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Processing and Secretion of a Virally Encoded Antifungal Toxin in Transgenic Tobacco Plants: Evidence for a Kex2p Pathway in Plants

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Ustilago maydis **is a fungal pathogen of maize. Some strains of *U. maydis* encode secreted polypeptide toxins capable of killing other susceptible strains of *U. maydis*. We show here that one of these toxins, the KP6 killer toxin, is synthesized by transgenic tobacco plants containing the viral toxin cDNA under the control of a cauliflower mosaic virus promoter. The two components of the KP6 toxin, designated α and β, with activity and specificity identical to those found in toxin secreted by *U. maydis* cells, were isolated from the intercellular fluid of the transgenic tobacco plants. The β polypeptide from tobacco was identical in size and N-terminal sequence to the *U. maydis* KP6 β polypeptide. Processing of the KP6 preprotoxin in *U. maydis* requires a subtilisin-like processing protease, Kex2p, which is present in both animal and fungal cells and is required for processing of (among other things) small secreted polypeptide hormones and secreted toxins. Our findings present evidence for Kex2p-like processing activity in plants. The systemic production of this viral killer toxin in crop plants may provide a new method of engineering biological control of fungal pathogens in crop plants.**

INTRODUCTION

One strategy for engineering pathogen resistance in crop plants is to create transgenic plants capable of systemically producing substances toxic to specific disease-causing pathogens. This approach is currently being pursued with bacterial polypeptides toxic to herbivorous insects (Gibbons, 1991) but has not yet been attempted with important crop pathogens, such as fungi or bacteria. We are interested in determining the feasibility of this strategy for producing plants that express viral polypeptides toxic to fungal pathogens. Killer toxins, such as those secreted by some virus-infected strains of *Ustilago maydis* (Koltin, 1988), provide a unique model for introducing fungal resistance into plants.

U. maydis, a fungal pathogen of maize, is the causative agent of corn smut. Interstrain inhibition can occur when different strains of *U. maydis* are grown together on media such that one strain inhibits the growth of a second, sensitive strain (Puhalla, 1968). Crosses and heterokaryon transfer experiments have shown that this inhibitory effect, the killer phenomenon, is cytoplasmically inherited (Day and Anagnostakis, 1973; Koltin and Day, 1976). The factors responsible for inhibiting the growth of sensitive *U. maydis* strains have been demonstrated to be secreted proteins produced by endogenous, noninfectious, double-stranded RNA viruses (Hankin and Puhalla, 1971; Koltin and Day, 1975).

The *U. maydis* virus (UmV) has three subtypes that have been characterized in detail. Each subtype produces a toxin with unique properties and unique specificity for sensitive *U. maydis* strains (Koltin and Day, 1975; Kandel and Koltin, 1978). These three subtypes are designated P1, P4, and P6 and their killer activities KP1, KP4, and KP6, respectively. At least one of the *Ustilago* killer toxins, KP6 (Ginsberg, 1992), appears to have a mode of action similar to that of the *Saccharomyces cerevisiae* killer toxin k1, which introduces new ion channels in cellular membranes (Martinac et al., 1990).

A *U. maydis* strain harboring one of the killer viral subtypes is resistant to that particular toxin but sensitive to the other toxins. A strain's sensitivity or resistance to a specific killer toxin is determined by three independent nuclearly encoded genes, each specific for only one of the toxin subtypes. Resistance is caused by recessive alleles of these genes and may be due to modification of membrane receptors for the toxin (Koltin and Day, 1975; Finkler et al., 1992). The *U. maydis* resistance genes are not common. A sample of the natural population of *U. maydis* reveals that only ~10% of the strains examined are resistant to any one of the killer toxins, and no strain has been found that is resistant to all three toxins (Koltin and Day, 1975; Day, 1981).

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The UmV toxins of *U. maydis* are particularly attractive as biological control agents because they have no known effects on plant or animal cells, presumably because specific receptor proteins are required for toxin binding and activity. We assume these are not present on the surface of nonfungal cells. The UmV toxins can be effective against a wide range of *Ustilago* species known as crop pathogens, including pathogens of maize, wheat, oats, and barley (Koltin and Day, 1975; Koltin, 1986). The UmV toxins should therefore be very useful as a means of introducing novel resistance to the Ustilaginales into plants, provided the toxins can be expressed at high levels, are correctly processed, and can be targeted to the intercellular spaces.

We have cloned and sequenced the UmV KP6 toxin gene and have characterized its expression in a KP6-resistant *U. maydis* strain (Kinal et al., 1991) and in heterologous yeast (*S. cerevisiae*) cells (Tao et al., 1990). The KP6 toxin consists of two distinct polypeptides, α and β, that are produced by processing a single propolypeptide. These two polypeptides are not covalently associated and are secreted separately from *U. maydis* cells (Peery et al., 1987). The secreted α and β polypeptides are predicted to be 78 and 81 amino acids in length, respectively (Tao et al., 1990). The KP6 preprotoxin appears to be processed in a manner very similar to that of the yeast virus ScV k1 and k2 preprotoxins (Bostian et al., 1983, 1984; Schekman, 1985; Boone et al., 1986; Zhu et al., 1987; Tao et al., 1990; Dignard et al., 1991). A major difference, however, is that the mature KP6 α and β polypeptides remain separate polypeptides, unlike the k1 α and β subunits, which are disulfide linked (Peery et al., 1987). Because the KP6 α and β polypeptides are not covalently linked, it has been possible to assay their biological activity separately by complementation assays in vivo and in vitro using UmV KP6 non-killer mutants that produce only one of the two polypeptides (Tao et al., 1993). These assays have indicated that killing requires two separate steps. Binding of α does not cause any toxic effects in itself but is required for β recognition, which then results in the death of the cells (Peery et al., 1987; Ginsberg, 1992).

Processing the KP6 preprotoxin and other known killer preprotoxins requires the activities of the signal peptidase and at least one specific protease, Kex2p (Tao et al., 1990; Park et al., 1994). Kex2p-like enzymes have been identified in eukaryotes as disparate as yeast, Drosophila, Xenopus, and mammals (Brennan and Peach, 1988; Thomas et al., 1988, 1990, 1991; Fuller et al., 1989; Germain et al., 1990; Seidah et al., 1990; Smeekens and Steiner, 1990; Bourbonnais et al., 1991; Korner et al., 1991; Brenner and Fuller, 1992; Hayflick et al., 1992). The Kex2p protease is very highly conserved; the human and yeast enzymes are interchangeable (Thomas et al., 1988). The major cellular function of these proteases is the processing of polypeptide prohormones. Despite its universality in other eukaryotes, Kex2p protease activity has not yet been demonstrated in plants.

The KP6 protoxin has no activity without processing (J. Tao and J.A. Bruenn, unpublished results). Therefore, the systemic production of functional UmV killer proteins for plant protection would require that the host plant be able to process the protoxin accurately. We generated transgenic tobacco plants containing the coding sequence of a KP6 preprotoxin cDNA under the constitutive control of the cauliflower mosaic virus (CaMV) promoter and analyzed the processing, secretion, and activity of the KP6 toxin produced by the transgenic plants. We show here that the transgenic plants produced and systemically secreted KP6 toxin and that the plant-produced preprotoxin was correctly processed to the α and β polypeptides. In addition, the toxin secreted by the plant cells possessed the same killer activity and specificity as the toxin produced by P6 UmV-infected *U. maydis*. Our findings present evidence for a Kex2p-like processing pathway in plants and suggest a new method for engineering crop plants resistant to fungal pathogens.

RESULTS

Isolation of Transgenic Plants Expressing the KP6 Toxin α and β Polypeptides

The α and β polypeptide components of the KP6 toxin in *U. maydis* are encoded by a 1234-bp double-stranded RNA segment, designated P6M2 (Figure 1A). A single transcript produces the 219–amino acid KP6 preprotoxin, which is then processed to produce the 78–amino acid α and 81–amino acid β polypeptides. In virally infected *U. maydis* cells, processing of the KP6 protoxin by Kex2p occurs after the Pro-Arg residues at position 27 and the Lys-Arg residues at positions 107 (to generate α) and 139 (to generate β). A cDNA clone encoding the entire KP6 protoxin was obtained from the vector p6-25-29, which contains bases 5 to 1051 of P6M2 in the SmaI site of pUC19. Nucleotides 120 to 776 of this toxin clone encode the complete 219–amino acid KP6 preprotoxin.

The KP6 cDNA expression vector pBI1P6-12 (Figure 1B) was constructed using the Ti plasmid vector pBI121 (Bevan, 1984; Jefferson et al., 1987). pBI1P6-12 is 12 kb in length and contains a P6M2 expression cassette consisting of the 1046-bp KP6 preprotoxin cDNA under the control of the CaMV 35S promoter and terminator as well as the right and left T-DNA borders and nopaline synthase–neomycin phosphotransferase gene for kanamycin selection in plants. The P6M2 expression cassette is 2.5 kb in length and is expected to produce a 1.2-kb transcript encoding the entire KP6 protoxin polypeptide.

Tobacco strain SR1 was used as the recipient for Agrobacterium infection by leaf disc transformation (Horsch et al., 1985). Leaves wounded in the presence of Agrobacterium strains that contained the plasmid pBI1P6-12 or, as a control, pBI121 gave rise to calli at a ratio of one per 50 wounds. Eighty percent of the calli developed into plantlets and rooted into hormone-free media in the presence of kanamycin. Four of five of the plants transformed with the control plasmid pBI121 were kanamycin resistant and showed β-glucuronidase (GUS) activity throughout the stem and leaf tissue when assayed using

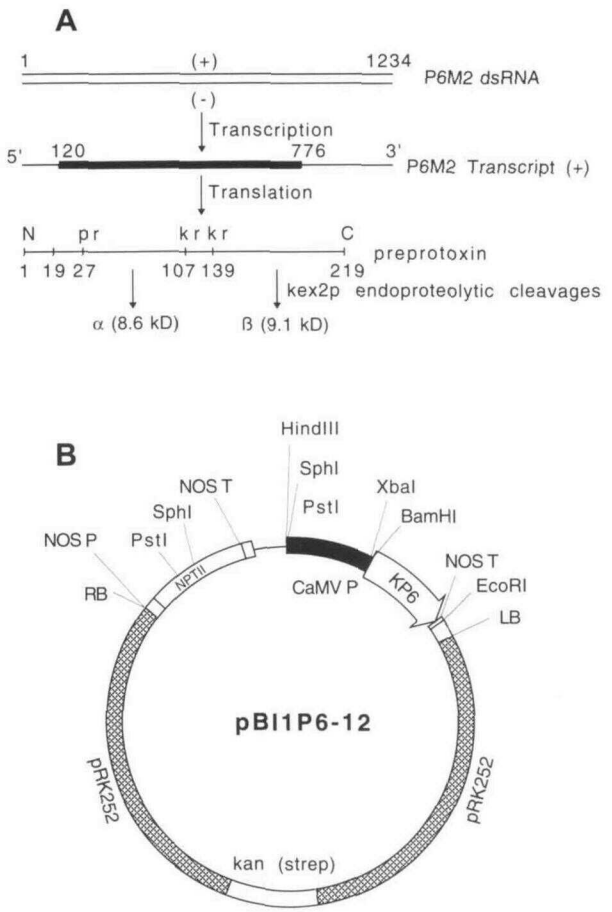

Figure 1. KP6 Toxin Expression and Expression Vector pBI1P6-12.

(A) *Expression of the KP6 toxin in U. maydis.* A 1234-base transcript, transcribed from the P6M2 double-stranded RNA (dsRNA) of the UmV P6 genome, is translated into the 219–amino acid preprotoxin. The preprotoxin is thought to be cleaved by signal peptidase after amino acid 19. Kex2p-like endoproteolytic cleavages occur after the Pro-Arg residues (pr) at position 27 and the Lys-Arg residues (kr) at positions 107 and 139. The C-terminal Lys and Arg of KP6 α are thought to be removed by a Kex1p-like cleavage (Tao et al., 1990), producing the mature α and β polypeptides. N, N terminus; C, C terminus; (+) and (−) indicate strands of dsRNA.

(B) Structure of pBI1P6-12. Construction of this vector for transformation of tobacco and transcription of the KP6 cDNA is described in Methods. CaMV P, CaMV promoter; kan (strep), kanamycin resistance gene from *Streptococcus*; LB, left border of the region integrated into plant genomes; NOS P, NOS promoter; NOS T, NOS terminator; NPTII, neomycin phosphotransferase gene from Tn5; RB, right border of the region integrated into plant genomes.

a histochemical staining procedure (Jefferson et al., 1987). Plants transformed with the KP6 toxin vector pBI1P6-12 were kanamycin resistant and showed no GUS activity.

Genomic DNA gel blot analysis was used to determine the presence of P6M2 cDNA in representative pBI1P6-12 primary transformant plants (F_0). The F_0 plants were self-fertilized, and the seed were harvested for the production of second generation (F_1) transgenic plants. The F_1 generation from one of the original pBI1P6-12 transformed plants, designated 12-2, was grown and analyzed in greater detail. Genomic DNA gel blot analysis for three of these F_1 generation plants, designated 12-2-8, 12-2-13, and 12-2-14, was performed. As a negative control, genomic DNA from the F_1 offspring of a self-fertilized plant transformed with the pBI121 vector was analyzed at the same time. Single-copy blot analysis indicated there was approximately one copy of the P6M2 fragment per genome in plant 12-2-14, consistent with a single integration event. The other two plants had multiple copies of the KP6 cDNA. It appears that a single copy of the integrating P6M2/T-DNA was present in one site in plant 12-2, whereas additional copies were inserted at a separate site on another chromosome. Transformant 12-2-13 appears to have received both P6M2/T-DNA–containing chromosomes.

Expression of KP6 cDNA in Tobacco

RNA gel blot analysis was used to determine if KP6 mRNA was transcribed from the P6M2 expression cassette in the transgenic plants. Total RNA was isolated from fully expanded leaves of the F_1 P6M2-transformed plants 12-2-8, 12-2-13, and 12-2-14. As controls, total RNA was isolated and analyzed from

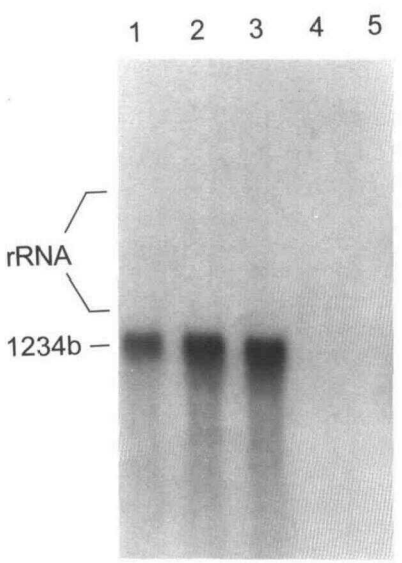

Figure 2. RNA Gel Blot of Tobacco RNA.

Twenty micrograms of total tobacco RNA per lane was electrophoresed on a 1.2% agarose–formaldehyde denaturing gel, transferred to nitrocellulose, and probed with radiolabeled DNA generated by random primed synthesis on a purified P6M2 cDNA fragment. Markers were the large tobacco rRNAs and denatured P6M2 (1234 bp), shown at left. Lane 1 contains RNA from plant 12-2-14; lane 2, RNA from plant 12-2-13; lane 3, RNA from plant 12-2-8; lane 4, RNA from a plant transformed with vector alone; lane 5, RNA from the parent plant (SR1).

the F_1 progeny of a plant transformed with p121 as well as from untransformed SR1 plants. Equal amounts of total RNA from each plant were electrophoresed on a denaturing agarose gel, transferred to nitrocellulose, and probed with ^{32}P-labeled gel-purified P6M2 cDNA. Figure 2 shows that F_1-transformed plants 12-2-14 (lane 1), 12-2-13 (lane 2), and 12-2-8 (lane 3) all demonstrated hybridization to the P6M2 probe. A single band of ~1.2 kb was detected in each of the P6M2 transformants, consistent with the expected size of the CaMV promoter transcript, including the P6M2 sequence. Total RNA from the original F_0 plants 12-2 and 12-3 also showed a 1.2-kb band that hybridized to the P6M2 probe (data not shown). As expected, the negative controls, RNA from a plant transformed with pBI121 (lane 4) or the SR1 parent plant (lane 5), did not hybridize to the P6M2-specific probe. Therefore, the P6M2 expression cassette produced the expected KP6 transcript in the original F_0 transformant plants and in the F_1 progeny.

Equal amounts of total RNA from each plant were loaded in each lane of the gel shown in Figure 2. However, less P6M2 mRNA was detected in plant 12-2-14 when compared with plants 12-2-13 and 12-2-8. The difference in transcript level among these sibling plants appears to correlate with differences in P6M2 copy number (as given earlier). It is also possible that, due to differences in P6M2/T-DNA integration sites, position effects might have contributed to differences in transcription from the P6M2 expression cassette in the sibling plants.

Synthesis of Functional KP6 Toxin in Tobacco

Because KP6 mRNA was present in the transformed plants, we asked if functional KP6 toxin was present as well. Leaf extracellular fluids were isolated using vacuum infiltration (Parent and Asselin, 1984; Carr et al., 1987) and then concentrated 200-fold. The total amount of intercellular protein was standardized by Coomassie Brilliant Blue R 250 staining intensity subsequent to SDS-PAGE. The presence of the KP6 toxin in the concentrated extracellular leaf extracts was determined using a functional killer assay. Concentrated extracts containing equal amounts of total protein from three F_1-transformed plants, and from control plants that do not express P6M2 mRNA, were placed onto KP6-sensitive and KP6-resistant lawns of *U. maydis*.

As shown in Figure 3A, the concentrated extracellular extracts from three F_1-transformed plants had easily detectable KP6 killer activity (Figure 3A, spots 1, 2, and 3). The concentrated leaf extracts from P6M2-transformed plants all produced a zone of inhibition, on the sensitive *U. maydis* lawn, of morphology similar to that observed with the authentic KP6 toxin secreted by virally infected *U. maydis* cells (Figure 3A, spot 7). The plant infiltration extracts had much less toxin activity than did media from wild-type KP6 *U. maydis* strains (see later discussion). There were some differences in the size of the kill zone produced by equal amounts of infiltration extract from different sibling plants. Plant 12-2-14 (Figure 3A, spot 3) had a weaker kill zone than its sibling plants 12-2-8 (Figure 3A, spot 1) and 12-2-13 (Figure 3A, spot 2). This is consistent with the results of DNA gel blot and RNA gel blot analysis indicating that 12-2-14 has fewer copies of the P6M2 expression cassette and lower levels of P6M2 transcript than the other two plants have.

Figure 3B demonstrates that the KP6 toxin synthesized in tobacco (Figure 3B, spots 1, 2, and 3), like the KP6 toxin produced in *U. maydis* (Figure 3B, spot 7), does not kill a lawn of KP6-resistant *U. maydis*. Therefore, the KP6 toxin produced in the transgenic plants maintained the same strain specificity

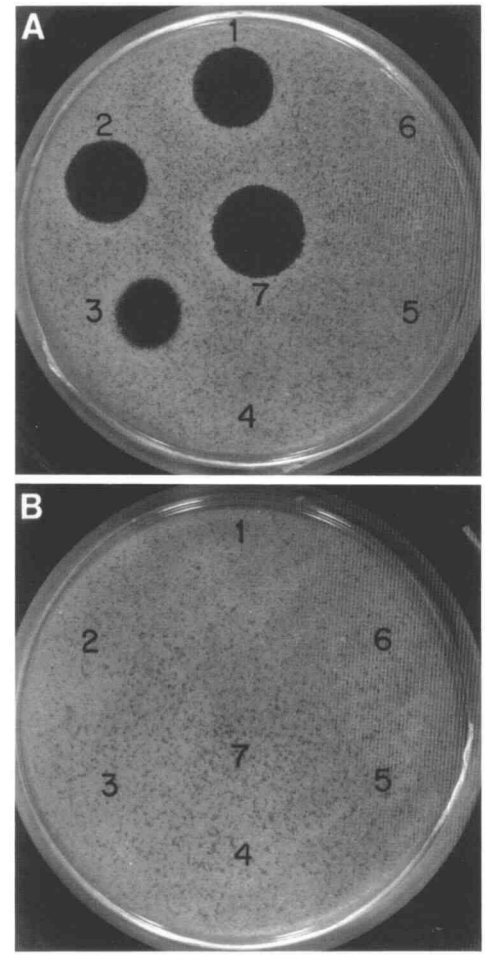

Figure 3. Killer Toxin Assay of Secreted Proteins from Tobacco Transformants.

Spot tests of concentrated toxin preparations came from the following: plant transformant 12-2-8, spot 1; plant transformant 12-2-13, spot 2; plant transformant 12-2-14, spot 3; the parent plant SR1, spot 4; a plant transformed with the vector without any cDNA insert, 121, spot 5; a plant transformed with a vector with the cDNA in the reverse orientation, 6P7, spot 6; *U. maydis* strain 75-1, the wild-type KP6 killer, spot 7.
(A) A KP6-sensitive *U. maydis* lawn (strain 54).
(B) A KP6-resistant *U. maydis* lawn (strain 75U1).

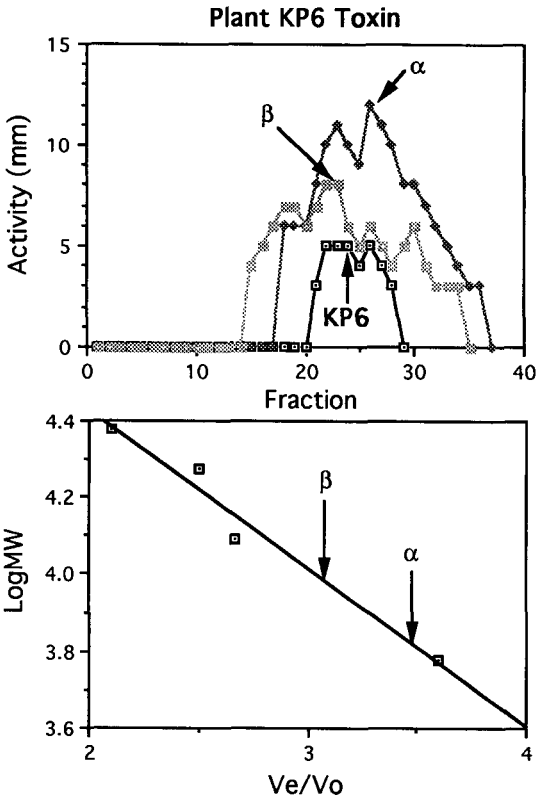

Figure 4. Sephadex G50 Chromatography of the Toxin Made in Tobacco.

Total KP6 activity was measured in a standard plate assay and quantified by the diameter of the kill zone. The activities of the α and β polypeptides were measured separately by complementation with added authentic *U. maydis* KP6 β or α polypeptides, respectively. The molecular masses of the α and β polypeptides were calculated from their elution positions, as shown in the lower curve: α had an apparent molecular mass of 6.7 kD and β an apparent molecular mass of 9.7 kD. Size markers were trypsinogen (24 kD), myoglobin (18.8 kD), cytochrome *c* (12.4 kD), and aprotinin (6 kD). The logarithm of the molecular mass (logMW) is given as a function of the ratio of elution to void volume (Ve/Vo). Arrows indicate elution position.

as that of the authentic KP6 toxin produced by virally infected *U. maydis* cells. The similarly prepared negative controls, spot 4 from the untransformed SR1 plant, spot 5 from the pBI121 (vector alone) transformed plant, and spot 6 from a plant with the P6M2 cDNA in reverse orientation in the expression vector, all failed to display any toxin activity on either lawn.

Although the transgenic plants produced significant amounts of killer activity with the same specificity as the authentic UmV KP6 toxin, we estimated that the transformed plants were ~0.03% as efficient in producing active secreted toxin, based on micrograms of toxin polypeptides recovered per gram wet weight of cells (see the following section). The lower relative killer activity in plant extracts may reflect the fact that infected *U. maydis* cells contain many copies of the P6M2 segment of the double-stranded RNA virus and therefore possess many more copies of the KP6 toxin gene than do transgenic tobacco cells. Alternatively, the lower relative activity in transgenic plants could be due to differences in processing or secretion of the toxin, to losses by proteolysis, or to less efficient recovery by plants from vacuum infiltration. It is probably not due to inhibition by other components of the plant extracts, because adding these components to authentic KP6 toxin has no effect on its activity (see the following section).

In contrast to these results, extracts of intracellular proteins isolated from the same leaf strips after vacuum infiltration, and similarly concentrated and assayed, had no detectable killer activity (data not shown). In the transgenic plants, as in *U. maydis* cells, the KP6 toxin may be processed and secreted very rapidly, so any slight amount of toxin remaining inside the cells would be below the level that could be detected by this functional killer assay. Alternatively, significant amounts of the unprocessed KP6 toxin remaining inside the plant cells are unlikely to have any killer activity and therefore would not be detected by this assay. In either case, our findings demonstrate that in the P6M2-transformed tobacco plants, as in P6M2-containing *U. maydis* cells (Peery et al., 1987; Koltin, 1988), KP6 killer activity could be detected only in secreted polypeptides.

Processing the KP6 Preprotoxin in Tobacco

Virally infected *U. maydis* strains show no killer activity unless the KP6 protoxin is processed into the α and β polypeptides (Peery et al., 1987; Tao et al., 1993). Therefore, the presence of killer activity in the extracellular leaf extracts implies that the KP6 protoxin is correctly processed as well as secreted in the transgenic tobacco plants. The specific endoprotease (Kex2p) necessary for processing the KP6 preprotoxin (Peery et al., 1987; Tao et al., 1990) has not previously been shown to occur in plants. We purified the KP6 β polypeptide to ascertain whether processing in tobacco was identical to that in *Ustilago.*

To generate the large amount of leaf material required for purifying the KP6 toxin, the F_1 plants 12-2-8 and 12-2-13 were selfed, and a large number of F_2 offspring were grown to maturity. The F_2 progeny were harvested separately and their intercellular fluid isolated as described previously. Toxin preparations from these tobacco plants were analyzed by gel filtration chromatography using Sephadex G50 (Sigma) according to methods previously developed for analyzing the authentic KP6 toxin (Tao et al., 1990). The column was calibrated with various protein standards (Figure 4) and with the authentic KP6 α polypeptide isolated from *U. maydis* strain NK13 (a mutant strain that produces only the α polypeptide) and the β polypeptide isolated from *U. maydis* strain NK3 (a mutant strain that produces only the β polypeptide). To determine whether plant proteins affected the migration of the KP6 peptides, authentic purified KP6 toxin was chromatographed in the presence and absence of extracts from the nontransformed control plants.

For the experiments shown in Figure 4, KP6 activity was used to determine the presence of the KP6 toxin in each of the column fractions. The α and β polypeptides are very similar in molecular mass and therefore have slightly different but overlapping migration rates in the sizing column. Because KP6 killer activity requires both polypeptides, the presence of one polypeptide can be assayed by adding the complementary polypeptide. Using this complementation assay, the location of α and β polypeptides in the eluant was determined (Figure 4). The broad activity peaks resulted from overlap in the peaks of the two polypeptides. In the overlapping region, both α and β were present in the same fractions and therefore showed activity even in the absence of complementing polypeptides. Results with the authentic KP6 toxin were essentially identical. Using this methodology, the sizes of the β and α polypeptides from the transgenic plants (as well as from *U. maydis*) were calculated to be 9.7 and 6.7 kD, respectively. These sizes compare with the calculated molecular masses of 9.1 kD (β) and 8.6 kD (α) and the sizes estimated by SDS-PAGE of 9 and 7.5 kD, respectively (Tao et al., 1990).

As shown in Figure 4, the migration of purified UmV KP6 was identical in the presence or absence of added plant extracts, indicating that components in the plant extracts do not affect the migration or the activity of the KP6 toxin polypeptides. Toxin from the transgenic plant extracts showed identical migration through the column, as did the authentic UmV toxin.

The presence and size of the β polypeptide synthesized in the tobacco transformants was also estimated by protein gel blot analysis, using a polyclonal antibody raised against the authentic KP6 β polypeptide. To prepare the extracts for protein gel blot analysis, intercellular fluid from the F_2 progeny of the toxin-producing plant 12-2-8 was first partially purified and concentrated by ammonium sulfate precipitation and CM-Sephadex C25 chromatography (Peery et al., 1987). The presence of functional KP6 α and β polypeptides in these concentrated effluents was confirmed by the killer plate assay using complementing peptides, as described earlier. As controls, the intercellular fluid from control plant SR1 and the authentic UmV KP6 toxin were treated identically and were run at the same time. The authentic KP6 α and β polypeptides isolated from *U. maydis* were shown to migrate through the CM-Sephadex C25 column with the same mobilities as the α and β polypeptides isolated from tobacco. Peak fractions identified by complementation assays as containing the plant-produced β polypeptide were loaded on an 18% SDS–polyacrylamide gel and electrophoretically transferred to nitrocellulose. The filter was probed with a rabbit polyclonal antibody raised against the SDS-PAGE–purified UmV KP6 β polypeptide.

Partially purified toxin from plant 12-2-8 has only one polypeptide that reacts with anti–KP6 β antibody, a polypeptide that comigrates with the authentic KP6 β polypeptide (Figure 5). Proteins isolated from control plants and purified and concentrated in the same manner showed no reaction to the KP6 β polypeptide–specific antibody (data not shown).

We further purified the KP6 β polypeptide from the transgenic tobacco plants by ammonium sulfate precipitation, CM-Sephadex C25 chromatography, denaturing Sephadex G50 chromatography, and affinity chromatography on an anti–KP6 β-polypeptide antibody column. We determined the resultant KP6 β polypeptide to be at least 60% pure. The size of the purified protein was determined by electrospray mass spectroscopy. The result is shown in Figure 6. The protein appeared to be ~85% pure and was 9126 D, in comparison with a size of 9127 D for the authentic KP6 β polypeptide and a calculated size of 9132 D based on the known sequence of the P6M2 cDNA (or 9126 D, assuming all three possible disulfides). This shows unequivocally that the predicted Kex2p cleavage, which creates the N terminus of the KP6 β polypeptide, occurs in exactly the same place in tobacco as in *Ustilago* and that no post-translational modifications of the β polypeptide other than this cleavage occur in either system. This result was confirmed by N-terminal sequence analysis, which demonstrated the same sequence (five residues determined) at the

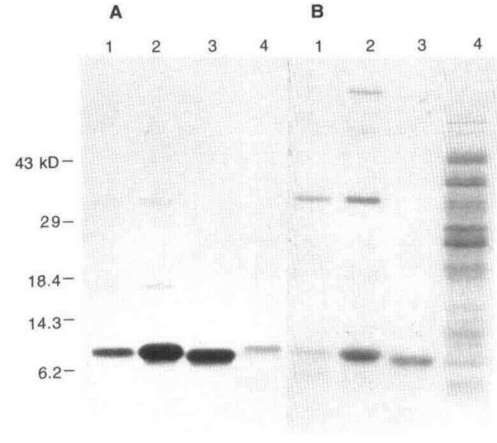

Figure 5. Protein Gel Blot Analysis of KP6 Toxin Produced in Tobacco.

An 18% SDS–polyacrylamide gel was run, and protein gel blot analysis was performed. **(A)** and **(B)** show identical gels.

(A) Protein gel blot.

(B) Gel blot following SDS-PAGE.

Lanes 1 contain proteins from the medium in which a KP6 killer strain (75-1) was grown. The proteins were precipitated with 70% saturated ammonium sulfate after removal of contaminants with precipitation with 40% saturated ammonium sulfate, and the pellet was dissolved in deionized water and dialyzed against water. Fifteen microliters of a sample concentrated 10 times was loaded. Lanes 2 contain the authentic KP6 β polypeptide purified by CM-Sephadex C25 chromatography. Five microliters of a sample concentrated 200 times was loaded. Lanes 3 contain the KP6 β polypeptide purified by ammonium sulfate precipitation, CM-Sephadex C25 ion exchange chromatography, and denaturing Sephadex G50 chromatography. Fifteen microliters of a sample concentrated 100 times was loaded. Lanes 4 contain concentrated KP6 toxin from the intercellular fluid of transgenic tobacco plants (12-2-8) that was partially purified by ammonium sulfate precipitation and CM-Sephadex C25 chromatography. Thirty microliters of a sample concentrated 6000 times was loaded. Molecular mass markers in kilodaltons are given at left.

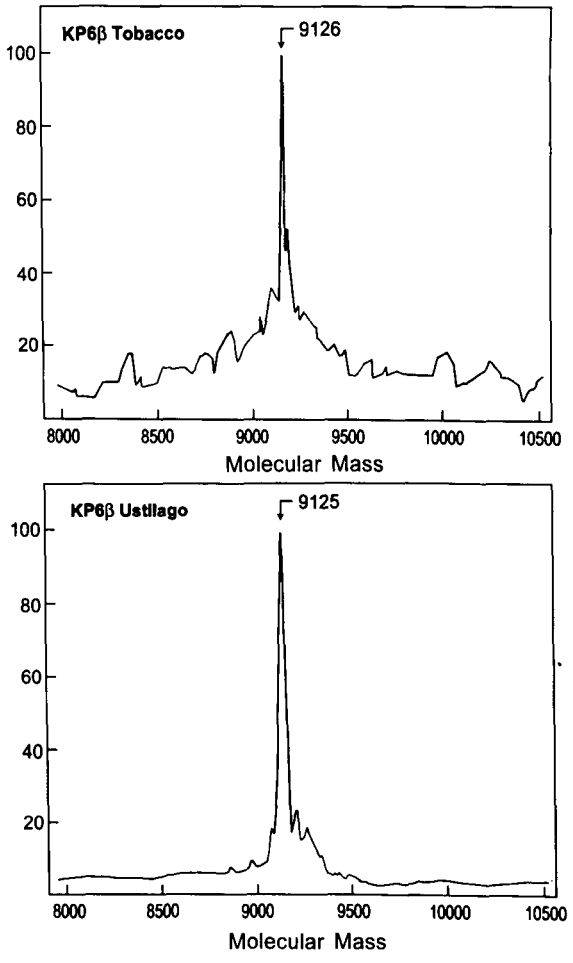

Figure 6. Mass Spectroscopy of Purified KP6 β Polypeptides.

Deconvoluted electrospray mass spectrographic analysis of the KP6 β polypeptide from tobacco and from *Ustilago* (courtesy of the Harvard Microchemical Facility, Cambridge, MA) is shown. The ordinate gives the amount of protein in arbitrary units, and the abscissa gives the molecular mass.

N terminus of the authentic KP6 β and the tobacco KP6 β polypeptides (W. Lane, personal communication). The sequence was the same as that expected to be produced by Kex2p-like cleavage (see Discussion).

We also believe that the functional form of the KP6 α polypeptide produced in tobacco is identical in size to the authentic KP6 α polypeptide, as indicated by Sephadex G50 sizing chromatography followed by complementation with the β polypeptide (Figure 4). Although the α polypeptide contains a possible site for N-linked glycosylation, the polypeptide is normally not glycosylated in *U. maydis*. However, the α polypeptide has been shown to be glycosylated when it is expressed in heterologous yeast cells (Tao et al., 1990). Our results indicate that the functional α polypeptide is not glycosylated in tobacco, because this would cause a migration difference on the column such that α would appear larger than β (Tao et al., 1990). Taken together, our results indicate that the KP6-specific toxin activity seen in transgenic plants expressing the P6M2 cDNA is the result of properly processed α and β polypeptides and that the N-glycosylation signal on the α polypeptide is, at least, not always utilized by the transgenic plants.

Finally, we have introduced mutant KP6 cDNAs containing mutations that prevent Kex2p processing in yeast in our standard expression vector construct into tobacco. The NK3 and NK13 preprotoxins are processed to form only the β or α polypeptides, respectively, in *Ustilago* (Tao et al., 1993), but they are not processed at all in yeast (J. Tao and J.A. Bruenn, unpublished data). We placed these cDNAs in our expression vector in transgenic tobacco plants and verified transcription, and we determined that, as in yeast (C.-M. Park and J.A. Bruenn, unpublished data), no toxin polypeptides are produced. Hence, the processing system in tobacco is very similar to that in yeast; this system is known to be the result of Kex2p cleavage (Park et al., 1994). These findings provide novel evidence for a Kex2p-like protease activity in plants that is capable of processing secreted polypeptides, such as the KP6 protoxin.

DISCUSSION

Transgenic tobacco plants produce, process, and secrete a *U. maydis* protein toxin, normally encoded by a virus that infects the fungal cells, when the viral toxin gene is placed under the control of the constitutive CaMV promoter. Transcripts encoding the 219–amino acid preprotoxin of the *U. maydis* KP6 killer toxin were translated in plant cells, producing a protein product that appears to have been recognized by a processing and extracellular targeting pathway not previously known to occur in plants. Two lines of evidence indicate that the KP6 protoxin produced in tobacco cells is processed by mechanisms analogous to those that process the toxin in *U. maydis*. First, correct processing of the protoxin is required for toxin activity, and the toxin polypeptides produced by the transgenic plants possess the same killer activity and specificity as that found in toxin secreted by *U. maydis* cells. The authentic KP6 α polypeptide complemented the tobacco β polypeptide, and the authentic KP6 β polypeptide complemented the tobacco α polypeptide. Second, the protoxin was post-translationally processed into two polypeptides that corresponded in size to the α and β subunits of the active toxin produced by virally infected *U. maydis*. In particular, the tobacco β polypeptide was shown to be exactly the same in size as the authentic KP6 β, within the margin of error of mass spectroscopy (~5 D at this mass). The N-terminal sequence of the tobacco β polypeptide also agreed with that predicted by Kex2p-like cleavage. These findings indicate that the *U. maydis* KP6 toxin has processing signals and extracellular targeting signals that can be recognized in both plants and fungi.

The Kex2p protease is required for processing the KP6 preprotoxin (Park et al., 1994) and has been identified in eukaryotes as disparate as yeast, insects, and mammals. We have previously demonstrated that secretion and processing of the KP6 toxin occur with complete fidelity in a heterologous fungal yeast system, except that the α polypeptide, which has one possible N-linked glycosylation site, is glycosylated in yeast but not in *U. maydis* (Tao et al., 1990). Mutants of *S. cerevisiae* that lack the Kex2p enzyme are incapable of producing active KP6 toxin (Park et al., 1994), thus confirming that the heterologous Kex2p enzyme can function and is, in fact, required to produce active KP6 toxin in yeast cells.

Although there have been no reports demonstrating the existence of the processing enzyme Kex2p in plants, the ubiquitous Kex2p processing of secreted proteins in other eukaryotes certainly prepares us for its existence in plants as well. The exact processing of the KP6 preprotoxin in transgenic tobacco plants provides novel experimental evidence that plants possess a protease with Kex2p-like activity responsible for processing secreted proteins.

The Kex2p-like activity in tobacco has precisely the same primary structure specificity observed with all Kex2p-processed fungal proteins, in which the recognition site is XNKR or XNPR, where X is a hydrophobic residue and N any residue (Park et al., 1994). The cleavage site creating KP6 β is IGKR*GKRPR, where the asterisk indicates the cleavage site after the first dibasic site. The next two possible basic sites (RGKR and KRPR) are not recognized in tobacco, yeast, or *Ustilago.* Even one fewer amino acid in the KP6 β polypeptide made in tobacco would have been easily detectable by mass spectroscopy, and the β polypeptide N terminus found by sequence analysis was the one predicted.

Clearly, however, there are secondary and/or tertiary structure cues utilized by Kex2p-like enzymes as well, and these must differ in tobacco and yeast, on the one hand, and *Ustilago*, on the other, because the NK3 and NK13 preprotoxins, which are processed only partially in *Ustilago* due to missense mutations outside the Kex2p recognition sites (Tao et al., 1993), are not processed at all in *S. cerevisiae* or in tobacco. This demonstrates that the processing system responsible for KP6 processing in tobacco must be very similar to that in yeast. Because we know that Kex2p is responsible for processing the KP6 preprotoxin in yeast (Park et al., 1994), we conclude that a Kex2p-like enzyme must exist in tobacco.

A normal function of the Kex2p-like processing activity in plants, and one major cellular function of these proteases, is the processing of small polypeptide prohormones. The single-known plant peptide with properties similar to those of the Kex2p-like–processed polypeptide hormones is systemin, a small polypeptide shown to be involved in triggering tomato plant defense mechanisms (Pearce et al., 1991). Preprosystemin contains a single possible Kex2p cleavage site, which happens to fall in the mature peptide and is therefore not cleaved during maturation. This site has the sequence PSKR (Pearce et al., 1991; McGuri et al., 1992) and by our previous predictions should not be cleaved. Whereas "preprosystemin" does not appear to be processed by any Kex2p-like events, the presence of the Kex2p-like processing site in the mature peptide has led to speculation that a Kex2p-like enzyme may play a role in its regulation (McGuri et al., 1992). A possible Kex2p-like protein (SBP50) that binds to systemin and may cause its cleavage has been identified (Schaller and Ryan, 1994). However, given the demonstrated specificity of the tobacco Kex2p-like activity, SBP50 is probably not responsible for processing the KP6 toxin. The tobacco Kex2p-like activity may imply the existence of a whole class of secreted polypeptides in plants so far undetected.

In addition to processing by a Kex2p-like protease, the KP6 preprotoxin was recognized by the tobacco signal recognition particle, allowing the heterologous secreted fungal protein to be correctly targeted to the extracellular space. This result was not surprising because the N terminus of the preprotoxin has a typical signal polypeptide sequence, with a signal peptidase cleavage site after an alanine residue at position 19 (von Heijne, 1985, 1986; Tao et al., 1990). Protein targeting and secretion have been studied in many plants; the best characterized secretion pathway is that which targets proteins to the vacuole (Chrispeels, 1991). We found no evidence for KP6 killer activity in extracts of intracellular proteins, which suggests that the KP6 polypeptides are not targeted to the plant cell vacuole. Because the fungal toxin polypeptides are correctly targeted to the outside of the cells in transgenic tobacco, these proteins are very useful as easily detectable heterologous marker proteins for investigating the steps involved in the extracellular secretion and targeting of plant proteins.

Tobacco plants are capable of correctly synthesizing, processing, and secreting KP6 toxin polypeptides with the same activity and specificity as the authentic KP6 toxin produced by *U. maydis.* We could not determine whether such transgenic plants are resistant to KP6-sensitive strains of *U. maydis*, because tobacco is not normally susceptible to *U. maydis* infection. However, many economically important crop plants, including maize, wheat, oats, and barley, suffer from Ustilaginales infections (Koltin and Day, 1975). If agriculturally important varieties of maize or other crop plants could be produced that possess KP6 toxin activity, increased resistance to many strains of smut might be obtained. Transgenic maize plants expressing genes for two different UmV toxins could be resistant to all but a fraction of a percentage of the natural *U. maydis* population. Nearly complete resistance for all *U. maydis* strains could be attained in a maize plant transgenic for all three known UmV toxins, given that the three resistance genes are independent and recessive (Day and Dodds, 1979). Because the UmV toxins have no toxic effects on cell types other those of the Ustilaginales, they would be safe for consumption and would have less environmental impact than the fungicides currently used to deter fungal pathogens.

Using tobacco as a model system, we determined that active antifungal toxin proteins can be synthesized and targeted to the outside of transgenic plant cells. If this methodology can

be applied to other agronomically important crop species, then the *U. maydis* UmV toxins may provide a novel means for the biological control of pathogenic fungi.

METHODS

Vector Construction

The vector pBI1P6-12 was constructed from the KP6 cDNA vector p6-25-29, containing bases 5 to 1051 of the P6M2 plus strand in the SmaI site of pUC19, and from the two CLONTECH (Palo Alto, CA) vectors pBI121 and pBI221, which were derived from the pBIN19 vector (Bevan, 1984; Jefferson et al., 1987). The pBIN19 vector has a core sequence from pRK252, a wide host-range vector for prokaryotes (Bevan, 1984). The vector pBI121 has a kanamycin resistance gene from *Streptococcus* for selection in bacteria and a neomycin phosphotransferase II gene from Tn*5* under the control of the nopaline synthase promoter and terminator for kanamycin selection in plants. It has a β-glucuronidase (*GUS*) gene under control of the cauliflower mosaic virus (CaMV) 35S promoter and terminator. In addition to these elements, pBI121 also has the Ti right and left borders for Ti-mediated integration into plant genomes. The KP6 cDNA, as a BamHI-KpnI fragment, was blunt ended and cloned into SmaI-SstI–cleaved and blunt-ended pBI221, replacing the *GUS* gene in this construct. The *GUS* gene cassette in pBI121 was then replaced with a HindIII-EcoRI fragment from the same region of pBI221, with the KP6 cDNA replacing the *GUS* gene. The resultant construct, pBI1P6-12, is diagrammed in Figure 1B. The total length of the vector is ~12 kb. Vector constructs were verified by restriction mapping and dideoxynucleotide sequencing.

Transformation

Transformation of *Escherichia coli* DH5α was done by the $CaCl_2$ method as described by Sambrook et al. (1989). Plasmid DNAs were isolated from the transformants and verified by restriction enzyme mapping and sequencing. Transformation of *Agrobacterium tumefaciens* LBA4404 was done by the freeze–thaw method (Holsters et al., 1978). Plasmid DNA was isolated from the Agrobacterium transformants, CsCl purified, restriction digest mapped, and sequenced to ensure the integrity of the transforming vectors. Transformation of *Nicotiana tabacum* was by the tobacco leaf disc transformation method as described by Horsch et al. (1985).

DNA Isolation

Plasmid DNAs were isolated from *E. coli* and Agrobacterium using the alkaline lysis procedure as described by Sambrook et al. (1989). DNA from Agrobacterium for sequencing was further purified by CsCl–ethidium bromide equilibrium gradient centrifugation. Genomic DNA was isolated from tobacco leaves as described by Dellaporta et al. (1983).

DNA Gel Blot Hybridization

Ten micrograms of tobacco genomic DNA was digested with five units of restriction enzyme in 20 μL of the appropriate buffers per μg of strate DNA at 37°C for 5 hr. Gel electrophoresis (in 0.8% agarose), depurination, transfer, and hybridization were performed as described previously (Klessig and Berry, 1983; Sambrook et al., 1989). The probe was P6M2 cDNA labeled by random primer labeling.

RNA Gel Blot Hybridization

Total plant RNA was isolated as described by Berry et al. (1985). Twenty micrograms of total RNA from tobacco was used in each lane of a formamide–formaldehyde denaturing gel and transferred to nitrocellulose as described previously (Berry et al., 1985; Sambrook et al., 1989). The probe used was the same as that used for the genomic DNA gel blot (as given earlier).

Killer Tests

Killer plate assays were performed as previously described (Tao et al., 1990). Strains used for lawn cells were 54, a KP6-sensitive strain, or 75U1, a KP6-resistant strain.

Gel Exclusion Chromatography

Dialyzed intercellular fluid from transgenic tobacco plants was concentrated 200 times by lyophilization, and 0.2 mL was loaded on a Sephadex G50 (Sigma) column 24 cm in height, with a bed volume of 19 mL. The void volume was determined with blue dextran and BSA (66 kD). Tris-EDTA (TE) (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) was used as the eluate buffer. Forty 0.5-mL fractions were collected, dialyzed, and concentrated 10 times by lyophilization. Five microliters of each concentrated fraction was then spotted onto lawns of *Ustilago maydis* strains 54 or 75U1, and complemented with 5 μL of medium from either *U. maydis* strain NK3 or NK13 concentrated 50 times on lawns of strain 54. As a positive control, 0.2 mL of concentrated (25 times) KP6 toxin in TE was run over the same column under the same conditions. The P6 column fractions, however, were not concentrated but were used directly for the same killer plate assay as the intercellular fluid fractions. To determine molecular size, 1 mg each of aprotinin (6 kD), cytochrome *c* (12.4 kD), myoglobin (18.8 kD), and trypsinogen (24 kD) in 0.2 mL of TE were loaded separately onto the same column under the same conditions. The eluate volumes of these molecular-size standards were determined spectrophotometrically by reading the absorbance of their column fractions at A_{280}.

Vacuum Infiltration

Tobacco plants were grown for 6 weeks up to 1.5 feet in height, and leaves were harvested. Up to 200 g of leaves could be harvested from each flat (30 × 40 cm^2). Intercellular fluid was extracted by the vacuum infiltration method (Carr et al., 1987). Each extraction consisted of 2.4 L of intercellular fluid from ~2.4 kg of leaves (12 flats). The extracted intercellular fluid was precipitated with 35% ammonium sulfate at 4°C overnight and centrifuged at 6000*g* for 20 min. The supernatant was precipitated again with 70% ammonium sulfate. After centrifugation at 8000*g* for 30 min, the pellet was dissolved in one-fiftieth of the original volume of double-distilled H_2O (ddH_2O), dialyzed extensively against ddH_2O at 4°C with frequent changes of water, and

lyophilized to dryness. The dry pellet was redissolved in 20 mL of 1 × column running buffer (25 mM acetic acid, pH 5.5, with NaOH).

Ion Exchange Chromatography

The CM-Sephadex C25 column (Sigma) had a bed volume of 150 cc (25 cm in height). The concentrated sample was loaded onto a column equilibrated with five volumes of running buffer (25 mM acetic acid, pH 5.5, with NaOH). After washing with five volumes of running buffer and three volumes of 0.1 M NaCl, fractions (10 mL each) were eluted with five volumes of a 0.2 to 0.6 M NaCl continuous gradient. Fractions were concentrated 12 times through dialysis and lyophilization and spotted on KP6-sensitive lawn plates, together with either *Ustilago* NK3 (active β polypeptide) or NK13 (active α polypeptide) concentrated culture supernatant, to determine the peak fractions (killer activity). The α and β polypeptide peak fractions were then pooled, extensively dialyzed against ddH_2O at 4°C, lyophilized to dryness, and dissolved in 400 μL of ddH_2O (~6000 times concentration).

SDS-PAGE and Protein Gel Blot Transfers

Five microliters of each sample, which had been partially purified and concentrated, was analyzed on an 18% SDS–polyacrylamide gel, as described previously (Diamond et al., 1989; Tao et al., 1990). Semidry transfer was made to a 0.1-μM nitrocellulose membrane at 0.8 mA/cm^2 for 1 hr. After treatment with a polyclonal anti-β antibody (1:500 dilution), the membrane was probed with protein A–conjugated alkaline phosphatase (1:1500 dilution), as directed by the supplier (Cappel, Organon Teknika Corp., Durham, NC).

Purification of Monoclonal Antibody

Anti–KP6 β monoclonal antibody was purified from ascites fluids by a combination of caprylic acid (Sigma) and ammonium sulfate precipitations as described previously (McKinney and Parkinson, 1987). Caprylic acid (2.5%) and ammonium sulfate (45%) were used for precipitations. The purity of the monoclonal antibody was 90% or higher, based on SDS-PAGE.

Immunoaffinity Chromatography

Activated CH-Sepharose 4B (Pharmacia Biotechnology) was used as a resin for the anti–β monoclonal antibody. Dry resin (0.3 g) was hydrated and rinsed with ice-cold 1 mM HCl. After filtration, the wet resin was suspended in coupling solution (for 5 mL, 380 mg of NaCl, 100 mg of $NaHCO_3$, 500 mg of monoclonal antibody) and rotated at room temperature for 60 min. Any remaining functional groups were completely blocked by adding 1 mL of 0.1 M Tris-Cl, pH 8.0, and rotating an additional 60 min. A column of 0.5 mL of bed volume was prewashed with three volumes of 0.1 M acetic acid, pH 4.0, and of 0.1 M Tris-Cl, 0.5 M NaCl, pH 8.0. These two washes were repeated two more times before sample loading. The plant intercellular fluid, partially purified and concentrated through ion exchange chromatography, was fractionated by G50 Sephadex chromatography under denaturing conditions (8 M urea). The purified KP6 β activity was dialyzed against ddH_2O and lyophilized. The monoclonal antibody affinity column was prerun with four volumes of running buffer (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA). The sample in running buffer was loaded and incubated for 10 min. Sample loading and incubation were repeated three more times. The column was washed with five volumes of running buffer and eluted with two volumes of 4.5 M $MgCl_2$. The eluted sample was dialyzed thoroughly against ddH_2O at 4°C and concentrated by lyophilization for analysis.

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