Plant Cell Reports (1987) 6:333–336

Plant Cell Reports

Kanamycin resistance during in vitro development of pollen from transgenic tomato plants

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Received May 26, 1987 – Communicated by I. Potrykus

ABSTRACT

Effects of kanamycin on pollen germination and tube growth of pollen from non-transformed plants and from transgenic tomato plants containing a chimaeric kanamycin resistance gene were determined. Germination of pollen was not affected by the addition of kanamycin to the medium in both genotypes. Kanamycin, however, severely affected tube growth of pollen from non-transformed plants, while pollen from plants containing the chimaeric gene were less sensitive and produced significantly longer tubes at kanamycin concentrations between 200-400 mgl-1. Apparently, this resistance for kanamycin correlates with the expression of the chimaeric gene during male gametophytic development.

ABBREVIATIONS

ATW, Agrobacterium Tomato Wageningen; KAN, Kanamycin; KANr, Kanamycin resistant; KANs, Kanamycin sensitive; mRNA, messenger RNA; NPT, Neomycin phosphotransferase.

INTRODUCTION

Male gametophytic functioning is involved with microsporogenesis, pollen germination, pollen tube elongation, and the transmission of gametes to the embryo sac. During these processes, the male haploid genome plays an important role. Willing and Mascarenhas (1984) estimated that mRNAs present in mature pollen grains of Tradescantia paludosa are the products of approximately 20,000 different genes. Large numbers of these mRNAs are translated into proteins during pollen germination and early pollen tube elongation (Mascarenhas and Mermelstein, 1981). Similar results were obtained using the pollen of Zea mays (Mascarenhas et al., 1984) and Nicotiana tabacum (Tupy, 1982). RNA synthesis and protein synthesis are independent during the early stages of in vitro pollen tube development, as was demonstrated by the use of specific inhibitors (see review Knox, 1984). These inhibition studies showed that RNA transcription in most plant species is not essential for germination and early pollen tube growth, whereas both processes are severely affected by translation inhibitors.

Most genes transcribed and translated during pollen development are also expressed in vegetative parts of the plant. Using RNA saturation hybridizations, Willing and Mascarenhas (1984) reported the expression of at least 64% of the pollen sequences in shoot tissues, and a maximum of about 60% of shoot mRNAs was also present in pollen of Tradescantia paludosa. Based on isozyme profiles, Tanksley et al. (1981) and Sari Gorla et al. (1986), found in Lycopersicon esculentum and Zea mays a comparable percentage of genetic overlap between the sporophytic and gametophytic phase of a plant.

The parallel expression of genes in the sporophyte and the gametophyte suggests that a selection pressure applied to the pollen is likely to alter the expression of genes in the sporophyte. Indeed, sporophytic gene expression is modified by chilling stress during pollen development in tomato species (Zamir et al., 1982) and after heavy metal stress during in vivo pollen germination in Silene dioica and Mimulis guttatus (Searcy and Mulcahy, 1985).

The development of Agrobacterium tumefaciens derived gene vectors have enabled the transfer of new genetic information into the cells of various plant species (Zambryski et al., 1983). The use of selectable marker genes which are constitutively expressed allows a convenient selection of transformed tissues (Horsch, 1985). Such an approach was also used by Koornneef et al. (1986), who introduced a chimaeric kanamycin resistance gene into tomato. The present study is undertaken to determine if this marker gene is also expressed in the gametophytic phase of the tomato plants and can be used for pollen selection.

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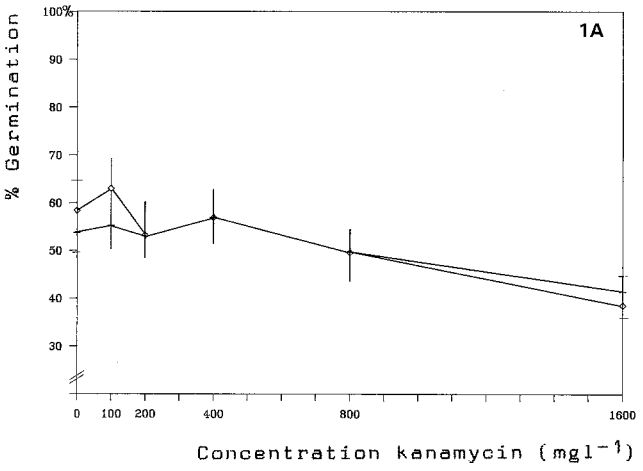
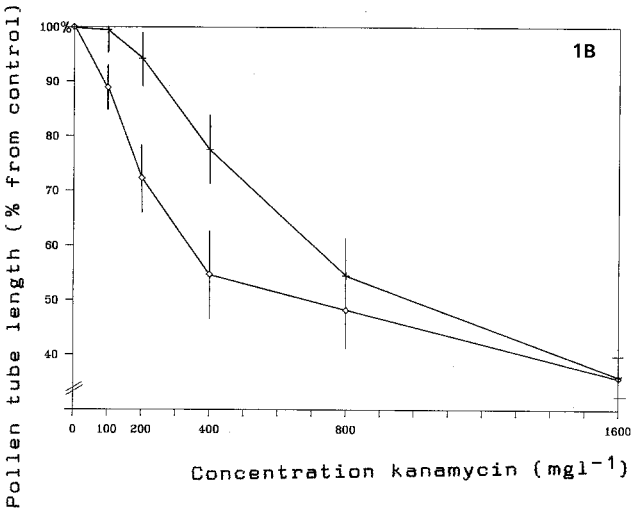
MATERIALS AND METHODS

Plant material. Tomato plants used in this study derived from material developed by Koornneef et al. (1986) and are indicated as MsK. This genotype is selected for outstanding regeneration capacity and originally derived from the cross Lycopersicon peruvianum x L. esculentum. Transformants were obtained following leaf disc transformation with either the Agrobacterium tumefaciens strain C58C1(pGV3850::1103neodim) (Czernilofsky et al., 1986) or the strain LBA 4404 (pAL4404, pAGS112) (Van den Elzen et al., 1985) as described by Koornneef et al. (1986). In between the T-region border sequences, both strains contain the NPT II gene from Tn5 under transcriptional control of the regulatory elements of the nopaline gene from A. tumefaciens. Expression of the chimaeric gene confers resistance of transformed plant cells to kanamycin (Hain et al., 1985). Transformed plants were named ATW and the transgenic character was confirmed both by Southern blot analyses and by segregation ratios of the KANr trait of seeds obtained from selfed plants (i.e., resistant : sensitive = 3 : 1) (Koornneef et al., 1986). As a control for kanamycin sensitivity, pollen of MsK plants and two commercial tomato cultivars (viz. Moneymaker and Delicates) were used.

In vitro pollen germination. Plants were grown in glasshouses at 18-22 °C, under daylight conditions. Pollen was collected from flowers at anthesis and germinated in solidified media containing 3 mM H_3BO_3, 1.7 mM $Ca(NO_3)_2$, 10% sucrose, 0.7% agar, with different concentrations of kanamycin sulphate (Sigma) at pH 5.8. Pollen was incubated for 2 h at 24 °C in the dark, and both stained and fixed with aceto-carmine (1%).

Germination and pollen tube length were determined for separate samples of 5-7 complete replicates. Per flower, and for each concentration, germination of at least 200 pollen grains was scored. Since stress induces the extrusion of short pollen tubes (Stanley and Linskens, 1974), pollen was recorded as germinated only when the pollen tube length was twice the diameter of the pollen grain. Pollen tube length was measured using an ocular micrometer for 50 tubes per flower at each concentration. The results were expressed as the mean ± standard deviation, significant differences among means ($P < 0.05$) were partitioned by an analysis of variance.





Figs. 1 A and B. Effects of increasing KAN concentrations (mgl-1) on germination and tube growth of pollen from non-transformed MsK (◇) and transgenic ATW (+) plants. Pollen from ATW plants, transformed with C58C1 and pollen from plants transformed with LBA 4404, behaved similar and are therefore combined. Germination percentages and tube lengths were determined for 5 (MsK) and 7 (ATW) complete replicates of pollen from different flowers. Results are expressed as mean ± standard deviation.
Fig. 1 A. Percentage of germination. Per flower, and for each concentration, germination of at least 200 pollen grains was scored.
Fig. 1 B. Pollen tube lenghts expressed as percentages of the control value (0 mgl-1 KAN). Per flower, and for each concentration, length of at least 50 pollen was scored.

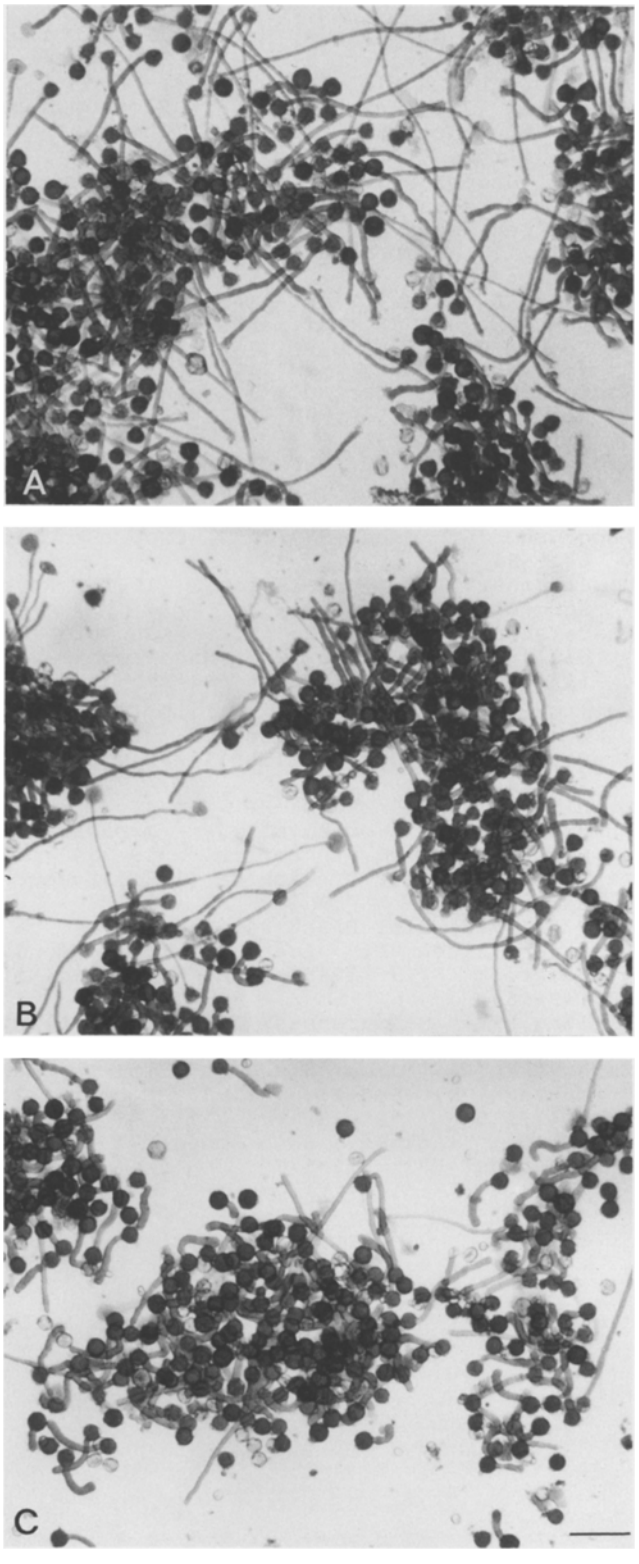


Fig. 2. ATW pollen, in vitro germinated at 0 mgl-1 KAN (A), 400 mgl-1 KAN (B) and 800 mgl-1 KAN (C). Bar = 0.1 mm.

RESULTS

The effects of increasing KAN concentrations on germination of pollen from MsK and ATW plants are presented in Fig. 1 A. Pollen germination of both genotypes was nct significantly affected by the addition of KAN to the medium.

Fig. 1 B shows the effects of the addition of KAN to the pollen germination medium on pollen tube length, a microscopic view of the results is presented in Fig. 2. After two hours of incubation, at 0 mgl-1, mean pollen tube length was 2.0 $\pm$ 0.3 · 10-4 m. Inhibition of tube growth of pollen from non-transformed (KANs) MsK plants was evident at 100 mgl-1 KAN, while at 400 mgl-1 KAN, the tube length was about 50% of the value obtained at 0 mgl-1. In contrast, inhibition of pollen tube growth of transformed (KANr) ATW plants was manifest at 200 mgl-1, while the 50% value occured at about 800 mgl-1. Significant differences between pollen tube lengths of MsK and ATW plants were obtained at 200 mgl-1 ($P < 0.01$) and 400 mgl-1 ($P < 0.05$). No different effects of KAN were observed between pollen tube growth of ATW plants transformed with the pGV3850::1103neodim construct or with the pAGS112 plasmid. Pollen tube growth of the cultivars Moneymaker and Delicates was similarily affected by KAN as pollen of MsK plants.

DISCUSSION

Tomato pollen tube growth is inhibited by the addition of KAN to the germination medium. Initial effects of KAN on pollen of non-transformed MsK plants were already apparent at 100 mgl-1. Similar KAN concentrations have been reported to inhibit MsK callus formation and seedling growth (Koornneef et al., 1986). The effects on vegetative growth, however, became evident only after several weeks in culture, while effects on pollen tube development were manifest 2 hours after pollen germination. Toxicity of KAN is due to interference with the translation system of the 70S ribosomes as present in mitochondria and chloroplasts of eucaryotic cells (Franklin and Snow, 1975). Since pollen tubes lack chloroplasts, the effect of KAN might indicate that an active mitochondrial translation is important during tomato pollen tube growth. Similar effects on pollen tube growth of several other species were obtained by Hoekstra (1979), using chloramphenicol, another inhibitor of 70S ribosomal protein synthesis. KAN did not significantly affect tomato pollen germination. For this, at least two explanations are possible: A) the antibiotic reagent is only gradually absorbed during pollen germination, or, B) mitochondrial translation is not essential for tomato pollen germination.

Pollen tube growth of plants containing the chimaeric resistance gene is less sensitive for KAN than pollen of non-transformed plants. Since ATW plants are heterozygous for the KANr trait, only half of the pollen population is expected to be resistant. Hence, the difference between pollen from ATW and MsK plants is even more evident considering the heterogenous character of the ATW pollen sample.

Resistance to aminoglycoside antibiotics such as KAN, is due to expression of the NPT II gene (Hain et al., 1985). During early tube growth in vitro, however, genes appear not to be transcribed (Knox, 1984). Probably, in pollen, mRNAs of the chimaeric gene are presynthesized concurrently with the other mRNAs during microsporogenesis, while KAN resistance is induced by gene translation during pollen tube elongation. The largest difference between pollen tube growth of ATW and MsK plants occured at 200-400 mgl-1 KAN. Here, tubes of ATW pollen are about 1.4 times longer than the pollen from MsK plants. Since the level of pollen resistance probably correlates with the amount of NPT present in the cells, the use of a stronger promoter may enhance selection efficiency.

The gametophytic phase of a plant is characterized by large populations and haploid genomes. Expression of selection sensitive genes by the male haploid genome opens the possibility to make pollinations with pre-selected pollen. For example, the toxin from Drechslera maydis prevents germination of pollen from maize plants susceptible to the disease but not of pollen from resistant plants (Laughnan and Gabay, 1973), and, compounds present in Alternaria brassicicola culture filtrates inhibit brassica pollen germination (Hodgkin and MacDonald (1986). However, linkage of agronomic important traits with a newly introduced marker gene, would be of general interest for the application of pollen selection methods in plant breeding programmes.

ACKNOWLEDGMENTS

We thank J. Jansen for helping with the statistical analyses, and J.J.M. Dons and A.G. Stephenson for critically reading the manuscript.

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