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Expression of a spider venom peptide in transgenic tobacco confers insect resistance

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ABSTRACT

Spider venom contains a mixture of peptide toxins, some able to kill insects specifically to those considered as important pest. In this study, a peptide toxin produced by the *Macrothele gigas* spider, Magi 6, was cloned and expressed in tobacco plants, as this toxin has been shown to constitute an effective insecticide. For this purpose, a genetic construction for the cDNA that codifies for Magi 6 was subcloned in a plant expression vector using the *35S* promoter and the 5′-end leader from tobacco mosaic virus, in order to transform tobacco leaf disks. The resulting plants demonstrated the presence of *Magi 6* gene in the tobacco genome using PCR, and transcription of the cDNA was verified by means of RT-PCR. The expression of the Magi 6 peptide in tobacco was demonstrated by Western blot, which exhibited the expected size, thus suggesting a correct processing of the signal peptide. No morphological alterations in the different transgenic lines were observed, nor any change in plant growth. Subsequently, experiments were carried out challenging detached leaves or whole plants with the herbivorous insect *Spodoptera frugiperda*. The bioassays indicated that the transgenic lines were significantly more resistant than the wild type plants. This work demonstrated that the expression of Magi 6 peptide in transgenic plants conferred resistance to insect attack and opens the possibility of employing this peptide to improve the resistance of diverse plants.

1. Introduction

Plagues are a serious threat to agriculture, causing huge losses to farming. For a number of decades chemical pesticides of diverse origin have been used to combat insects, many of which are neurotoxic and carcinogenic, as well as acting as persistent contaminants. Furthermore, insect plagues have become resistant to many chemical pesticides after many years of use.

A possible alternative solution to the problem posed by agrochemicals is biological control or genetically modified plants, in order to decimate insect plagues. To date, the most common organism used as a bioinsecticide is *Bacillus thuringiensis* (*Bt*), which codifies for the insecticidal Cry proteins (Bates et al., 2005). The Cry δ-endotoxins form crystals, which are proteolitically processed and dissolved in the insect intestine, binding to the cellular membranes of the epithelium, in order to form ion channels that induce cell osmotic lysis and cause death to the insect (Bravo et al., 2007).

During the last decade, the employment of transgenic plants expressing Cry proteins has represented one of the most important advances in agricultural biotechnology (Ferry et al., 2006). Even though cases of insects resistant to transgenic plants expressing *cry* genes have not been detected in the field, it is recommendable that a variety of strategies are developed over the long term, in order to avoid that insects become resistant. One option is

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pyramiding the expression of various Cry genes in plants (Maqbool et al., 2001; Kurtz et al., 2007) or combining the use of a variety of molecules to express hybrid Cry proteins that manifest greater toxic effect on insects (Naimov et al., 2003; Mehlo et al., 2005). Other examples of genes different from the *Bt* toxins, for the production of transgenic plants resistant to insects are esculentin from amphibians, chicken avidin and protease inhibitors from both animals and plants (Christeller et al., 2002; Ponti et al., 2003; Yoza et al., 2005; Abdeen et al., 2005). The overexpression of a *Myb* transcription factor gene involved in the synthesis of secondary metabolites provides resistance to insect plagues in maize (Johnson et al., 2007). Recently, insect resistance of tobacco expressing a poisonous toxin from the *Hadronyche versuta* spider was reported (Khan et al., 2006).

The venom from insectivorous spiders represents a complex mixture of molecules and peptides with a wide range of mechanisms for activity at a biochemical level, thus having a great biotechnological potential for improving resistance to insects. An important constituent of the spider venom consists of 4–10 kDa strong ligand peptides, that are tightly folded by means of various intramolecular disulfide bridges and which include a great diversity of antagonists, acting on the ion channels of excitable membranes (Olivera et al., 1994; Grishin, 1999).

Among the great number of known spider species, the *Macrothele* genus has been little studied and it is distributed in Iriomote a southern Japanese island. In a previous work, it was described the purification and amino acid sequencing of 6 peptides (Magi 1–6) that are found in *M. gigas* poison (Corzo et al., 2003). Also, ten putative peptides (Magi 7–16) whose sequence was deduced from the cDNA of the venom glands of such spider were described (Satake et al., 2004). The Magi 1–6 peptides were fractionated by cation-exchange chromatography and some of them act as neurotoxins or have site-specific affinity for sodium channels. The six peptides were injected into lepidoptera larvae *Spodoptera litura*, where both Magi 4 and 6 represented those inflicting greatest lethality to insects (Corzo et al., 2003).

In order to extend these observations and explore the possible use of Magi 6 as a bioinsecticide, in this work the cDNA of Magi 6 was cloned and expressed in transgenic tobacco plants; moreover, their resistance to insect attack was analyzed.

2. Materials and methods

2.1. Gene constructs and plant transformation

A 275-bp fragment from *Magi 6* cDNA without leader sequence was amplified using Expand™ High Fidelity PCR System (Roche) by PCR with forward (5′-CATGCCATGGCTT GCTGAAGGAAATGCAGC-3′) and reverse (5′-GGGGTACCA TCAACATCTCATGTTGCAGAGTACG-3′) primers. The PCR program consisted of 20 cycles of amplification (94 °C, 0.5 min; 55 °C, 0.5 min; 72 °C, 0.5 min). The added CCATGG sequence to create the *NcoI site* resulted in two extra amino acids, proline and tryptophan, after the initiation codon (underlined). The cDNA was digested with NcoI and KpnI and cloned in a pBluescriptKS-derived plasmid (pJLΩ27) containing the tobacco mosaic virus 5′-end leader sequence (Dowson Day et al., 1993). The XbaI–KpnI insert was excised and subcloned in pBin19 vector (Bevan, 1984), containing the 0.8 kb *35S* promoter and 0.3-kb *NOS* polyadenylation site. The construct was introduced by electroporation in *Agrobacterium tumefaciens* LBA4404 strain and used to transform *Nicotiana tabacum* L. cv. Petite Havana SR1 (Horsch et al., 1985). Regenerated tobacco transgenic plants were selected on Murashige and Skoog medium (Sigma) (Murashige and Skoog, 1962) containing 100 $\mu g\,ml^{-1}$ kanamycin (Sigma) and solidified with 0.8% phytagar (Sigma) before being transferred to pots with soil and grown in the greenhouse.

2.2. Genomic PCR and expression analysis

Genomic DNA from tobacco was isolated with a DNA isolation kit (Puregene Gentra Systems) and 100 ng were used for a PCR reaction using same oligonucleotides as described above corresponding to Magi 6 cDNA. The same PCR program was used except that amplification was of 35 cycles and one final cycle at 72 °C for 5 min. RT-PCR experiments were performed using 2 μg of total RNA extracted from tobacco plants using TRIZOL reagent according to manufacturer's instructions (Invitrogen), and an oligo dT for 1st strand cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen) was used. PCR was conducted using oligonucleotides corresponding to the 275 bp Magi 6 cDNA (described above) and the same program except that only 25 cycles were used which corresponded to the linearity phase of the exponential reaction after comparison of the PCR products at different cycles. PCR products were resolved in 1X Tris-acetic acid-EDTA, 1% agarose gels stained with ethidium bromide.

2.3. Western blot

Protein extracts were prepared by homogenization of 100 mg plant tissue in a buffer containing 50 mM Tris-HCl pH 7.0. Protein concentration was assayed using a protein determination kit (Bio-Rad) based on the Bradford method (Bradford, 1976). Proteins were precipitated overnight with acetone and small proteins fractionated in a Microcon YM-10 column (Millipore). Equal amounts of protein were loaded per lane and separated in a 15% SDS-PAGE, and transferred onto nitrocellulose Hybond-C membrane (Amersham). Membranes were blocked with TBST buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20), containing 5% fat-free milk powder, incubated overnight at 4 °C with Magi 6 mouse anti-serum diluted 1:100, washed 2× with TBST at 25 °C, and incubated with a secondary antibody conjugated to horseradish peroxidase diluted 1:1000. Immune complexes were detected by the chemioluminescence detection assay (Amersham).

2.4. Insect toxicity bioassays

Experimental assays on tobacco leaves were conducted using *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae from neonatal to 6 instar. Five groups of ten larvae