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A simple, nondestructive spraying assay for the detection of an active kanamycin resistance gene in transgenic tomato plants

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Summary. A simple, nondestructive kanamycin spraying assay for detecting neomycin phosphotransferase II activity in tomato has been developed. This assay does not require the use of tissue culture or biochemical methods, but allows transgenic and non-transgenic tomato plants to be distinguished directly at the plant level in the greenhouse. Its potential applications in large-scale genetic analyses are discussed.

Key words: Kanamycin resistance – *Lycopersicon esculentum* – Neomycin phosphotransferase II – Screenable marker – Selectable marker

Introduction

A selectable marker often used to identify transformed plants is the gene coding for the bacterial Tn5 neomycin phosphotransferase II (NPTII) enzyme that confers resistance to the antibiotics kanamycin, neomycin, and G-418 (for a review, see Fraley et al. 1986). Several approaches for detecting the NPTII gene or its product in transgenic plants have been followed thus far (Horsch et al. 1984; McDonnell et al. 1987; Reiss et al. 1984; Schreier et al. 1985; Chyi et al. 1986; Reynaerts et al. 1988). Alternatively, transgenic plants have been screened for the activity of a reporter gene, located on the same T-DNA as the NPTII gene (Chyi et al. 1986; McCormick et al. 1986). All these assays, however, suffer from being laborious and time-consuming, in particular when large populations of plants need to be screened. Testing seedlings or leaf tissue on selective media for kanamycin resistance (Horsch et al. 1984) requires sterile conditions, whereas radiolabel transfer assays (McDonnell et al. 1987; Reiss et al. 1984; Schreier et al. 1985), Southern blot analysis (Chyi et al. 1986), and immunoblot analysis (Reynaerts et al. 1988), apart from being cumbersome, involve the handling of radiolabel. In addition, when seedlings are selected for resistance, the sensitive individuals are lost to further analysis.

Two observations led us to develop a simple, non-selective assay for kanamycin resistance, which alows transgenic and non-transgenic plants to be identified visually in the greenhouse. One observation was that kanamycin-sensitive leaves, which are in full contact with kanamycin-containing medium, bleach completely due to inhibition of chloroplast-associated protein synthesis. Secondly, we noticed that kanamycin-sensitive tomato seedlings, following germination on selective medium, can be rescued by transferring the apex, plus a very short part of the hypocotyl, to non-selective medium. Such an explant will root, whereas a similar explant with a rather long hypocotyl part will not root. This suggests that transport of kanamycin through sensitive tomato seedlings/tissue occurs over rather short distances and that meristems are not killed. In this paper we describe a non-destructive spraying test for kanamycin resistance which is based on local bleaching of sensitive leaves.

Materials and methods

Genetic materials

For plant transformations, *Agrobacterium tumefaciens* strain LBA 4404 (pAL 4404, pAGS 112) (Hoekema et al. 1983; Van den Elzen et al. 1985) was used. The plasmid pAGS 112 is a binary type T-DNA vector with a *PNOS/NPTII/OCS3'* gene fusion between its T-region borders (Van den Elzen et al. 1985). Leaf discs were transformed essentially as described by Horsch et al. (1985) with some modifications (Koornneef et al. 1987). ATW 1-KK is a transgenic tomato plant which is homozygous for the chimaeric NPTII gene. This plant was obtained as follows: a primary transformant of genotype MsK93 (Koornneef

et al. 1986) was crossed with *Lycopersicon esculentum* genotype VF11, whereupon a kanamycin-resistant progeny plant was crossed with *L. esculentum* genotype LA 1166; one kanamycin resistant plant, obtained from the selfed progeny of a resulting hybrid, was named ATW 1-KK. The selfed progeny of ATW 1-KK did not segregate sensitive seedlings, showing its homozygous character. Southern blot analysis showed that ATW 1-KK contains one T-DNA copy per haploid genome (data not shown). Tomato transformant ATW4094 was obtained by transforming a hybrid of *L. esculentum* LA 1189 and *L. esculentum* LA 1665. The genetic characteristics of the lines LA 1166, LA 1189, and LA 1665, which are homozygous for a number of recessive mutations, have been described by Rick (1982).

Spraying test for kanamycin resistance

Seeds of *L. esculentum* VF11 and seeds obtained by selfing ATW 1-KK were germinated in soil in the greenhouse, 18 seeds per tray of 30×20 cm. After 3 weeks, when the third leaf had emerged, the young plants were sprayed from above with either water, 100 mg/l, or 400 mg/l kanamycin sulphate solution. This was done with a spray gun, once a day for 3 consecutive days. During this period, the plants were not watered otherwise. A total of approximately 100 ml was sprayed per tray. One week after spraying, leaves were scored for the appearance of chlorotic spots.

Seeds obtained by selfing tomato transformant ATW 4094 were germinated and the plants were sprayed with 100 mg/l kanamycin sulphate solution, as described above. Ten days after scoring the plants of this population for chlorosis, they were assayed for NPTII activity with a radiolabel transfer assay (McDonnell et al. 1987), using 15 mg of tissue from a non-chlorotic leaf which had emerged after spraying and which showed a healthy appearance. The phosphocellulose filter was exposed for 24 h to a Kodak XAR-50 X-ray film at $-80\,^{\circ}C$ using an intensifying screen. Control leaf material was taken from five tomato plants which had been independently transformed with *A. tumefaciens* strain LBA 4404 (pAL 4404, pAGS 112), and from five untransformed *L. esculentum* LA 780 plants (Rick 1982).

Results and discussion

Kanamycin-sensitive leaves exhibit chlorosis upon incubation in vitro on kanamycin-containing growth medium. To test whether the appearance of chlorotic symptoms allows for large-scale identification of transgenic plants under greenhouse conditions, 162 non-transgenic kanamycin-sensitive (cultivar VF11) and 162 homozygous transgenic, kanamycin-resistant (ATW 1-KK) tomato seeds were germinated in soil in the greenhouse. Each population was then divided into three groups of 54 seedlings each, which were sprayed with either water (control), 100 mg/l kanamycin, or 400 mg/l kanamycin. One week after spraying, leaves of all non-transgenic plants sprayed with either 100 mg/l or 400 mg/l kanamycin exhibited chlorotic spots, whereas chlorotic spots could be detected neither on the transgenic plants, nor on the non-transgenic plants sprayed with water (Fig. 1). Although the intensity of chlorosis was somewhat higher in leaves sprayed with 400 mg/l kanamycin, all non-transgenic, kanamycin-sensitive plants could be easily scored as chlorotic, following treatment with 100 mg/l kanamycin. An important observation was that chlorosis remained restricted to those leaves that had actually been sprayed. No chlorotic spots could be detected on leaves emerging after the spraying period. These leaves showed a normal phenotype, and all seedlings, either resistant or sensitive, developed into normal, healthy plants following the treatment with kanamycin.

To test whether kanamycin-resistant plants could be distinguished from kanamycin-sensitive plants when

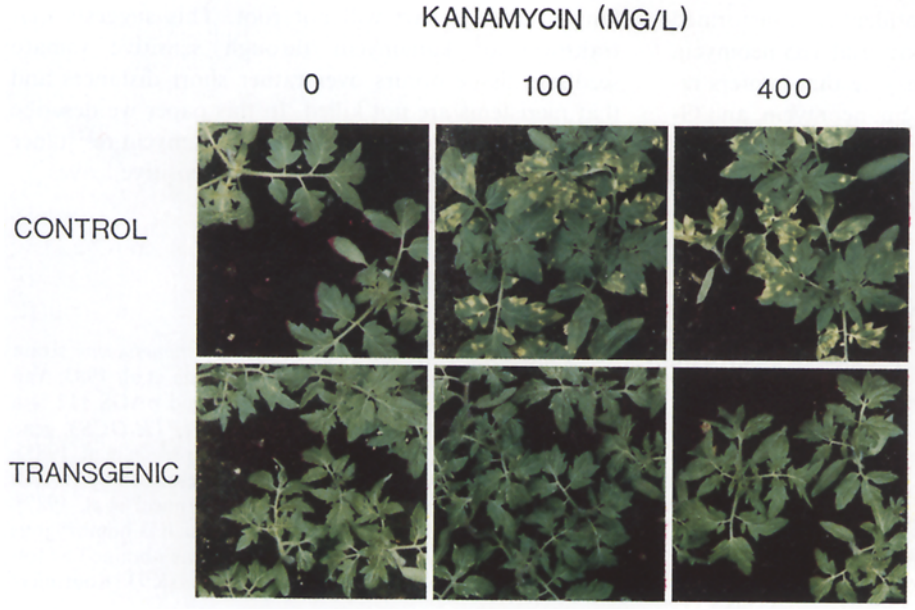

Fig. 1. The effect of spraying young tomato plants with kanamycin. Three-week-old kanamycin-sensitive, non-transgenic control plants and kanamycin-resistant transgenic plants were sprayed with either water, 100 mg/l, or 400 mg/l kanamycin sulphate solution. The plants were photographed 9 days after spraying. The transgenic plants were derived from a tomato plant transformed with *Agrobacterium tumefaciens* strain LBA 4404 (pAL 4404, pAGS 112) and were homozygous for the NPTII gene

present in a segregating population, 100 young tomato plants, obtained by selfing a primary transformant, were sprayed with 100 mg/l kanamycin. Of these, 77 developed into non-chlorotic and 21 into chlorotic plants, consonant with the 3:1 segregation ratio expected if the parental transformant contained one kanamycin resistance locus ($X_1^2 = 0.667$; $0.3 < p < 0.4$). It should be mentioned that segregation of the kanamycin-induced chlorotic phenotype could also be observed among segregants homozygous recessive for chlorophyll mutations *yv* and *l* (Rick 1982).

These results strongly indicate that kanamycin application to the surface of leaves allows unambiguous identification of young tomato plants which express the NPTII gene. To establish whether the response to spraying reflected the activity of the NPTII gene, 20 non-chlorotic and 10 chlorotic plants were randomly chosen from the segregating population and tissue from a healthy, non-chlorotic leaf, which had emerged after the spraying period, was assayed for NPTII activity using the radiolabel transfer assay of McDonnel et al. (1987).

All the extracts from the 20 non-chlorotic plants contained NPTII activity, whereas no NPTII activity could be detected in any of the 10 chlorotic plants (Fig. 2), indicating absolute correlation in this sample between the response to spraying and the presence of an active kanamycin resistance gene. Similar results as shown for young plants were obtained with 2-month-old tomato plants.

Since the spraying assay for detecting an active NPTII gene in transgenic plants is nondestructive and, furthermore, facilitates the scoring of large populations of young plants, several new applications of the NPTII gene become feasible.

By using large populations, which segregate for morphological markers with known map positions and for kanamycin resistance, linkage analyses should allow introduced NPTII genes to be positioned very accurately on the classical linkage map (R. Weide, J. Hille, P. Zabel and M. Koornneef, in preparation). When the NPTII gene is found to be closely linked to a gene of interest, it could serve as a convenient dominant selectable marker for that gene, or even as a starting point for chromosome walking. If a recessive genotype is desired, tagging the recessive allele with NPTII facilitates repeated backcross programs which are required to introduce this allele into a specific genetic background. An interesting example of an economically important trait which might be tagged with a NPTII gene is male sterility. When the NPTII gene is tightly linked to the dominant fertile allele of a male sterility gene, the kanamycin-sensitive F_1 plants from msms × Msms crosses will be male sterile, whereas the kanamycin-resistant F_1 plants will be fertile. Thus, application of the spraying assay to young plants bypasses the conventional screening of mature, flowering plants for male sterility, prior to their transfer to a hybrid seed production field (see also Singleton and Jones 1930 and Jorgensen 1987).

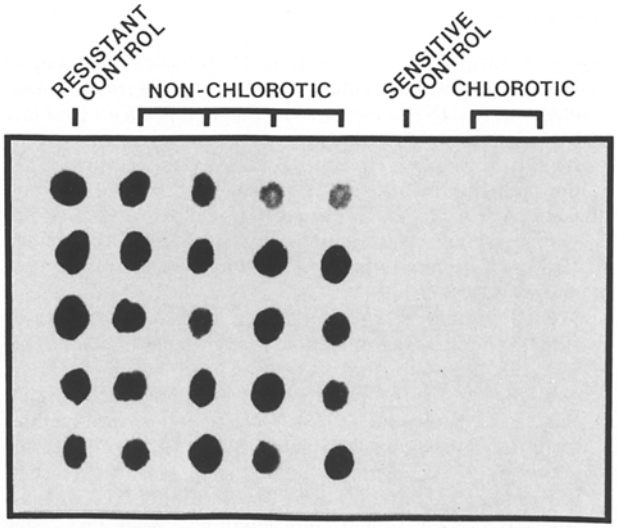

Fig. 2. Autoradiogram showing the activity of the NPTII gene in young tomato plants which exhibited a chlorotic or non-chlorotic phenotype after spraying with kanamycin. Three-week-old tomato plants, obtained from selfing the primary transformant ATW4094 and segregating for kanamycin resistance, were sprayed with 100 mg/l kanamycin sulphate solution. Three weeks after spraying, 20 non-chlorotic and 10 chlorotic plants were randomly chosen from the population and assayed for NPTII activity with a radiolabel transfer assay (McDonnell et al. 1987), using 15 mg of tissue from a non-chlorotic leaf, which had emerged after spraying and which showed a healthy appearance. ATW4094 and five kanamycin-resistant control tomato plants had been independently transformed with *Agrobacterium tumefaciens* strain LBA 4404 (pAL 4404, pAGS 112). Kanamycin-sensitive control leaf material was taken from five untransformed tomato LA 780 plants

In addition to its applications as a gene tag, the NPTII gene may serve as a target for endogenous transposable elements, which have not yet been identified in tomato. Spraying large numbers of seedlings, heterozygous for a single NPTII gene, could reveal the presence of an individual in which the activity of the kanamycin resistance gene has been lost due to insertion of such an element. The viability of the sensitive plant will allow subsequent molecular analysis of the inactivated NPTII gene and cloning of the putative transposable element.

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References

Chyi Y-S, Jorgensen RA, Goldstein D, Tanksley SD, Loaiza-Figueroa F (1986) Locations and stability of *Agrobacterium*-mediated T-DNA insertions in the *Lycopersicon* genome. Mol Gen Genet 204:64–69

Fraley RT, Rogers SG, Horsch RB (1986) Genetic transformation in higher plants. CRC Crit Rev Plant Sci 4:1–46

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of the Vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179–180

Horsch RB, Fraley RT, Sanders PR, Lloyd A, Hoffmann N (1984) Inheritance of functional foreign genes in plants. Science 223:496–498

Horsch RB, Fry JE, Hoffmann NL, Wallroth M, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1232

Jorgensen RA (1987) Synthetic linkages and genetic stability in transgenic plants: Implications for breeding. In: Nevins DJ, Jones RA (eds) Tomato biotechnology. Alan Liss, New York, pp 179–188

Koornneef M, Hanhart C, Jongsma M, Toma I, Weide R, Zabel P, Hille J (1986) Breeding of a tomato genotype readily accessible to genetic manipulation. Plant Sci 45:201–208

Koornneef M, Jongsma M, Weide R, Zabel P, Hille J (1987) Transformation of tomato. In: Nevins DJ, Jones RA (eds) Tomato biotechnology. Alan Liss, New York, pp 169–178

McCormick S, Niedermeyer J, Fry J, Barnason A, Horsch R, Fraley R (1986) Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rep 5:81–84

McDonnell RE, Clark RD, Smith WA, Hinchee MA (1987) A simplified method for the detection of neomycin phosphotransferase II activity in transformed plant tissues. Plant Mol Bio Rep 5:380–386

Reiss B, Sprengel R, Will H, Schaller H (1984) A new sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. Gene 30:211–218

Reynaerts A, De Block M, Hernalsteens J-P, Van Montagu M (1988) Selectable and screenable markers. In: Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual. Kluwer, Dordrecht, Sect A9 pp 1–16

Rick CM (1982) Tomato Genet Coop Rep 32:3–7

Schreier PH, Seftor EA, Schell J, Bohnert HJ (1985) The use of nuclear-encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant chloroplasts. EMBO J 4:25–32

Singleton W, Jones D (1930) Heritable characters of maize. 35. Male sterile. J Hered 21:266–268

Van den Elzen P, Lee KY, Townsend J, Bedbrook J (1985) Simple binary vectors for DNA transfer to plant cells. Plant Mol Biol 4:149–154