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Screening of Wound-Responsive Genes Identifies an Immediate-Early Expressed Gene Encoding a Highly Charged Protein in Mechanically Wounded Tobacco Plants

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In order to identify genes that are temporally and spatially regulated during wound response, a cDNA population in mechanically wounded tobacco leaves was screened by the fluorescence differential display method. Of 28 clones initially identified to have altered levels of transcripts within 3 h of wounding, eight were characterized. Although each clone showed a unique pattern of transcript accumulation, one distinct clone was further characterized because of its immediate-early response. Its transcripts began to accumulate 10 min after wounding, reached a maximum level within 1 h and disappeared after 2 h. The response, which occurred repeatably and systemically, was observed by the treatment with propionic acid or erythrosin B, indicating that cytosolic acidification could be one of the signals for immediate-early response of this gene. The cDNA encodes a polypeptide of 513 amino acids with a relative molecular mass of 60,952. The putative polypeptide is rich in lysine (K), glutamic acid (E) and aspartic acid (D), which constitute up to 70% of total amino acids, and was therefore designated as KED. The KED polypeptide is composed of a highly hydrophilic N-terminal region and a relatively hydrophobic C-terminal region, suggesting that KED may function through electrostatic interactions with cellular components.

Key words: Charged protein — Fluorescence differential display — *Nicotiana tabacum* — Systemic response — Wounding.

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, including genes encoding extensins for reinforcement of the cell wall, PAL and CHS for phenylpropanoid synthesis, ACC synthase and ACC oxidase for ethylene synthesis and proteinase inhibitors (PI) against insect feeding (Green and Ryan 1972, Corbin et al. 1987, Kende 1993). Since PI transcripts accumulate locally and systemically after wounding, PI genes have been widely used as markers for wound signal transduction pathways.

These observations indicate that, in the plant kingdom, diverse defense networks may function as a combination of different signals with a set of defense-related genes. However, in order to clarify the general aspects of the early response to wounding, it is essential to understand the relationship between wound signals and the temporal and spatial expression of wound-responsive genes. To this end, we have employed the fluorescence differential display (FDD) method, which allows the time course of transcript accumulation of a set of genes to be easily and simultaneously displayed. In this report, we describe the identification of eight wound-responsive genes and have studied the properties of one of them because of its early temporal response to wounding.

Abbreviations: CHX, cycloheximide; FDD, fluorescence differential display; JA, jasmonic acid; LNA, α-linolenic acid; MeJA, jasmonic acid methyl ester.

The nucleotide sequences reported in this paper have been submitted to the GenBank, EMBL, DDBJ under accession numbers AB009881 (A1 cDNA), AB009882 (A5), AB009883 (*KED* cDNA), AB009884 (A8), AB009885 (C13 cDNA), AB009886 (C14), AB009887 (C19) and AB009888 (C20 cDNA).

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Materials and Methods

Plant material 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 mature leaves of approximately 2-month-old healthy plants into pieces with a pair of scissors. To investigate systemic response, intact leaves of healthy plants were cut across the midrib with a pair of scissors. The resulting leaf fragments were floated on a buffer solution (0.05% 2-(*N*-morpholino)ethanesulfonic acid (MES)-KOH buffer, pH 5.7) and replaced to the buffer solution with chemical compounds. The compounds tested were diluted to their final concentration from 100- or 1,000-fold concentrated stock solutions prepared as follows: α-linolenic acid (LNA) in water with 0.1% Triton X-100, propionic acid (PA) and 1,2-*bis*-(*o*-aminophenoxy)ethane *N,N,N′,N′*-tetraacetic acid (BAPTA) in water, CHX and staurosporine (STAU) in DMSO (from Sigma), MeJA in *N,N* dimethyl-formamide, abscisic acid (ABA) in ethanol (from Wako Pure Chemicals Industries, Ltd.) and okadaic acid in DMSO (from Life Technologies, Inc.). The samples were harvested at an appropriate time point, immediately frozen in liquid nitrogen and stored at −80°C.

Fluorescence differential display—The fluorescence differential display (FDD) method was performed according to the described method (Ito et al. 1994, Ito and Sakaki 1996) with a modification. 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., U.S.A.). 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 s, 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 insert co-migrated at the same position as the original wound-responsive products was 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 hybridization analysis—Total RNA was isolated by the ATA method (Gonzalez et al. 1980), fractionated on 1% formaldehyde 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 × Denhardt's, 3 × SSC, 50% formamide, 10% dextran sulfate, 0.1 mg ml^{-1} denatured salmon sperm. The membrane was washed with 0.5 × 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 cDNAs for *WIPK* and *DIN1* were provided by Y. Ohashi and A. Watanabe, respectively. The cDNAs for *PI-II, ODC* 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′-GAGCAAAGCAACACGGATACAAC-3′ for A1, 5′-CATTACTCCATCGTGTGCCTTCAGGTC-3′ for A7, 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—Total RNA samples were extracted from tobacco leaves harvested 0, 15, 45, 90 and 180 minutes after wounding, and cDNAs were synthesized. Using two fluorescence-labeled anchored primers in combination with 32 arbitrary primers, 64 independent FDD-PCRs were performed. From the differences in displayed patterns, 28 cDNA fragments were initially found to change their levels within 3 h after wound treatment (data not shown). Among them, eight cDNA fragments were cloned, sequenced and further characterized by RNA gel blot analysis (Fig. 1). Full length cDNAs of several clones (A1, A7, C13 and C20) were obtained by 5′-RACE, and their sequences were determined. Results of sequence analyses are summarized in Table 1.

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. The level of A5 transcripts gradually decreased within 6 h of wounding and remained at a low level thereafter. A7 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. The A7 cDNA encodes a 513-amino-acid protein which shows no similarity to any gene product so far reported. Because of its early temporal response to wounding, A7 was further characterized in the present study. 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 (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

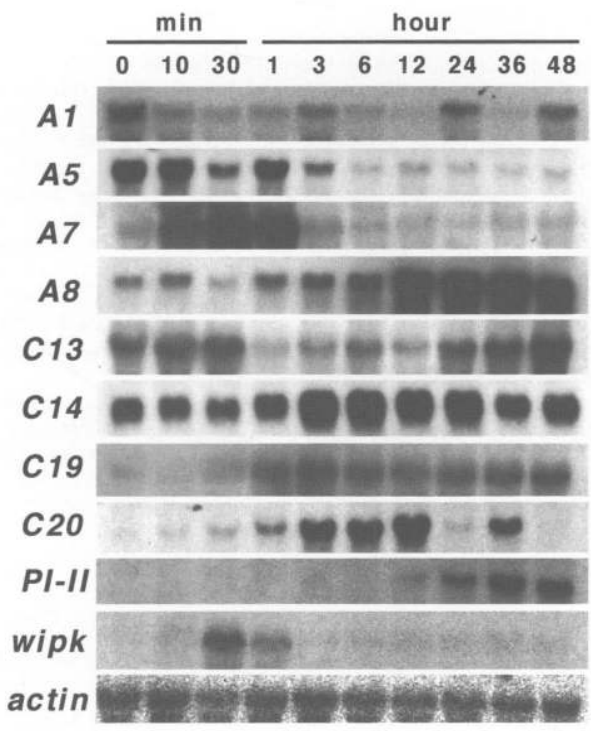

Fig. 1 Time course analyses of accumulation of transcripts of wound-responsive genes. Prior to experiments, 2-month-old tobacco plants were transfered into a controlled environmental room at 23°C under continuous light for a week. Healthy leaves were detached and wounded by cutting into pieces with a pair of scissors, and floated on water. The wounded leaves were harvested at the indicated time intervals. An RNA blot containing 35 μg of RNA per lane was subjected to hybridization with the indicated probes.

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 (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) (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 (accession numbers X52395, Y11553, L12245, X80342 and L12245).

Highly charged protein encoded by KED—A nearly full-length cDNA of A7 was obtained and its putative amino acid sequence was determined (Fig. 2A). The deduced polypeptide was rich in lysine (K; 34.7%), glutamic acid (E; 25.0%) and aspartic acid (D; 12.5%), which constituted up to 70% of total amino acids. This amino acid composition makes the product of the A7 gene, which was designated as *KED*, 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) (Fig. 2B). Another distinct feature of the KED polypeptide is that acidic amino acids form small clusters, and so do basic amino acids. These clusters alternate (Fig. 2C).

Properties of KED—Southern hybridization indicated that one copy, or at most two copies, of *KED* is present in the genome of *N. tabacum* (Fig. 3A). *KED* transcripts were rapidly accumulated in wounded leaves of *Nicotiana syl-*

Table 1 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*[i]
A5	219[b]	nd	nd	No significant similarity found
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*[j]
C13	247	1,540[e]	317	No significant similarity found
C14	286[f]	nd	nd	57% Identity with proteinase inhibitor II of *L. esculentum*[k]
C19	708[g]	nd	nd	90% Identity with chloroplast carbonic anhydrase of *N. tabacum*[l]
C20	408	844[h]	205	44% Identity with 21 kDa protein precursor of *M. sativa*[m]

[a–m] Accession number: [a]A1 AB009881, [b]A5 AB009882, [c]A7 AB009883, [d]A8 AB009884, [e]C13 AB009885, [f]C14 AB009886, [g]C19 AB009887, [h]C20 AB009888, [i]U32511, [j]L13439, [k]X94946, [l]L19255, [m]Y11553.
nd: not determined.

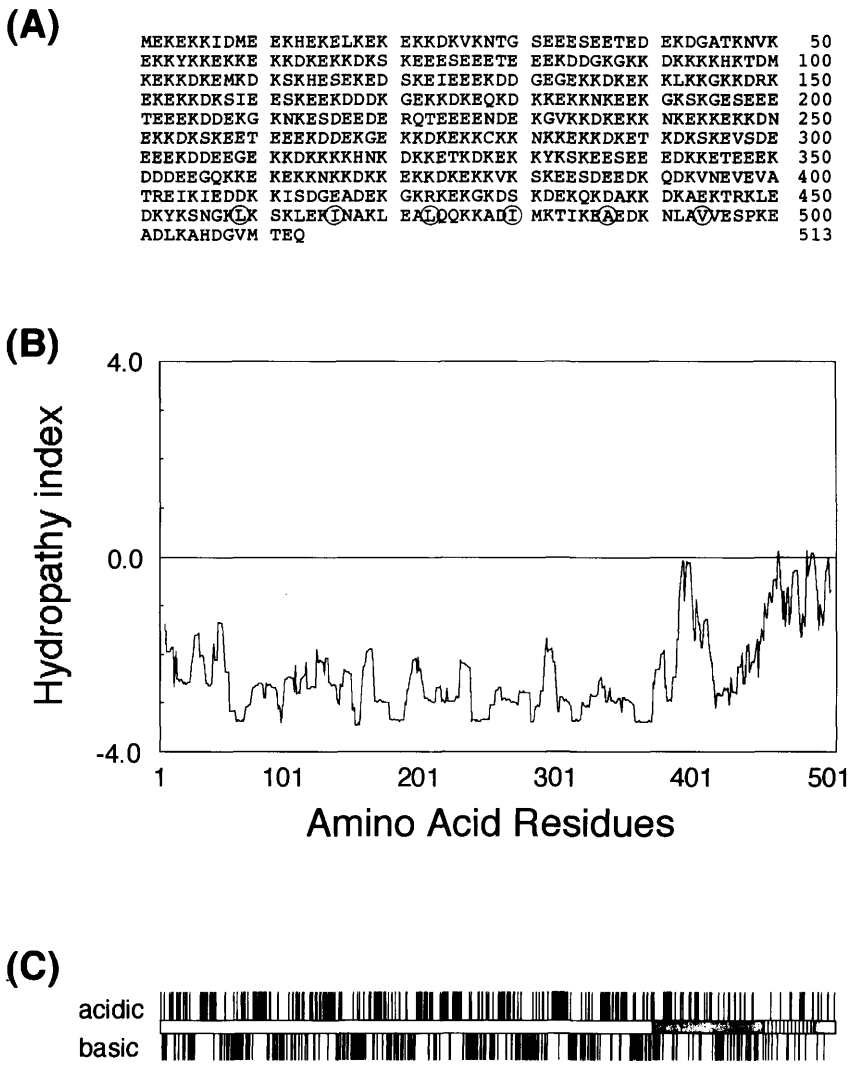

Fig. 2 Amino acid sequence and structural features of the *KED* gene product. (A) The derived one-letter amino acid sequence is shown. The circles indicate hydrophobic residues in the putative leucine zipper motif. The accession number is AB009883. (B) Hydropathy plot of the KED polypeptide. Hydropathy analysis was performed using a window of nine amino acids (Kyte and Doolittle 1982). (C) 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 shaded. The putative leucine zipper motif is indicated by a striped box.

vestris, 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*. 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 (Fig. 3B). 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. Under non-stressed condition, *KED* transcripts were not observed in leaves, roots, flowers and even in BY-2 cultured cells (data not shown).

Response of KED to chemicals—Healthy tobacco leaves were cut into pieces and floated on a buffer solution, and accumulation of *KED* transcripts was examined by northern assay (Fig. 4). *KED* 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, *KED* transcripts again accumulated (Fig. 4A). This clearly shows that the induction of *KED* transcripts by wounding is temporary but repeatable. Instead of the second wounding, leaf pieces were transferred to a buffer solution containing

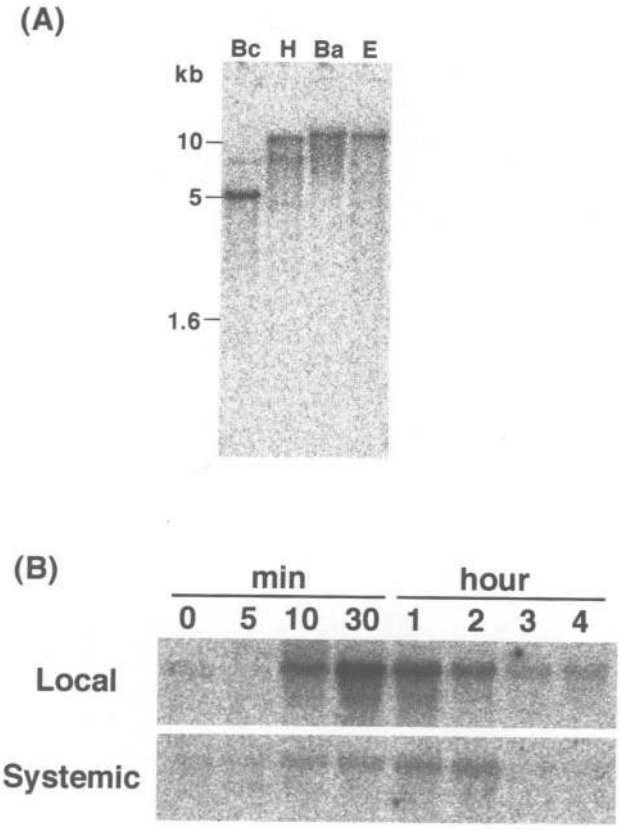

Fig. 3 Hybridization analysis of *KED*. (A) Genomic DNA analysis. Genomic DNA (10 μg) digested with one of *Bcl*II (Bc), *Hin*dIII (H), *Bam*HI (Ba) or *Eco*RI (E) were fractionated on 0.8% agarose gel, transferred to a nylon membrane and probed with ^{32}P-labeled *KED* cDNA fragments. DNA size is indicated on the left. (B) 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 RNA per lane was subjected to hybridization with a ^{32}P-labeled *KED* cDNA probe.

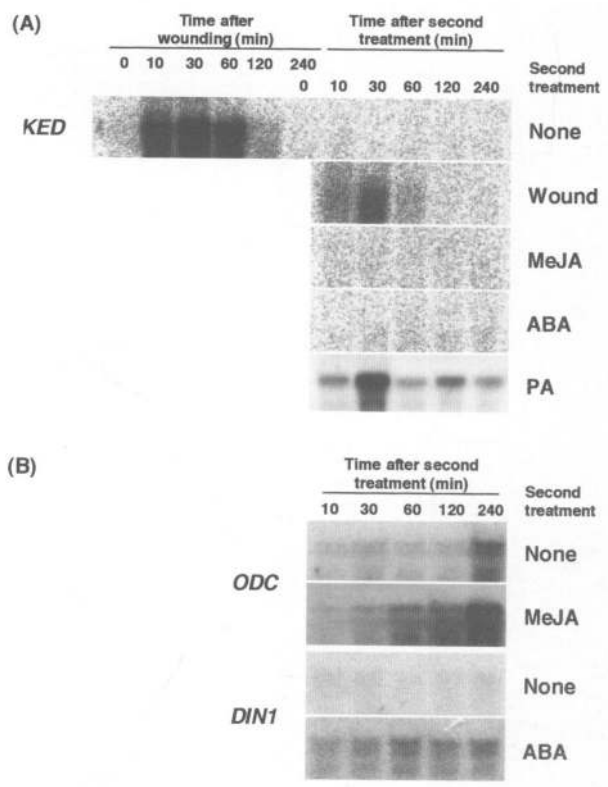

Fig. 4 Effect of chemicals on *KED* transcript accumulation. Healthy leaves were wounded by punching, and leaf pieces were floated on a buffer solution. At 4 h after the first wounding, leaf pieces were cut by a pair of scissors (Wound), or were transferred to a buffer solution containing one of 50 μM methyl jasmonate (MeJA), 100 μM abscisic acid (ABA) or 10 mM propionic acid (PA). Untreated samples served were as the control (Control). The leaf pieces were harvested at the indicated time intervals. An RNA blot containing 18 μg of RNA per lane was subjected to hybridization with ^{32}P-labeled cDNA probes for *KED* (A), *ODC* (B) and *DIN1* (B).

MeJA, LNA or ABA, and transcript accumulation was examined 4 h after the first wounding. No accumulation of *KED* transcripts was observed (Fig. 4A, data not shown for LNA). In contrast, transcripts for *ODC* which responds to MeJA (Imanishi et al. 1998) and for *DIN1* which responds to ABA (Azumi and Watanabe 1991), were markedly induced (Fig. 4B). The results suggest that *KED* activation is not mediated by octadecanoids or by ABA. Effect of propionic acid (PA), which changes ion fluxes, was then examined. The resulting cytoplasmic acidification induced transient accumulation of *KED* transcripts, which was sustained for up to 4 h after treatment (Fig. 4A). When cytoplasmic Ca^{2+} concentration was increased by Ca^{2+} ionophore A23187, the levels of *KED* transcripts did not change (data not shown).

Effects of metabolic inhibitors—Leaf pieces were transferred 3.5 h after the first cutting to a buffer solution containing various metabolic inhibitors, preincubated for 30 min and wounded again by cutting. Treatment with erythrosin B, which is a powerful inhibitor of H^+-ATPase and therefore causes cytosolic acidification, induced *KED* transcripts (Fig. 5). The second wound response, however, appeared not to be significantly affected in the presence of erythrosin B (Fig. 5). Accumulation of *KED* transcripts was not inhibited by CHX treatment, clearly indicating that new protein synthesis is not required for *KED* activation. However, CHX treatment alone induced and sustained accumulation of *KED* transcripts (Fig. 5). This is consistent with many other genes, including mammalian oncogenes and early auxin-, salicylic acid- and low temperature-responsive genes (Herschman 1991, Abel and

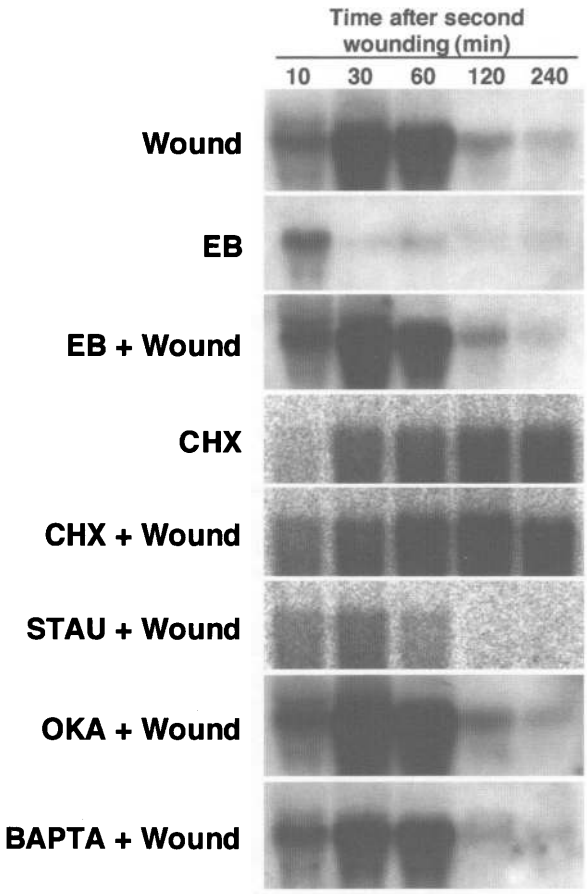

Fig. 5 Effect of chemicals on wound-induced *KED* transcript accumulation. Healthy leaves were wounded by punching, and leaf pieces were floated on a buffer solution. At 3.5 h after the first wounding, leaf pieces were transferred to a buffer solution containing 50 μM erythrosin B (EB), 50 μM cycloheximide (CHX), 1 μM staurosporine (STAU), 0.1 μM okadaic acid (OKA) or 5 mM BAPTA (BAPTA). After further incubation for 30 min, the leaf pieces were cut again by a pair of scissors (Wound). The leaf pieces were harvested at the indicated time intervals. An RNA blot containing 18 μg of RNA per lane was subjected to hybridization with a ^{32}P-labeled *KED* cDNA probe.

Theologis 1996, Horvath and Chua 1996, Berberich and Kusano 1997). Leaf pieces were also wounded in the presence of staurosporine, okadaic acid or Ca^{2+} chelator BAPTA. None affected accumulation of *KED* transcripts (Fig. 5). Ca^{2+} channel blocker La^{3+} was also not effective (data not shown).

Discussion

Wound-responsive genes identified by FDD—In order to understand the general aspects of plant response to wound stress, it is desirable to catalog as many as possible of the genes whose expression is induced and to determine their temporal and spatial expression patterns, from which functional interactions among the various gene products can be hypothesised. In the present study, 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. 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, C14 and C20); (ii) genes so far not known to be related to wounding (A1 and C19); and (iii) unidentified genes (A5, C13 and A7 or *KED*). The gene products of the first group are obviously involved in defense, and will not be discussed further. The genes of the second group are worthy to note. 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. Transcript levels for Ins-1P 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 (Smart and Fleming 1993, Abu-Abied and Holland 1994, Ishitani et al. 1996). 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 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). 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. The accumulation pattern of A7 (*KED*) transcripts is distinct,

showing a rapid increase within a few minutes after wounding followed by a rapid decrease 1 h later. Analysis of the KED polypeptide revealed that there are none of the clusters of hydrophobic amino acids that are necessary for protein folding, suggesting that the KED is unable to form a firm tertiary structure. Search of protein sequence data bases revealed that no similar proteins have been reported so far. Dehydrins, however, are known to be extremely hydrophilic with abundance of charged and polar amino acid residues (Close 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 1997). 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.

Signals for immediate-early wound-response—The *KED* transcripts are rapidly increased and transiently accumulated on wounding. The response is repeatable, and occurs 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 wounded site to the whole plant. It is generally considered that MeJA, its precursor LNA, and/or ABA serve as the signal molecule for wound signal transmission (Farmer and Ryan 1990). In the case of *KED*, however, none of these compounds were effective. Moreover, induction of *KED* transcripts was independent on protein phosphorylation, calcium channels or protein synthesis. These observations suggest that *KED* is transcriptionally activated not through conventional signal transduction pathways, but by some independent factor(s).

In search of such factors, we identified propionic acid, which decreases cytoplasmic pH, to induce transient accumulation of *KED* transcripts. Erythrosin B, which acidifies cytosols by blocking H^+-ATPase, also induced transient accumulation of *KED* transcripts. These results strongly suggest that acidification is one of signals for *KED* activation. In tobacco cells, cytosolic acidification was reported to activate MAP kinases and to induce expression of defense genes (Lapous 1998, Tena and Renaudin 1998). Alkalinization of cell culture medium is rapidly, transiently and repeatedly induced by oligogalacturonides, which are one of the inducers of wound-responsive genes in tobacco cell (Mathieu et al. 1998). When soybean cells were treated with CHX, medium alkalinization and oxidative bursts were induced (Tenhaken and Rubel 1998). The wound- and CHX-induced *KED* transcript accumulation was not influenced by NADPH oxidase inhibitor, diphenylene iodonium chloride (DPI) (unpublished observation), suggesting again that wound-induced *KED* transcript accumulation is mediated not by oxidative bursts but by cytosolic acidification. However, there is no clear evidence showing association of cytosolic pH change with wounding. This may be experimentally shown by the use of recently developed caged proton for pH modulation and the use of fluorescent pH indicator for the direct measurement of cytosolic pH.

Taken together, we propose that physical changes in the cellular environment, especially cytosolic pH, may trigger *KED* transcription, and that the *KED* gene product, with its characteristic repeated charged domains, contributes to improve cellular homeostasis damaged by wound stress.

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