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Short communication

High-level secretion of a virally encoded anti-fungal toxin in transgenic tobacco plants

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Abstract

Ustilago maydis killer toxins are small polypeptides (7–14 kDa) which kill susceptible cells of closely related fungal species. The KP4 toxin is a single polypeptide subunit with a molecular weight of 11.1 kDa. In this work, a transgenic tobacco plant was constructed which secretes the KP4 toxin at a high level. The KP4 toxin expressed in this transgenic plant was of the same size and specificity as the authentic *Ustilago* KP4 toxin. The expression level was at least 500 times higher than that of the KP6 toxin expressed in plants. Transgenic crop plants producing the KP4 toxin could be rendered resistant to KP4-susceptible fungal pathogens.

Ustilago maydis is a smut fungus which infects the ears and leaves of corn plants and induces tumor-like galls, finally resulting in black-colored smut formation. Some natural isolates of *U. maydis* are persistently infected by a double-stranded RNA (dsRNA) virus, the *U. maydis* virus (UmV). UmV is non-infectious and propagates only by vertical transmission through mitosis, meiosis, and mating [16]. Some UmV strains encode anti-fungal killer toxins. The killer toxins may confer a selective advantage on their host cells in nature, by preventing infection of the same plant with *U. maydis* varieties lacking resistance to killer toxins [5, 18].

The three killer subtypes, P1, P4, and P6, secrete KP1, KP4, and KP6 killer toxins respectively. Each killer toxin has a distinct specificity. One subtype can kill the other two killer subtypes and sensitive cells but is resistant to its own killer toxin, and vice versa. There are also three independent resistance genes, which confer resistance to each killer toxin [17]. The resistance to killer toxins is determined by recessive alleles of these genes, probably due to mutations of toxin membrane receptors [8, 18].

UmV viral killer toxins are small polypeptides with molecular weights of 7–14 kDa. They consist of one or two subunits and are secreted into the extracellular space. They are stable in culture medium, probably due to disulfide-stabilized secondary and tertiary structures.

The KP6 toxin-encoding dsRNA, P6M2, has been cloned and expressed in *Ustilago*, yeast, and plant cells [13, 14, 21]. The KP6 toxin consists of two small polypeptides, α (8.6 kDa) and β (9.1 kDa). The KP4 toxin consists of a single

polypeptide of 105 amino acids with a molecular weight of 11.1 kDa [19]. The structure of the KP4 toxin has recently been determined to 0.19 nm (1.9 Å) resolution and found to be composed of a five-stranded anti-parallel β-sheet with two anti-parallel α-helices lying at 45° to these strands [9]. The KP4 toxin appears to act by inhibiting calcium channels in susceptible cell membranes [9].

One strategy for conferring pathogen resistance to crop plants is to introduce genes whose expression produces compounds toxic to the disease-causing pathogens but uninjurious to the plants. Tobacco serves as an easily manipulable model system to test the feasibility of this strategy. We have previously shown that the KP6 toxin is expressed, processed, secreted, and active in tobacco plants [13]. Immunological assays, N-terminal sequence analysis, and mass spectroscopy have shown that the KP6 toxin is expressed and processed into its mature form in tobacco plants. However, the expression level was diminished in comparison with that in *U. maydis*, such that killer activity could be detected only from the concentrated intercellular fluid of plant leaves.

We have shown that the KP4 toxin is more efficiently expressed than the KP6 toxin in a heterologous system, *Saccharomyces cerevisiae* [19], suggesting that the KP4 toxin might be more efficiently expressed than the KP6 toxin in plants. In the current study, we constructed a transgenic tobacco plant expressing the KP4 killer toxin under the control of a cauliflower mosaic virus (CaMV) 35S promoter. The KP4 toxin expressed in tobacco plants had the same size and specificity as the authentic *Ustilago* KP4 toxin. The expression level was at least 500 times higher than that of the KP6 toxin in tobacco plants and comparable to that of the *Ustilago* KP4 toxin. The expression level was so high that a small piece of leaf secreted enough of the KP4 toxin to kill sensitive cells. Therefore, KP4 expression in plants could be practically applied to control fungal pathogens if KP4-sensitive fungal species are identified in economically important crop plants.

The *U. maydis* virus KP4 killer toxin is encoded by a medium-size dsRNA segment, P4M2. P4M2 cDNA has been cloned and expressed in yeast cells [19]. P4M2 dsRNA consists of 1006 bp and is the only M genome segment present in the P4 killer strain (77–1) currently used in our laboratory. The KP4 preprotoxin includes 127 amino acids and has a predicted molecular weight of 13.6 kDa. The N-terminal signal peptide is cleaved after Ser-22 by signal peptidase, resulting in the mature KP4 toxin of 105 amino acids with a molecular weight of 11.1 kDa (Fig. 1A).

A full length P4M2 cDNA was cloned into the unique *Bam*HI site of pGEM-7Zf(+) vector, resulting in the plasmid 7Z-P4M2-1, in which the P4M2 cDNA is oriented in the same direction as the *lacZ* gene in the vector. The P4M2 cDNA expression vector was constructed by transferring a *Bam* HI fragment of the P4M2 cDNA from 7Z-P4M2-1 into the Ti plasmid pBI121 using pBI221 as an intermediate cloning vector. The resulting pBI121-P4C expression vector is 12 kb in length and has the P4M2 cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (NOS) terminator (Fig. 1B). The P4M2 cDNA expression cassette (2.6 kb) can be integrated into the plant genome via the left and right T-DNA borders. The expected size of the P4M2 transcript generated from the expression cassette is about 1200 bases (1006 bases of P4M2 plus about 200 bases of the NOS terminator sequence).

The *Nicotiana tabacum* strain SR1 was infected with *Agrobacterium* transformants containing the P4M2 cDNA expression vector by the leaf disk method as described [11]. One or two calli were usually obtained per 50 wounds. The calli, which developed into plantlets and rooted into hormone-free MS media supplemented with kanamycin, were grown in soil under green house conditions up to 30 cm in height before killer activity assays. The leaves were harvested and used directly for protein and nucleic acid preparation.

A KP4 F_0 plant (a primary transformant, designated P4C) was self-fertilized, and the seeds were used to obtain F_1 plants. Four F_1 plants were chosen for further analysis (designated P4C-1, -2, -3, and -4). Plant genomic DNAs iso-

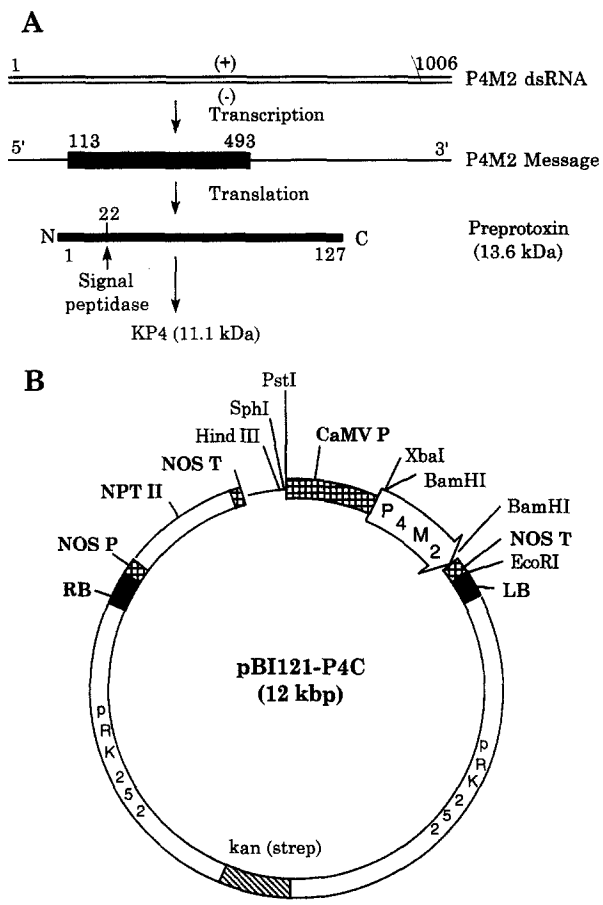

Fig. 1. Expression of KP4 toxin and structure of P4M2 cDNA expression vector pBI121-P4C. A. Expression of KP4 toxin in *Ustilago maydis* cells. A 1006 base message is transcribed from the M2 double-stranded RNA of the UmV P4 viral genome and translated into a 127 amino acid preprotoxin with a molecular mass of 13.6 kDa. The preprotoxin is cleaved by signal peptidase after Ser-22 to yield mature KP4 toxin (11.1 kDa, 105 amino acids). KP4 preprotoxin processing does not require Kex2p activity, unlike processing of the KP6 preprotoxin [19]. Numbers indicate base positions; N, N-terminus; C, C-terminus. B. Structure of P4M2 cDNA expression vector pBI121-P4C. The P4M2 cDNA expression vector pBI121-P4C was constructed by transferring a complete UmV P4M2 cDNA fragment (KP4 toxin gene) to the two Clontech vectors pBI121 and pBI221. Plasmid 7Z-P4M2-1 has a complete P4M2 cDNA of 1006 bp in length in the unique *Bam* HI site of pGEM-7Zf(+). The two Clontech vectors pBI121 and pBI221 were derived from the pBI19 vector [2, 12]. pBI121 has a kanamycin resistance gene from *Streptococcus* for selection in *E. coli* and a neomycin phosphotransferase II (NPT II) gene from Tn*5*. The plasmid pBI221 is an intermediate cloning vector, in which a 3.0 kbp *Hin*dIII/*Eco* RI fragment of the pBI121 (CaMV 35S promoter-*GUS* gene-nopaline synthase terminator) is cloned into pUC19. The *Bam*HI fragment of the P4M2 cDNA was blunt-end ligated into the *Sma* I/*Sac* I double-digested and blunt-ended pBI221, replacing the *GUS* gene in the vector. The *GUS* gene cassette (3.0 kb) in pBI121 was then replaced with a *Hin*dIII (partial digestion)/*Eco* RI fragment (2.6 kb) of pBI221 containing the P4M2 cDNA, resulting in the final pBI121-P4C (12 kb). All intermediate and final vector constructs were verified by restriction digestion and DNA sequencing. CaMV P, cauliflower mosaic virus 35S promoter; NOS P and NOS T, nopaline synthase promoter and terminator, respectively; RB and LB, right and left borders of the region integrated into plant genomes; kan (strep), kanamycin resistance gene from *Streptococcus*; NPT II, neomycin phosphotransferase gene from Tn*5*.

lated from the four KP4 F_1 progeny plants and SR1 plant were digested with *Bam*HI to detect the P4M2 cDNA and with *Xba* I to characterize integration events. *Bam* HI should cut out the 1 kb P4M2 cDNA fragment from the integrated expression cassette in any integrant. The pBI121-P4C expression vector has a unique *Xba*I site at the 3′ end of the CaMV promoter. Therefore, the sizes of DNA fragments generated from *Xba* I digestion should vary depending on integration sites.

Figure 2 shows one such Southern blot analysis. A fragment with an expected size of about 1000 bp was detected from all KP4 F_1 genomic DNA samples but not from SR1 sample when digested with *Bam* HI (Fig. 2A). This result confirmed that transgenic plants with the KP4 toxin gene have integrated P4M2 cDNA in their genomic DNAs. Samples digested with *Xba* I showed various band patterns depending on insertion sites (Fig. 2B). A fragment of about 4.5 kb was detected from P4C-1, -3, and -4, but not from P4C-2. A larger fragment of about 12 kb was also detected from P4C-2 and -4. The P4C-4 showed both fragments. These Southern results indicate that two integration events occurred at two different sites on two different chromosomes in the F_0 P4C transformant. The intensity of each band resulting from *Xba* I digestion (Fig. 2B) is about the same. From the band intensities relative to the P4M2 cDNA internal control, we calculate 1–2 copies of the P4M2 cDNA per plant genome. P4C-1, -2, and -3 progeny plants each appear to have a single copy of the integrated P4M2 cDNA and P4C-4 two copies.

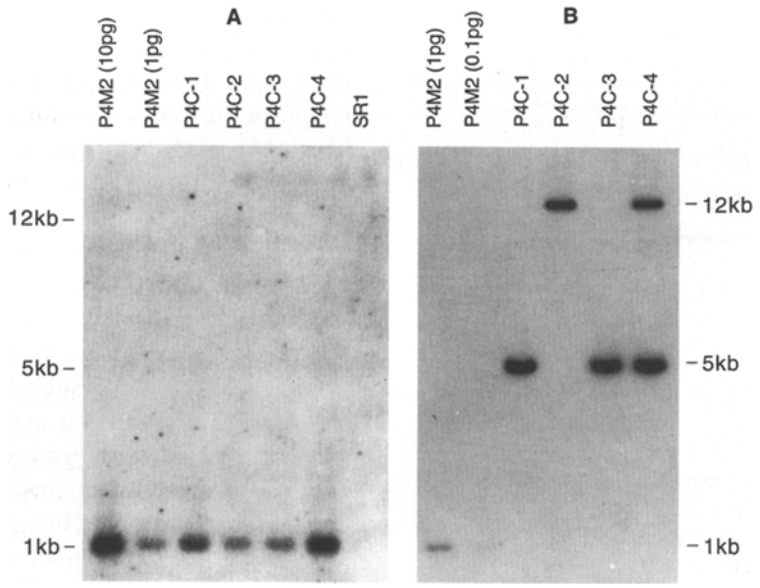

Fig. 2. Southern blot analysis of tobacco genomic DNA. *Nicotiana tabacum* strain SR1 was used as a recipient for *Agrobacterium tumefaciens* (strain LBA4404) infection as described [11, 13]. Transformation of *A. tumefaciens* strain LBA4404 was performed by the freeze-thaw method as described [10]. Transformation of *N. tabacum* strain SR1 was performed by the leaf disk method as described [11]. The recipient plants, grown under sterile conditions on MS medium (BRL), were used for *Agrobacterium* infection. Plasmid DNA was isolated from *E. coli* and *A. tumefaciens* cells by the alkaline lysis method as described [20]. Plant genomic DNA was routinely isolated from 2 g of tobacco leaves as described [6]. Plant genomic DNA was digested with appropriate restriction enzymes (5 units of enzyme per μg DNA, for 5 h at 37 °C), extracted with phenol/chloroform, and ethanol-precipitated. 20 μg of digested genomic DNA was electrophoresed on a 0.8% agarose gel. Transfer to a nitrocellulose membrane (0.45 μm, Millipore) and hybridization were performed as previously described [15]. The probe used was a T7 RNA polymerase *in vitro* transcribed, minus-sense P4M2 riboprobe prepared as described [20] using α-^{32}P-UTP (specific activity 3000 Ci/mmol, ICN). Genomic DNA was digested either with *Bam* HI (A) to detect P4M2 cDNA inserts or with *Xba* I (B) to characterize the insertion events. Lane SR1 is genomic DNA of SR1 plant digested with *Bam* HI. P4C-1 to P4C-4 in each panel are four different F_1 progeny plants of KP4 F_0 transformant P4C. Markers were 1 kb ladder (BRL) and a complete P4M2 cDNA fragment (1006 bp) (pg per lane given).

To determine whether the P4M2 cDNA is efficiently transcribed in transgenic plants, total cellular RNA was isolated from fully grown plant leaves, and northern blot analysis was performed. A 15 μg portion of each RNA sample was denatured with formaldehyde and electrophoresed on a 0.8% formamide-formaldehyde denaturing agarose gel. Total cellular RNAs of the *Ustilago* P4 killer (77–1) and nonkiller (77NK) strains were also analyzed on the same gel as controls. RNA was transferred to a nitrocellulose membrane and probed with ^{32}P-labeled, *in vitro* transcribed minus-sense P4M2 riboprobe.

As shown in Fig. 3, all four F_1 plants produced a P4M2 mRNA with an expected size (about 1150 bases = P4M2 of 1006 bases + NOS terminator sequence of about 150 bases). A smaller transcript of about 500 bases was also detected in all F_1 plants. Two transcripts with different sizes are also detected in *Ustilago* P4 total RNA; one of about 1000 bases and the other of about 350 bases, which are consistent with the sizes of the plus strands of the genomic dsRNAs M2 and L, respectively. L is derived from the 3′ end of the M2 plus strand [4, 7, 21], apparently by processing of the viral plus strand followed by replication within viral particles. This processing of the plus strand occurs in *S. cerevisiae* as well as in *U. maydis* (C.-M. Park and J.A. Bruenn, unpublished), and present results indicate that it takes place in tobacco as well. The smaller P4M2 transcript from tobacco is about 150 bases larger than

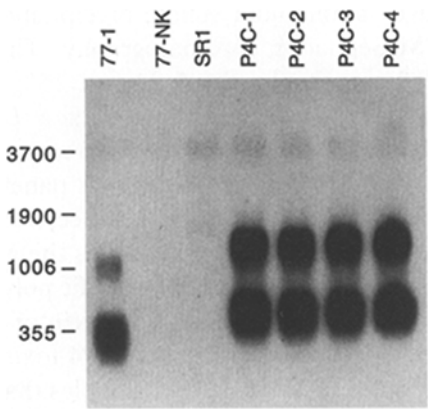

Fig. 3. Northern blot analysis of tobacco total RNA. Total cellular RNA was isolated from *Ustilago* cells as follows. Cycloheximide was added to a final concentration of 100 μg/ml to 5 ml YPD culture at $OD_{600} = 4–6$, and the culture was cooled on ice for 10 min. YPD is yeast extract (1%), peptone (2%), and dextrose (2%). After centrifugation at 5000 × *g* for 5 min, the cell pellet was suspended in 1.5 ml of 100 μg/ml cycloheximide solution. After transfer to an Eppendorf tube and recentrifugation, the pellet was resuspended in 0.5 ml of ice-cold lysis buffer (50 mM Tris.Cl pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$, 100 μg/ml cycloheximide). The mixture was vortexed with an equal volume of glass beads for 15 s and cooled for 20 s on ice four times. An equal volume of phenol and SDS to a final concentration of 0.2% were added. After brief vortexing, the mixture was centrifuged for 3 min. The supernatant was further extracted with an equal volume of phenol/chloroform (phenol/chloroform/isoamyl alcohol 25:24:1) two more times and ethanol precipitated at −70 °C until use. Cellular RNA was isolated from tobacco leaves as described [1] and stored in ethanol at −70 °C until use. Five grams of tobacco leaves was routinely used to isolate about 1 mg of total cellular RNA. A 15 μg portion of plant total cellular RNA was denatured with formaldehyde and electrophoresed on a 0.8% formamide-formaldehyde denaturing agarose gel. *Ustilago* P4 killer and non-killer cellular RNAs were used as positive and negative controls, respectively. Transfer to a nitrocellulose membrane and hybridization were essentially as described [1]. An *in vitro* transcribed, minus-sense P4M2 riboprobe was used as in Southern hybridization. The denatured 1 kb ladder (BRL) was used as a size standard. Lanes 77–1 and 77NK, *Ustilago* P4 killer and non-killer cells, respectively; lane SR1, SR1 plant; lanes P4C-1 to P4C-4, four different F_1 progeny plants of the KP4 F_0 transformant P4C. Size markers were a denatured 1 kb ladder (BRL) and P4M2 cDNA (1006 bases). Large and small rRNAs were also used as internal size markers.

the UmV L transcript, consistent with the 3′ addition of NOS terminator sequences. As in *Ustilago* and yeast, the 5′ portion of the processed mRNA is missing.

The intensity of signals was similar in all four F_1 plants, despite the results of the Southern hybridization (above) and toxin activity assays (below). The Southern hybridization indicated that P4C-4 has twice as many copies of the P4M2 cDNA as the other F_1 plants, and the toxin assays also showed that P4C-4 produced more toxin (see below).

Intercellular fluid was isolated from leaves of transgenic tobacco plants and concentrated by differential ammonium sulfate precipitation (40–65% saturation) and C25 CM-Sephadex ion exchange chromatography. The killer activity of the KP4 toxin secreted from tobacco plants was so strong that the intercellular fluid without further concentration showed detectable killer activity (data not shown). In contrast, the SR1 intercellular fluid did not show any detectable activity even after concentrating 500 times. Of the 4 F_1 plants, P4C-4 showed the strongest activity, as expected from the Southern (Fig. 2) but not from the northern (Fig. 3). Given the strong KP4 toxin activity, we expected leaf tissue itself to show detectable killer activity.

Small leaf disks (8 mm in diameter) were cut out and directly spotted without further treatment on KP4-sensitive lawn plates. As shown in Fig. 4, the leaf disks secreted enough of the KP4 toxin to kill sensitive cells. On the other hand, the leaf disk of the KP6 plant, spotted in the same way, did not show any detectable activity (data not shown). The KP6 intercellular fluid must be concentrated at least 200 times to show detectable killer activity [13]; see below. This difference is significant because in *Ustilago*, there is approximately twice as much extracellular KP6 toxin as KP4 toxin. It is unlikely that the difference between the KP4 and KP6 toxin activities in transgenic plants is due to a positional effect during integration events, since all, independent parental transformants expressing KP4 or KP6 tested gave the same results.

The killer specificity of the KP4 toxin secreted in tobacco transformants was determined using killer plate assays. Leaf disks (0.8 cm in diameter)

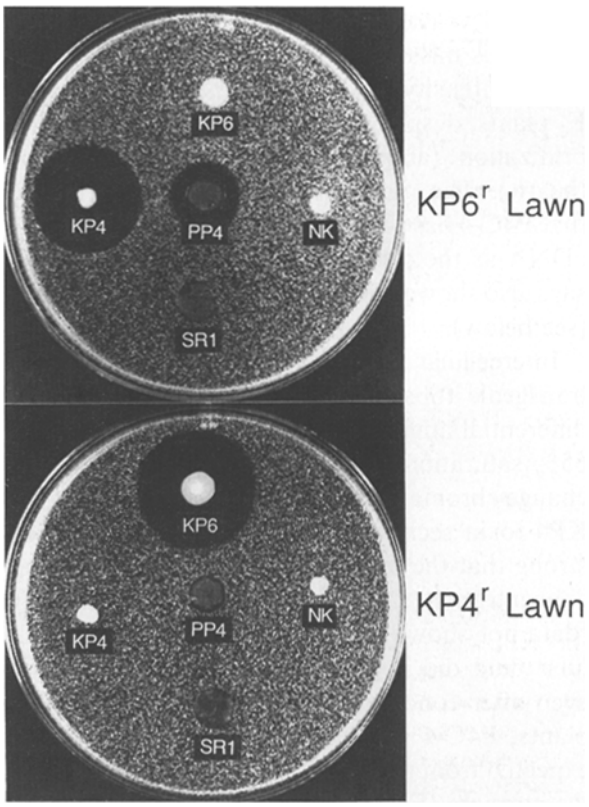

Fig. 4. Killer plate assay of KP4 toxin secreted from tobacco transformants. Killer activity was assayed by spotting protein samples or cell pellets on lawn plates as previously described [21]. Leaf disks (8 mm in diameter) of parent and transformant plants were spotted, along with *Ustilago* cells, on KP4-sensitive (strain 75U-1, KP6-resistant, upper plate) and KP4-resistant (strain 54, KP6-sensitive, lower plate) lawns. KP6, *Ustilago* P6 cells; KP4, *Ustilago* P4 cells; NK, *Ustilago* P4 non-killer cells (77NK); SR1, parent plant SR1; PP4, a KP4 plant transformant (P4C).

of a KP4 F_0 transformant (P4C) and parent SR1 plant were spotted on KP4-sensitive (strain 75U-1, $KP6^r$) and -resistant (strain 54, $KP6^s$) lawns (Fig. 4). The toxin from the KP4 plant (spot PP4) showed the same specificity as toxin from the *Ustilago* P4 killer cells (spot KP4). Both *Ustilago* P4 cells and the KP4 plant disks inhibited the growth of the KP4-sensitive lawn but not that of the KP4-resistant lawn.

The size of the KP4 toxin expressed in tobacco transformants was estimated by western blot analysis. Intercellular fluid extracted from tobacco leaves was concentrated and partially purified by ammonium sulfate precipitation and C25 CM-Sephadex chromatography. The concentrated sample was loaded on a 15% SDS-urea-polyacrylamide gel, along with a *Ustilago* KP4 sample (Fig. 5, right panel), and western blot analysis was performed (Fig. 5, left panel). The result showed that the KP4 toxin secreted from tobacco plants had the same size as the authentic *Ustilago* KP4 toxin. A KP4-specific polyclonal antibody detected only a single protein of identical size from both systems. The KP4 toxin band was not detected from protein samples that were isolated from the medium of Ustilago P4 non-killer cells or from intercellular fluid from the control plant (SR1) concentrated in the same manner as from the KP4 toxin-producing plants. The size of the KP4 toxin produced in plants and in *Ustilago* is the same, showing that the toxin is not glycosylated in plants.

Even though the KP6 toxin is expressed successfully in tobacco plants, the expression level is so low that a piece of leaf, when directly spotted on lawn plates, does not show any detectable activity. The KP6 intercellular fluid must be concentrated more than 200 times for any detectable killer activity [13]. The amounts of KP4 and KP6 toxins secreted from tobacco plants were more quantitatively compared. Intercellular fluids were extracted from 5 g of leaves from the KP4 expressing plants and 2500 g of leaves from the KP6 expressing plants and concentrated to 400 μl final volumes. When the same amount of each concentrated sample was spotted on a lawn of strain 18 ($KP1^sKP4^sKP6^s$), the KP4 sample showed slightly higher killer activity than the KP6 sample (data not shown). This result showed that KP4 activity was at least 500 times higher than KP6 activity in tobacco plants. This may be due to the fact that the KP4 toxin is not glycosylated in either *Ustilago* or yeast cells [19, 21], while the KP6 α polypeptide is glycosyolated in heterologous systems but not in *Ustilago* [21]. In spite of the high expression level of the KP4 toxin, the KP4 plants grew as well as the parent SR1 and KP6 plants. The expression level of the UmV KP4 toxin may be adequate for biological control of susceptible fungal pathogens. Probably only a

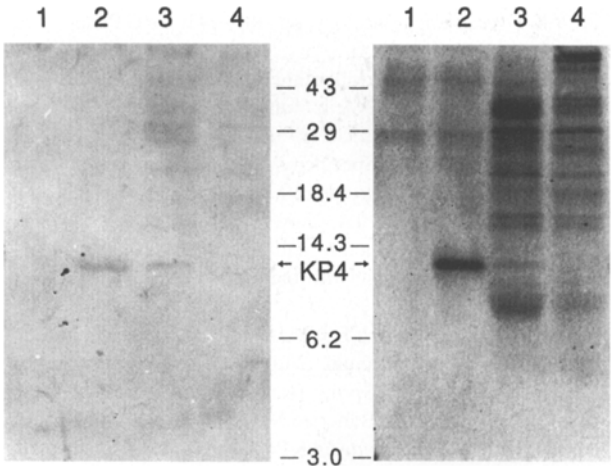

Fig. 5. Western blot analysis of KP4 toxin secreted from tobacco transformants. Intercellular fluid was extracted from transgenic tobacco plants by the vacuum infiltration method as described [3, 13]. It was then precipitated with 40% saturated ammonium sulfate at 4 °C overnight and centrifuged at 8000 × *g* for 30 min. The supernatant was reprecipitated with 65% saturated ammonium sulfate at 4 °C overnight and centrifuged at 8000 × *g* for 30 min. The pellet was dissolved in double-distilled water (ddH_2O) and dialyzed thoroughly against ddH_2O at 4 °C. The dialyzed sample was adjusted to 25 mM acetic acid pH 5.5 with 1M NaOH, for C25 CM-Sephadex ion exchange chromatography. The *Ustilago* KP4 toxin was prepared in the same way from the YPD culture supernatant. The running buffer used for C25 CM-Sephadex was 25 mM acetic acid, adjusted to pH 5.5 with NaOH. Running conditions were as previously described [13] with one modification: the column was run at room temperature and eluted with 5 volumes of a 0.2–0.4 M NaCl continuous gradient. For the KP4 intercellular fluid (5 ml from 5 g of leaves), the ammonium sulfate fractioned sample (2 ml) was run on a 20 ml column. Fractions were spotted directly on KP4-sensitive lawns to identify the peak fractions. The peak fractions were pooled and precipitated with 70% saturated ammonium sulfate as described above, and the pellet was dissolved in a small volume of ddH_2O and dialyzed. To further concentrate the KP6 and SR1 intercellular fluids, the concentrated sample was run again on a G50 size exclusion column as described [13]. Protein samples were analyzed on a 15% SDS-urea-polyacrylamide gel as described [19]. The gel was extensively polymerized before use (at least 24 h). Western transfers and immunological assays were as described [19]. Polyvinylidine difluoride membrane (PVDF, Millipore) was used for western transfers, and detection was by protein A conjugated with alkaline phosphatase as described by the supplier (Cappel, Organon Teknika Corp., Durham, NC). The right panel is a 15% SDS-urea-polyacrylamide gel, and the left panel is a western blot analysis. In both panels: lane 1, *Ustilago* P4 non-killer (strain 77NK); lane 2, *Ustilago* P4 killer (strain 77–1); lane 3, a KP4 plant transformant (P4C); lane 4, parental plant SR1. Size markers in kDa are shown in the middle. The KP4 toxin is indicated by an arrow.

small proportion of wild varieties of *U. maydis* are resistant to the KP4 toxin [5].

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