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12. A 2.18-kb P_{SAG12} fragment from the Eco RV site (at 2073 bp upstream of the transcription start site) to an Nco I site (created at the *SAG12* translation start codon by oligomutagenesis) was fused to the open reading frame of *GUS* and *IPT* (*8*) at their translation start codon, resulting in plasmids containing P_{SAG12}-*GUS* and P_{SAG12}-*IPT* chimeric genes, respectively. The P_{SAG12}-*IPT* fusion flanked by Spe I sites was recloned into a binary vector at an Xba I site, resulting in the plasmid pSG529 (Fig. 1). A Pst I–Xba I fragment containing P_{SAG12}-*GUS* was inserted into a binary vector to create pSG514.
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14. Shoot tips (a 25-mm region) from 4-week-old P_{SAG12}-*IPT* transgenic and wild-type tobacco plants were reciprocally grafted by the wedge grafting method [R. J. Garner, *The Grafter's Handbook* (Cassell, London, 1988)]. Twelve grafted plants were analyzed.
15. Leaves of 11-week-old transgenic and wild-type plants grown in a greenhouse with a 16-hour photoperiod at ~26°C were measured for net CO_2 uptake rate with a LI-6400 infrared monitor (Li-Cor). The internal light source was set at 1500 μmol m^{-2} s^{-1} and at a temperature of 26°C. Six plants of each genotype were measured.
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17. After 6 months growth the seed pods and the remainder of the above-soil parts were harvested separately and dried at 55°C until no further weight change was observed (7 to 10 days).
18. Seedlings were started in sterile culture and transplanted when the cotyledons had expanded into 3-liter clay pots containing Jiffy-Mix soil (Jiffy Products of America) saturated with Peters 20-20-20 fertilizer at the concentration of 473 parts per million of nitrogen (Peters Fertilizer Products, W. R. Grace & Co.) and grown in a greenhouse (*15*). The plants were sub-irrigated as needed with water.
19. Leaves that had just reached full expansion at the same positions of P_{SAG12}-*IPT* or wild-type plants were cut from the plants, immediately inserted into a jar filled with water, and maintained in a growth chamber at 23°C and 70% relative humidity under 120 μmol m^{-2} s^{-1} of continuous light. Four leaves for each genotype were tested.
20. At the indicated ages (expressed as days after the emerging leaf was 3 mm in length) a 1-cm^2 leaf disk was harvested with a cork borer from the distal part of leaf number 7 (counted from bottom). Three plants of each genotype were sampled each time. GUS activity in leaf disks was assayed with 4-methylumbelliferyl-β-D-glucuronide as substrate according to standard protocol [R. A. Jefferson, *Plant Mol. Biol. Rep.* **5**, 387 (1987)].
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Tobacco MAP Kinase: A Possible Mediator in Wound Signal Transduction Pathways

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A complementary DNA encoding a mitogen-activated protein (MAP) kinase homolog has been isolated from tobacco plants. Transcripts of the corresponding gene were not observed in healthy tobacco leaves but began to accumulate 1 minute after mechanical wounding. In tobacco plants transformed with the cloned complementary DNA, trans inactivation of the endogenous homologous gene occurred, and both production of wound-induced jasmonic acid and accumulation of wound-inducible gene transcripts were inhibited. In contrast, the levels of salicylic acid and transcripts for pathogen-inducible, acidic pathogenesis–related proteins were increased upon wounding. These results indicate that this MAP kinase is part of the initial response of higher plants to mechanical wounding.

One of the severest environmental stresses to which plants may be subjected is wounding, which may come about through such diverse causes as mechanical injury or pathogen or herbivore attack. To cope with such stresses, plants have developed multiple self-defense systems, activating a set of genes that are mostly involved in wound healing (*1*). Well-known examples are the genes encoding proteinase inhibitor (PI)-I and PI-II, which accumulate not only in wound sites but also in distal unwounded tissues to defend the plant body against, for example, insect proteases (*1*). The transcriptional activation, by wounding, of genes encoding PI is therefore systemic, and it is generally considered that the endogenous activator of these genes is jasmonic acid (JA) along with its methyl ester (MeJA) (*2*–*6*). The biosynthesis pathway of JA and MeJA has been proposed to involve a lipid-based signaling system including lipases, linolenic acids, lipoxygenases (*2*, *6*–*8*), and phosphorylation of proteins (*2*, *3*). However, the molecular mechanism responsible for activation of these pathways in response to wound stress remains unknown. Here, we report identification and characterization of a MAP kinase homolog that may function in the initial step of wound signal transduction pathways.

To identify genes that are involved in mechanical wounding or lesion formation after pathogen attack of tobacco plants, we isolated a particular complementary DNA (cDNA) clone (DS22) by differential hybridization (*9*). Northern (RNA) hybridization analyses revealed that DS22 transcripts of 1.8 kilobases (kb) in size are absent in healthy leaves of tobacco plants but accumulate upon mechanical wounding. Indeed, DS22 transcripts began to accumulate as early as 1 min after wounding, reaching a maximum level within an hour and rapidly declining thereafter (Fig. 1). The accumulation of DS22 transcripts, however, was not limited to wounded leaves, but rapidly expanded into unwounded adjacent leaves (Fig. 1). This observation was confirmed by experiments showing that DS22 transcripts reach nearly a maximum level in leaves 1 min after the stem was crosscut at the basal position. Almost the same amounts of DS22 transcripts were observed in lower leaves adjacent to the cut stem and in much more distant upper leaves (*10*). Crosscutting of petioles or leaf apex also induced a rapid accumulation of DS22 transcripts (*10*). Thus, the DS22 gene response to wounding is systemic.

The cDNA of DS22 contains a 1725–base pair open reading frame encoding a polypeptide of 375 amino acids with a relative molecular weight of 42,858 (Fig. 2A). The putative amino acid sequence is similar to those of MsERK1, a mitogen-activated protein (MAP) kinase homolog from alfalfa (71% similarity), ATMPK1 from *Arabidopsis* (54%), and ERK2 from rat (46%) (Fig. 2A). The similarity is especially high throughout the 11 conserved kinase domains, and amino acids TEY (Thr, Glu, and Tyr, residues 201 to 203) that are required for MAP kinase activation are well conserved (*11*), which indicates that DS22 is a member of the MAP kinase subfamily. That DS22 encodes a protein kinase was confirmed by autophosphorylation activity of the overexpressed products in a bacterial system (Fig. 2B). We therefore designated

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DS22 as the protein WIPK (wound-induced protein kinase).

The genomic organization of the gene encoding WIPK in the tobacco genome was determined by Southern (DNA) hybridization analyses. When genomic DNA was digested with appropriate restriction endonucleases and hybridized with a WIPK cDNA probe, almost exclusively one or two hybridization signals were observed (Fig. 2C). Although interpretation of such data is not simple because of the amphidiploid nature of *Nicotiana tabacum* between *N. tomentosiformis* and *N. sylvestris* and because of the presence of introns, the simple hybridization pattern suggests that the gene encoding WIPK is present as a single copy.

To study further the function of WIPK gene products, we introduced a sense-oriented 1.2-kb fragment from the coding region into tobacco plants under the control of the 35S cauliflower mosaic virus promoter (*12*). Among more than 50 antibiotic-resistant transformants, five were selected on the basis of transgene expression. Northern hybridization analyses showed that although the introduced 1.2-kb cDNA was expressed in these plants, the endogenous 1.8-kb WIPK transcripts did not accumulate after wounding, as they would in a normal wild-type plant (Fig. 3A). Further analyses with a myelin basic protein (MBP) phosphorylation assay (*13*) showed that transgenic plants constitutively exhibit a low level of WIPK activity, but wounding did not increase kinase activity, whereas the MBP kinase activ-

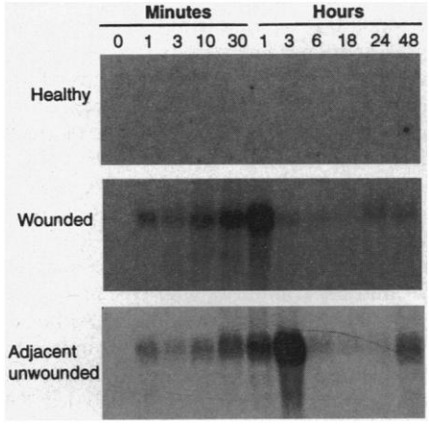

Fig. 1. Accumulation of DS22 transcripts after mechanical wounding. Well-expanded upper leaves of 50-day-old tobacco plants were wounded by gentle rubbing of the upper epidermis of leaves with wet Carborundum. Wound treatment was completed within 15 s. The wounded and adjacent unwounded leaves were harvested at the indicated time intervals and used for total RNA extraction (*28*). For controls, total RNA was isolated from healthy leaves of untreated intact plants. Northern (RNA) blots containing 20 μg of RNA per slot were subjected to hybridization with a ^{32}P-labeled DS22 cDNA probe (*29*). The amounts of loaded RNA in each lane were confirmed to be equal by rehybridization with a ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe after the previous probes were stripped off (*10, 30*).

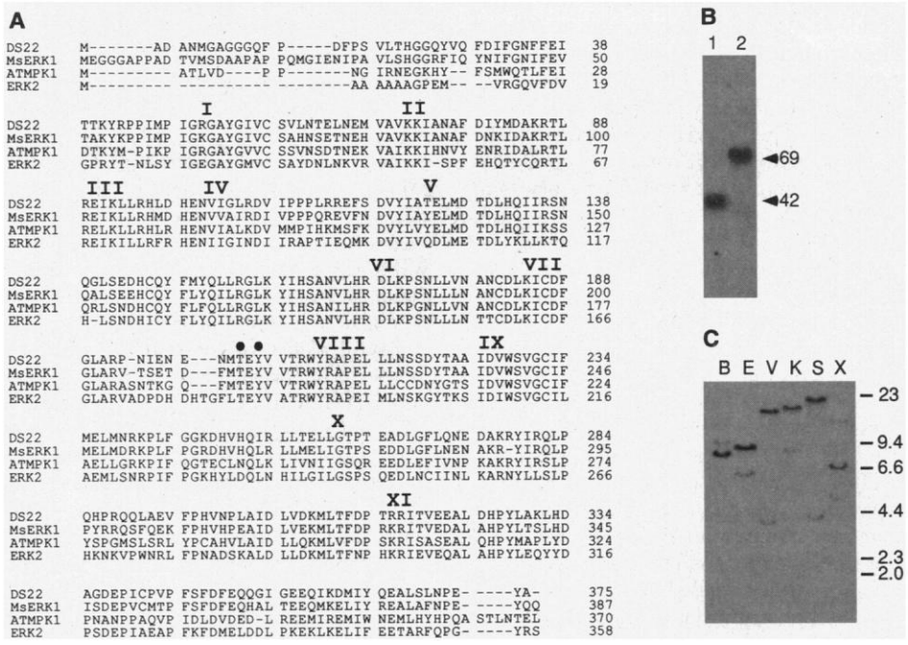

Fig. 2. (**A**) Alignment of the predicted amino acid sequences of DS22 with *Arabidopsis* ATMPK1 (*27*), alfalfa MsERK1 (*31*), and rat ERK2 (*32*). Hyphens indicate gaps introduced to maximize alignment. Roman numerals indicate the 11 major conserved subdomains of protein kinases. Closed circles show threonine and tyrosine residues that are phosphorylated in activated MAP kinase (*11*). Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The nucleotide sequence data will appear in the European Molecular Biology Laboratory, GenBank, and DDBJ (DNA Data Bank of Japan) nucleotide sequence databases under the accession number D61377. (**B**) WIPK protein, expressed in *Escherichia coli* as a GST fusion protein (GST-WIPK) (*33*), was extracted from cells and purified by glutathione-Sepharose-agarose affinity chromotography. The resulting protein was incubated with 0.1 mM [γ-^{32}P]ATP and subjected to electrophoresis (*34*). *Xenopus* 42-kD MAP kinase (lane 1) was used as a control (*34*). Relative molecular mass is indicated in kilodaltons. Note that WIPK activity is located at 69 kD (lane 2) instead of the native 46 kD because of GST fusion. (**C**) Tobacco genomic DNA (10 μg) was digested with Bam HI (B), Eco RI (E), Eco RV (V), Kpn I (K), Sac I (S), and Xba I (X), fractionated by agarose gel electrophoresis, blotted to nylon membrane (Hybond-N+, Amersham), and subjected to hybridization with ^{32}P-labeled WIPK cDNA at 65°C for 16 hours in ×5 SSC solution containing 0.5% SDS, 0.5% Ficoll, 0.5% polyvinylpyrolidone, 0.5% bovine serum albumin, and salmon sperm DNA (100 μg/ml). The filter membrane was washed with ×0.1 SSC containing 0.1% SDS at 65°C and subjected to autoradiography. Size markers are indicated in kilobase pairs.

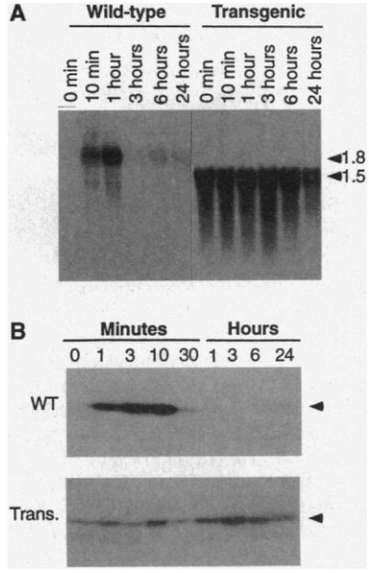

Fig. 3. WIPK transcription and MBP kinase activity in wild-type and transgenic plants. Well-expanded upper leaves of 50-day-old wild-type or transgenic (TMP1S-13 line) tobacco plants were wounded as described in Fig. 1. (**A**) The wounded leaves were harvested at the indicated time intervals and used for total RNA isolation (*28*). Northern blots were subjected to hybridization with ^{32}P-labeled WIPK cDNA (*29*). In the transgenic plants, the 1.5-kb RNA consists of a 1.2-kb WIPK cDNA fragment and a 300-bp nopaline synthase terminator region originating from the vector (*12*). Size markers are indicated in kilobases. (**B**) The wounded leaves of wild-type (WT) and transgenic (Trans.) plants were harvested at the indicated time intervals, and crude protein fractions were prepared for MBP kinase assay (*35*). Arrowheads indicate the relative molecular mass of 46 kD.

ity in wild-type plants is newly and transiently induced by wounding (Fig. 3B). Indeed, despite the fivefold higher levels of transcripts from the WIPK transgene, the MBP phosphorylation activity was less than half that of wounded wild-type plants. These results suggest that in transgenic plants, the endogenous gene encoding WIPK is silenced by the introduction of a foreign gene and that translation of introduced gene transcripts could also be partly repressed. This might be due to trans inactivation or cosuppression phenomena recently found in various transgenic plants (*14*).

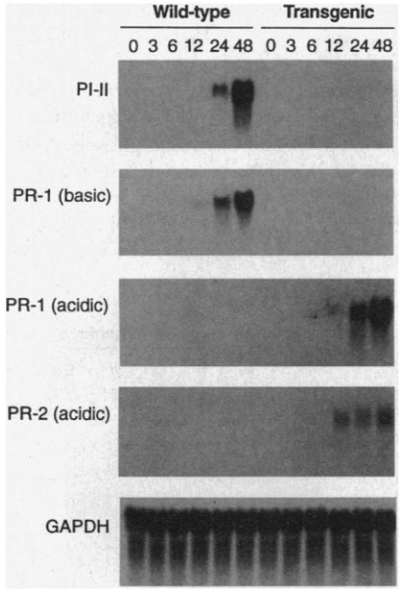

Fig. 4. Accumulation of transcripts for PR proteins in transgenic plants. Well-expanded upper leaves of 50-day-old wild-type and transgenic (TMP1S-13 line) tobacco plants were wounded as described in Fig. 1. Wounded leaves were harvested at the indicated time intervals and used for total RNA isolation (*28*). Northern blots were subjected to sequential hybridization with ^{32}P-labeled cDNAs first for acidic PR-1 (*36*), then after the probe was stripped off, for acidic PR-2 cDNA (*16*), followed by basic PR-1 (*37*) and PI-II (*38*) cDNAs (*29*). To standardize RNA loading, the blots were finally subjected to hybridization with GAPDH cDNA (*30*). Time scales are expressed in hours.

In normal wild-type plants, wounding generally induces an accumulation of transcripts for a set of genes encoding, for example, PI-II and basic pathogenesis-related (PR)-1 protein (Fig. 4) (*15*). In transgenic plants, however, no such transcript accumulation was observed even after extensive wounding (Fig. 4). Instead, the transgenic plants responded to wounding by accumulating transcripts for acidic PR-1 and PR-2 proteins, a response normally induced by pathogen attack but not by simple mechanical wounding (Fig. 4) (*15, 16*).

Because it is believed that JA induces production of PI-II and basic PR proteins (*2, 4–6, 17*), and salicylic acid (SA) induces acidic PR proteins (*18*), we measured the JA and SA content of wild-type and transgenic plants after wounding. Although wild-type plants responded to wounding by producing JA and MeJA after 3 hours, the transgenic plants produced much less JA and MeJA, even up to 48 hours after wounding (Fig. 5, A and B). In contrast, SA and its sugar conjugate, salicylic acid β-glucoside (SAG), began to accumulate 6 hours after wounding of transgenic plants. Such production of SA and SAG was not observed in wounded wild-type plants (Fig. 5, C and D). These data thus indicate that the response of transgenic plants to wounding is the converse of that of wild-type plants in terms of SA and JA production.

MAP kinases contribute to cellular signal transduction pathways by phosphorylating various proteins in various cellular compartments (*19*). The substrates are classified into three major groups: other protein kinases, transcription factors within the nucleus, and cell surface proteins (*19*). Among these diverse and specific targets of MAP kinases, cytoplasmic phospholipase A_2 ($cPLA_2$), a cell surface protein, is of particular interest in connection with our work here. Upon phosphorylation of Ser^{505} by the MAP kinase, the enzymatic activity of $cPLA_2$ greatly increased in comparison with that in unphosphorylated controls (*20*). Because $cPLA_2$ cleaves phospholipids to release arachidonic acid, which is the precursor of prostaglandins and leucotrienes, it has been suggested that one of the functions of MAP kinases is activation of pathways controlling inflammatory and other disease responses in mammals (*20, 21*).

The chemical structures of MeJA and its precursors are similar (*2*) to those of prostaglandins, signaling molecules for inflammation in mammals. Furthermore, the mammalian inflammation response is suppressed by aspirin, or acetylsalicylic acid, which also inhibits JA and MeJA biosynthesis in plants (*5, 22*). Therefore, it has been proposed that the wound response in plants is mediated by a lipid-based signal transduction pathway, in which the wound signal is initially transmitted to protein kinases that then activate lipases and the following pathways (*2, 3*). Our results show that within a minute, mechanical wounding enzymatically activates a 46-kD protein kinase and that this activation is accompanied by rapid accumulation of transcripts encoding a MAP kinase homolog, WIPK (*23*). Furthermore, the involvement of WIPK in JA and MeJA biosynthesis could be directly demonstrated by experiments with transgenic plants showing that when the endogenous gene encoding WIPK is silenced by the introduced gene, the levels of endogenous JA and MeJA do not increase after intensive wounding. We therefore speculate that WIPK is one of the kinases predicted (*2*) to regulate $cPLA_2$ metabolism, although the target $cPLA_2$-like protein or proteins have not yet been identified in higher plants. Regulation of WIPK action, however, appears to be complicated, as the transgene encoding WIPK was expressed at a low but steady rate in the transgenic plants. By contrast, the wound response of a normal plant requires expression of WIPK to be rapidly but transiently increased. Another curious aspect of the transgenic plants is that they produced SA and SAG after wounding. We have observed similar behavior in transgenic plants expressing a *rab*-related guanosine triphosphate binding protein gene from rice and have shown that MeJA actually inhibits SA biosynthesis in these transgenic plants (*24*). Thus, the simplest explanation for abnormal SA production in WIPK transgenic plants is that the wound-triggered SA synthesis, which is normally suppressed by JA and MeJA synthesized within 3 hours in wounded wild-type plants, is released by their absence. Involvement of a protein phosphorylation cascade in plant defense signaling pathways has long been predicted (*25*) and recently confirmed (*26*). WIPK raises the possibility that the plant wound response is regulated by a well-tuned signal network in which individual signals appropriately cross talk under the control of protein phosphorylation.

Note added in proof: After submission of this manuscript, a report that supports our results appeared (*26a*).

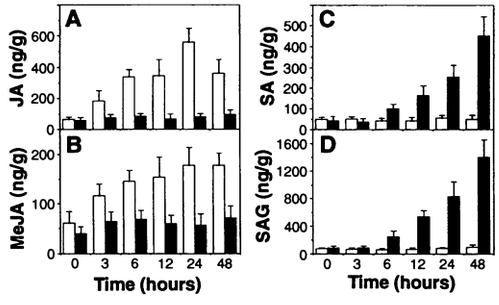

Fig. 5. Accumulation of SA, SAG, JA, and MeJA in wounded wild-type and transgenic tobacco plants. Well-expanded upper leaves of 50-day-old wild-type (open bars) and transgenic (closed bars) tobacco plants were wounded as described in Fig. 1. The wounded leaves were harvested at the indicated time intervals and used for quantitation of JA (**A**), MeJA (**B**) (*39*), SA (**C**), and SAG (**D**) (*40*). The amount of each compound is expressed in nanograms per gram of fresh weight of leaf tissue. Values are the means of three measurements (each with duplicated samples), and standard deviations are shown by error bars.

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9. Mature leaves of 50-day-old tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were inoculated with tobacco mosaic virus (TMV) suspended in 10 mM phosphate buffer (pH 7.0, 10 μg/ml) and incubated at 30°C for 48 hours, then at 20°C for appropriate time intervals. Polyadenylated [poly(A)$^+$] RNA was prepared as described [F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Wiley, New York, 1987)] from total RNA that was isolated from leaves harvested 3 hours after the temperature shift. Complementary DNA was synthesized (Pharmacia kit), inserted into the Eco RI site of Lamda ZAP II vector (Stratagene), and cloned in *Escherichia coli* according to the manufacturer's instructions. Approximately 10^7 recombinants were obtained, and 10^5 clones were differentially screened with radioactively labeled single-strand cDNA probes synthesized from poly(A)$^+$ RNA of leaves harvested either 51 hours (plus probe) or 99 hours (minus probe) after inoculation. A 30-μl labeling reaction mixture contained 1 μg of poly(A)$^+$ RNA, 50 mM tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 20 mM dithiothreitol (DTT), 1.5 mM each of deoxynucleoside triphosphates (dNTP) except for 4 μM deoxycytosine triphosphate (dCTP), oligo$(dT)_{12-18}$ (0.1 mg/ml), 200 μCi of [α-^{32}P]dCTP, and 200 units of avian myeloblastosis virus reverse transcriptase (Gibco-BRL). After incubation at 37°C for 1 hour, unincorporated nucleotides were separated by Sephadex G-50 (Pharmacia) and labeled probe DNA was used for hybridization. A total of 20 plates, each containing approximately 5×10^3 clones, were used to prepare pairs of replica nylon membranes (Hybond-N; Amersham), one of which was hybridized with the plus probe and the other with the minus probe. Hybridization was performed at 65°C for 16 hours in ×6 standard saline citrate (SSC) solution containing 0.1% SDS, 0.1% Ficoll, 0.1% polyvinylpyrolidone, 0.1% bovine serum albumin, and salmon sperm DNA (100 μg/ml). Filters were washed once in ×2 SSC and 0.1% SDS at 25°C and then three times in ×0.1 SSC and 0.1% SDS at 65°C. The filters were exposed to XAR film (Kodak) with an intensifying screen at −80°C for 48 hours. Among 10^5 clones screened, six hybridized with only the plus probe. RNA blot hybridization revealed that among these six clones, only one responded to mechanical wounding to accumulate the transcripts. The cDNA, designated as DS22, was excised with helper phage and recircularized to generate a subclone in the pBluescript SK$^-$ phagemid vector according to the manufacturer's instructions (Stratagene). The DS22 sequence was determined for both strands with nested exonuclease III deletion fragments with the use of an automated DNA sequencer (Applied Biosystems model 373A).
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12. The WIPK cDNA, cloned in pBluescript SK$^-$, was digested with Ase I (position 108) and Ava II (position 1324). The resulting fragment was repaired with the Klenow fragment, ligated to the Sma I site of the pUC18 vector, and propagated in *Escherichia coli*, strain JM109. The recombinant plasmid was isolated and digested with Bam HI (5′ end) and Sac I (3′ end) to generate a 1.2-kb cDNA fragment that contains the complete WIPK open reading frame with an extra 13 bp from the 5′ untranslated region and 82 bp from the 3′ untranslated region. The fragment was ligated, in the sense orientation relative to the 35*S* cauliflower mosaic virus promoter, to the binary vector, pBI121 (Clonetech), which had previously been digested with Bam HI and Sac I. The resulting plasmid was introduced into *Agrobacterium tumefaciens* LBA4404 [G. Ooms, P. J. J. Hooykaas, M. Moolenaar, A. Schilperoort, *Gene* **14**, 33 (1981)] by electroporation [S. Wen-Jun and B. G. Forde, *Nucleic Acids Res.* **17**, 8385 (1989)]. Transformation was done with *Nicotiana tabacum* cv. Samsun NN leaf disks, and resulting transformants were selected by kanamycin resistance [M. Ohshima, H. Itoh, M. Matsuoka, T. Murakami, Y. Ohashi, *Plant Cell* **2**, 95 (1990)]. Among 50 kanamycin-resistant clones, five were selected because of their high expression of the transgene. The kanamycin resistance assay of F_1 progeny revealed that all five parental transformants contained more than two copies of the gene encoding WIPK. Phenotypes of parental and progeny plants were apparently normal, although at the juvenile stage lateral buds appeared.
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29. Twenty micrograms of total RNA per slot was fractionated by denaturing agarose gel electrophoresis and blotted to Hybond-N (Amersham), which was subjected to hybridization with the probe labeled with [α-^{32}P]dCTP (Amersham) by the random priming method as described [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989)]. Blots were washed twice for 15 min in ×2 SSC and 0.5% SDS at 25°C and three times for 20 min in ×0.1 SSC and 0.1% SDS at 65°C and exposed to XAR film (Kodak) with an intensifying screen at −80°C for 48 nours.
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33. To generate a glutathione-S-transferase (GST)–WIPK fusion gene, WIPK cDNA (1.2-kb DNA fragment) cloned in the pUC18 vector was digested with Bam HI (5′ end) and Eco RI (3′ end), and the resulting fragment was ligated to the Bam HI and Eco RI sites of the pGEX vector [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)]. The construct, pGEX-WIPK, was sequenced around the junction between the GST and WIPK regions to confirm that the respective coding regions had been joined in-frame. The pGEX-WIPK fusion was expressed in *Escherichia coli*, strain JM109, by incubating with 0.1 mM isopropylthio-β-D-galactoside for 12 hours. Cells were pelleted, washed, and homogenized with MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, and 4 mM NaH_2PO_4, pH 7.3) and disrupted by sonication, and after addition of Triton X-100 to a final concentration of 1% (v/v), the lysate was subjected to centrifugation. The supernatant was incubated with glutathione-Sepharose (GSH) 4B beads (Pharmacia) for 6 hours at 4°C. After the beads were washed with excess MTPBS and poured into a column, fusion proteins were eluted with a solution containing 50 mM tris-HCl (pH 8.0) and 10 mM reduced glutathione (Sigma). Free GSH was removed by dialysis against MTPBS for 16 hours at 4°C.
34. For the autophosphorylation assay, 500 ng of purified fusion protein, GST-WIPK, or *Xenopus* 42-kD MAP kinase (Santa Cruz Biotechnology) was incubated in a 10-μl solution containing 20 mM tris-HCl (pH 7.5), 1 mM DTT, 20 mM $MgCl_2$, and 0.1 mM [γ-^{32}P]adenosine triphosphate (ATP) at 30°C for 30 min. After an equal volume of ×2 SDS gel loading buffer [100 mM tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol] was added, the mixture was heated at 90°C for 2 min and subjected to SDS–polyacrylamide gel electrophoresis [U. K. Laemmli, *Nature* **227**, 680 (1970)]. The gel was dried and exposed to XAR film (Kodak) at −80°C for 16 hours.
35. For the MBP kinase assay, tissues were scraped with a scraper into 1 ml of an extraction buffer containing 20 mM Hepes-KOH (pH 7.6), 2 mM $CaCl_2$, 100 mM KCl, 1 mM magnesium acetate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 20 mM β-glycerophosphate at 0°C. The homogenate was subjected to centrifugation first at 20,000*g* for 30 min and then at 100,000*g* for 4.5 hours at 4°C. The supernatant was immediately used as a crude extract. Aliquots (50 μg of proteins) of the extracts were assayed for detection of MBP kinase activity as described (*27*).
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39. For quantitation of JA and MeJA, 10 g of leaf material was used for each assay. Quantitation of JA and MeJA was performed essentially as described [R. A. Creelman, M. L. Tierney, J. E. Mullet, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4938 (1992)], except that [$^2H_2$](±)-JA and [$^2H_2$](±)-MeJA were used as internal standards [H. Nojiri *et al.*, *Plant Cell Physiol.* **33**, 1225 (1992)] to estimate the recovery rate of JA and MeJA during the extraction procedure by adding 2 μg of each to the first extraction step. After ether extraction, samples were further purified by isocratic (89% hexane, 10% ethyl acetate, and 1% acetic acid) silica gel (μBondasphere silica, 5-μm, 3.9 mm by 19 cm; Waters) high-performance liquid chromatography (HPLC) at a flow rate of 1 ml/min. Fractions equivalent to MeJA and JA, for which retention times were 5 and 10 min, respectively, were collected, evaporated to dryness, dissolved in 50 μl of ethyl ace-

tate, and analyzed by gas chromatography–mass spectrometry selected ion monitoring (GC-MS-SIM). Before analysis, JA was methylated with diazomethane to give MeJA. The GC-MS instrument was a Hewlett-Packard quadrupole mass spectrometer (HP 5970B) fitted to an HP 5890 gas chromatographer equipped with an Ultra-1 fused silica capillary column (25 m by 0.2 mm, inner diameter, 0.33 μm phase thickness; Hewlett-Packard). Each sample was injected in the splitless mode at 120°C. After a 2-min isothermal pause at 120°C, the column temperature was programmed to 280°C at 16°C/min. The column pressure of carrier gas (He) was 40 kPa. GC-MS-SIM was performed by monitoring the mass-to-charge (*m/z*) ratios 226, 224, 195, 193, 153, and 151. The electron energy was 70 eV. The retention time of MeJA was approximately 8 min. The amounts of JA and MeJA in the original extracts were determined from the ratio of peak areas for *m/z* $224(^2H_0)/226(^2H_2)$. All data were corrected for losses.

40. For quantitation of SA and SAG, 2 g of leaf material was used for each assay. Quantitation of SA and SAG was performed essentially as described [J. Malamy, J. Hennig, D. F. Klessig, *Plant Cell* **4**, 359 (1992)]. HPLC was performed on a μBondasphere 100 Å, 5-μm C-18 (3.9 mm by 15 cm) column maintained at 40°C. Isocratic separation was performed at 1 ml/min with 23% (v/v) methanol in 20 mM sodium acetate, pH 5.0. Fluorescence detection was done with a Model RF-550A (Shimadzu, Tokyo, Japan). The detection limit was 10 pg of SA. All data were corrected for losses.

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Tissue Plasminogen Activator Induction in Purkinje Neurons After Cerebellar Motor Learning

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The cerebellar cortex is implicated in the learning of complex motor skills. This learning may require synaptic remodeling of Purkinje cell inputs. An extracellular serine protease, tissue plasminogen activator (tPA), is involved in remodeling various nonneural tissues and is associated with developing and regenerating neurons. In situ hybridization showed that expression of tPA messenger RNA was increased in the Purkinje neurons of rats within an hour of their being trained for a complex motor task. Antibody to tPA also showed the induction of tPA protein associated with cerebellar Purkinje cells. Thus, the induction of tPA during motor learning may play a role in activity-dependent synaptic plasticity.

Long-term memory requires the synthesis of new proteins, which are thought to bring about structural changes in synaptic connections within the brain (*1*). The cerebellar cortex has been implicated as an important area of plasticity for motor learning (*2*, *3*). An increase in the number of synapses per Purkinje cell in the molecular layer of the cerebellar cortex is seen in rats trained to perform complex acrobatic tasks (*4*). In contrast, the number of parallel fiber synapses on Purkinje neurons does not change in animals subjected to forced or voluntary exercise (*4*). Thus, the synaptic changes are related to the learning of a specific motor task and not merely to increased synaptic activity. These studies (*4*) support the Marr-Albus mathematical models of cerebellar learning, in which climbing fibers are postulated to modulate mossy fiber inputs to Purkinje cells (*2*, *5*). Synaptic changes have also been seen in Purkinje cells of rabbits subjected to a classical conditioning eyeblink response (*6*). Thus, structural changes in synaptic connections appear to accompany cerebellar learning.

Molecules that may participate in synaptic remodeling are the plasminogen activators (PAs), secreted serine proteases that primarily serve to cleave the zymogen plasminogen into the active protease plasmin (*7*). In the absence of plasminogen, PAs can act directly to cleave matrix molecules such as fibronectin and to activate latent growth factors, including scatter factor–hepatocyte growth factor (*8*). The PA-plasmin system plays a role in tissue remodeling, cell migration, and tumor cell invasion (*7*, *9*).

Tissue plasminogen activator (tPA) is the primary PA in the brain (*10*). During embryonic and neonatal development, tPA levels are high in brain regions undergoing extensive cell migration or tissue remodeling. Similarly, PAs are secreted by neurons during axonal growth and regeneration and then bind with high affinity to the surface of the growth cone, where they are poised to facilitate nerve outgrowth through a tissue matrix (*11*). In general, tPA levels are reduced in the mature brain, where the dentate gyrus and cerebellum show the most tPA activity (*12*).

We investigated the involvement of tPA in the consolidation phase of the learning of complex motor tasks. We used a runway task in which rats had to learn to traverse a runway by placing their paws on aluminum pegs that protruded horizontally from the walls of the runway, a task that is dependent on cerebellar function (*13*) (Fig. 1, inset). Initially, the rats were shaped and trained on a regular (REG) peg pattern; then, after a 2-week break, they were tested for their performance on an irregular (IRR) peg pattern. Their speed in crossing the runway was measured for 20 trials per day. The greatest improvement in performance (that

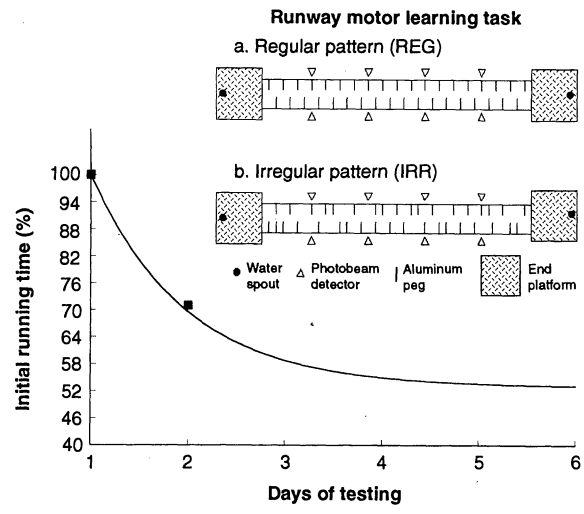

Fig. 1. Motor learning curve and apparatus. Thirty 3-month-old male Fischer rats were trained (*19*) on a complex motor task that involved learning to negotiate a runway consisting of aluminum pegs placed in the specific pattern depicted in the inset on the upper right. The solid line represents a learning curve on the IRR pattern for normal control animals from previous experiments (*13*); the squares represent the rats that were studied for tPA levels. It can be observed from the learning curve that a majority of the improvement in running times (that is, learning) takes place between days 1 and 2. Smaller improvements are observed between days 2 and 5. Rats brains were analyzed for tPA mRNA at varying intervals after training on the IRR task, starting at 1 hour after training on day 1. Two rats were examined after training on day 2, and the improvement in running times for these rats was similar to that of the 10 rats that had been previously observed and that fit the solid curve (*13*).

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