulation of cytosolic pH is involved in the early stages of defense response. Alkalinization of cell culture medium is rapidly, transiently and repeatedly

induced by oligogalacturonides, which stimulate wound-responsive genes (Mathieu et al., 1998; Spiro et al., 1998). Rapid and prolonged medium alkalinization has been observed in tobacco cells treated by various elicitors (Bourque et al., 1998; Zhang et al., 1998). Elicitor treatment induces cytosolic acidification, and also defense-related gene expression (Mathieu et al, 1996; Lapous et al, 1998). Drop in intracellular pH was also found to activate MAP kinases rapidly and transiently (Tena and Renaudin, 1998). The current experiments revealed that cytosolic pH modification by erythrosin B, fusicoccin, and propionic acid indeed induced immediate-early transcript accumulation, suggesting that this is one of the factors in induction of early wound responses.

The proton-pump ATPase of the plant plasma membrane is involved in many physiological functions, including the uptake and efflux of ions and metabolites, loosening of the cell wall, stomata movement, and intracellular pH regulation (Michelet and Boutry, 1995; Sze et al, 1999). The activity of H^+-ATPase is inhibited and activated by erythrosin B and fusicoccin, respectively. However, neither of these was here found to affect wound-induced transcript accumulation of the tested genes. These results suggest that plasma membrane H^+-ATPase is not a major factor controlling the early wound response.

Propionic acid induced cytosolic acidification rapidly and strongly, which presumably was sustained for a prolonged time period because of the high concentration applied. Continuous erythrosin B treatment may gradually reduce cytosolic pH and a number of experiments have suggested that the intensity and duration of pH change could be related to the mode of transcript accumulation of certain genes. For examples, rapid and transient pH change was induced within 2 hr by oligogalacturonides, while rapid and sustained pH change

was observed over several hours in elicitor-treated cells (Mathieu et al., 1996; Mathieu et al., 1998; Spiro et al., 1998; Bourque et al, 1998; Zhang et al., 1998). These observations indicate that wounding and similar stimulation might cause an initial rapid pH change that might switch on the defense response if maintained for a extended period.

Fusicoccin was recently reported to increase not only transcripts of PR-genes but also the salicylic acid level in tomato cells (Robert and Bowles, 1999; Schaller and Oecking, 1999), indicating that other effects caused by modulation of H^+-ATPase activity must be taken into consideration. Transcripts of *PHI-1* were originally reported to accumulate rapidly on phosphate treatment (Sano et al., 1999). In a cell suspension of *Catharanthus roseus*, phosphates induced transient cytosolic acidification (Sakano et al., 1992), suggesting that *PHI-1* gene expression may be regulated by phosphate and/or proton levels. It is likely that delayed accumulation of *PHI-1* transcripts by fusicoccin treatment is due to increased salicylic acid and/or phosphate produced by activated ATPase.

On the basis of the present findings and the literature, I propose that pH changes in the cellular microenvironment caused by wounding trigger the transcript accumulation of immediate-early wound-responsive genes. To date, however, there is no clear evidence of such an association between cytosolic pH change and wounding. It is to be hoped that experimental evidence can be obtained using the recently developed caged proton for pH modulation and fluorescent pH indicators for direct measurement of cytosolic pH.

Concluding Remarks

In order to understand molecular events that take place during the early stage of wound response, I performed following experiments. (1) Identification of early wound-responsive genes by FDD screening; (2) Investigation of function of immediate-early wound-responsive gene products; (3) Characterization of promoters of representative two genes; (4) Pharmacological analysis of signals and components involved in the immediate-early wound response. In consequence, several clues were obtained to gain a better understanding of the early wound response. (1) Nearly 200 genes are differentially expressed within 3 hr after wounding; (2) Transcripts of a set of genes are rapidly and transiently accumulated; (3) Among them, *A7* and *C10* encode a highly charged protein KED and a new WRKY transcription factor WIZZ, respectively; (4) *WIZZ* is rapidly and transiently activated at the transcriptional level; (5) Protein phosphorylation/dephosphorylation and cytosolic pH change are involved in transcript accumulation of the immediate-early wound responsive genes.

Taken together, I propose the following hypothetical model. Upon wounding, cytosolic pH changes quickly. This pH change activates two signaling pathways, MAP kinase cascade and so far uncharacterized one. These lead to immediate-early gene expression followed by defense response and/or wound healing (Figure 25). A rapid cellular pH change is assumed to be one of the earliest wound-signals and probably affects membrane potential, providing rapid systemic signal(s). The pH change may activate signal transduction pathways, which is either dependent or independent on protein phosphorylation. The MAP kinase cascade, including WIPK and SIPK, is activated and induce *WIZZ* and *WIPK* transcript

accumulation. WIZZ binds TTGAC(C/T) sequence found in defense-related gene promoters and modulates their expression. Several hours after wounding, defense response such as cell wall reinforcement and interference of digestion by herbivores is developed by up-regulation of many defense-related genes including *A8*, *C14*, *C20*, and *PI-II*. Factor(s) in other pathway, which is not mediated by protein phosphorylation/dephosphorylation, is unknown. KED and PHI-1 are postulated to be involved in cellular homeostasis and phosphorylation, respectively. Although further studies of uncharacterized other immediate-early wound-responsive genes will provide more information about early wound response, current study clearly adds a novel information to the molecular feature of early wound response in higher plants.

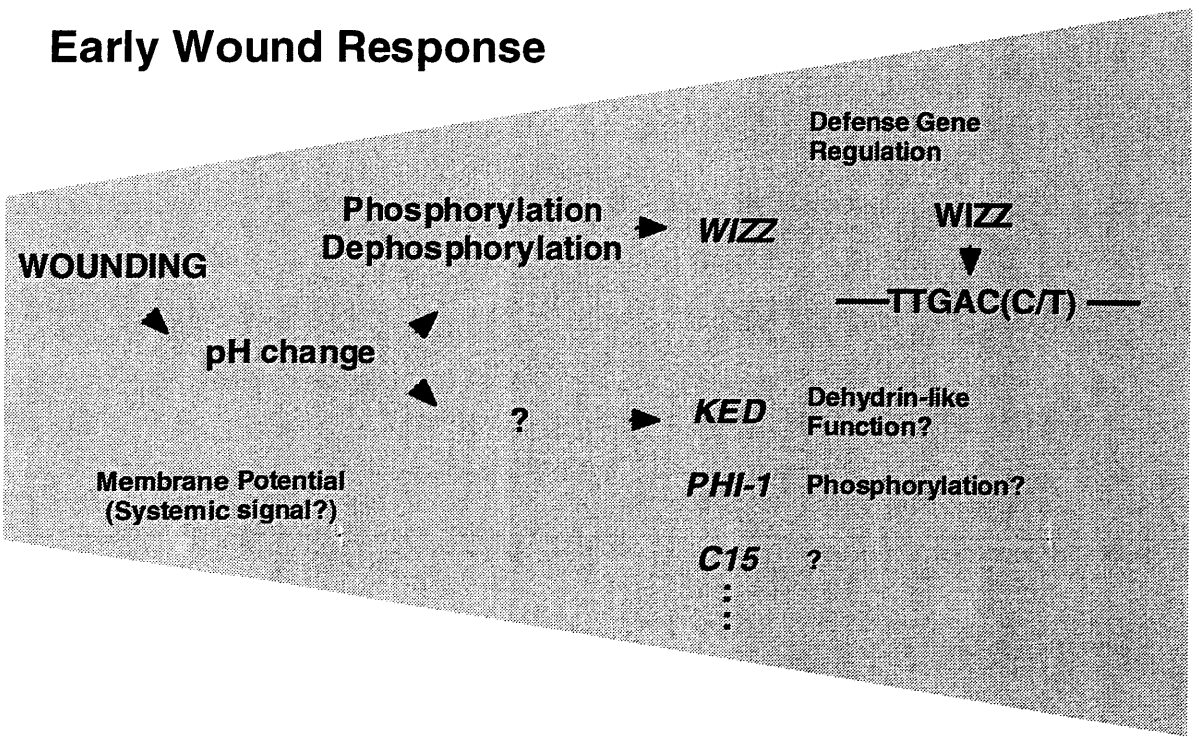

Figure 25. Model for molecular events during early stage of wound response.

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Hara, K., Yagi, M., Koizumi, N., Kusano, T., and Sano, H. (2000). Screening of wound-responsive genes identifies an immediate-early expressed gene encoding a highly charged protein in mechanically wounded tobacco plants. Plant Cell Physiol. (in press).

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