Abstract Tomato black ring virus (TBRV) belongs to the nepoviruses, an important genus of phytoviruses characterized by isometric particles and by their transmission by longidorid nematodes. As for all other nepoviruses, the coat protein (CP) of TBRV is encoded by the 3'-terminal part of the viral RNA2 (positions 2801–4334). A hybrid gene driving the expression of a truncated form of the TBRV CP was constructed. It contains a frameshift deletion at position T4065 so that in the encoded protein the last 90 amino acids of the wild-type CP are replaced by 52 amino acids encoded by a different reading frame of the viral RNA. This hybrid gene was introduced into the genome of Nicotiana tabacum cv ‘Xanthi’ plants. When compared to control plants, progeny of some transformants expressing the mutated CP gene (CPm+ plants) showed resistance against TBRV infection. This resistance is characterized by a delay in the appearance of symptoms, a reduction in the number of infected plants and a reduction in virus accumulation.

Key words Transgenic plants · Coat protein · Resistance · Nepovirus

Abbreviations ELISA: Enzyme linked immunosorbent assay · GUS: β-glucuronidase · ISEM: Immunosorbent electron microscopy · PCR: Polymerase chain reaction

Introduction

Since Powell-Abel et al. (1986) demonstrated that tobacco plants expressing the tobacco mosaic virus (TMV) coat protein (CP) were protected against TMV infection, similar results have been obtained with plant viruses from more than ten genera (for reviews see Beachy et al. 1990; Wilson 1993). Such CP-mediated resistance has been demonstrated to be effective against the nepoviruses: arabis mosaic virus (ArMV, Bertioli et al. 1992), grapevine chrome mosaic virus (GCMV, Brault et al. 1993), grapevine fanleaf virus (GFLV, Bardonnet et al. 1994) and, more recently, strawberry latent ringspot virus (SLRV, Kreiah et al. 1996). It has also been observed that a phenomenon of heteroencapsidation can occur in transgenic plants expressing a wild-type CP and infected by another virus (Candelier-Harvey and Hull 1993; Lecoq et al. 1993). Moreover, due to this heteroencapsidation phenomenon, the infecting virus can acquire new properties (Lecoq et al. 1993). In our laboratory, Hiriart (1995) observed that such an heteroencapsidation phenomenon can occur in transgenic plant expressing a nepovirus (GCMV) CP. The transformation of plants with a truncated CP gene could be a way to avoid such heteroencapsidation and the ensuing risks.

For these studies, tomato black ring virus (TBRV), which systemically infects tobacco (Nicotiana tabacum cv ‘Xanthi’), was used. TBRV is a member of the genus Nepovirus, within the family Comoviridae (Mayo and Martelli 1993). Nepoviruses (for a review see Mayo and Robinson 1996) are characterized by isometric particles and by their nematode transmission. Their genome consists of two separately encapsidated, single-stranded RNAs of positive polarity. Each RNA is bound to a genome-linked protein (VPg) at its 5’ end and is polyadenylated at its 3’ end.

In this paper we describe the production of tobacco plants (Nicotiana tabacum cv ‘Xanthi’) transformed with a truncated CP gene of TBRV strain ED (TBRV-
ED). The R1 and R2 progenies were obtained, checked for transgene expression and evaluated for their resistance to virus infection. Several transgenic lines were found to be resistant to TBRV-ED, but not to two closely related nepoviruses, TBRV-S and GCMV.

**Materials and methods**

**Plants and viruses**

TBRV-ED(–) is derived from TBRV-ED, a gift from Dr. A. Murant (Dundee, Scotland), by elimination of its satellite RNA (Le Gall et al. 1995a). The TBRV-S(–) (Doz et al. 1980) and the grapevine chrome mosaic virus (GCMV), two closely related nepoviruses, were also used. The three viruses were propagated under greenhouse conditions in Chenopodium quinoa. They were purified (Doz et al. 1980) and stored at –80°C until their use for plant inoculation. Tobacco (Nicotiana tabacum cv ‘Xanthi’) was used in the transformation experiments.

Recombinant plasmid constructions

Plasmid pBS6 is a derivative of pBluescript (Stratagene) containing the cDNA sequence of the TBRV-ED RNA2 from nucleotide 284 up to the 3′ end. In this plasmid, the sequence encoding the coat protein (positions 2801–4334, Le Gall et al. 1995a) contains a frameshift mutation (deletion of a T at position 4065) so that the protein encoded by this sequence (CPm) differs from the wild-type CP by the absence of the 90 carboxy terminal amino acids and their replacement by 52 amino acids encoded by a different reading frame of the viral RNA (Fig. 1). PCR was used to isolate this mutated CP gene and to add an initiation codon in an optimal context for plant cells (Lütcke et al. 1987). The upstream and downstream oligonucleotides used for the PCR were respectively 5′-cctggagaatcggagggt and 5′-cctggagaagtgtgtggt. The amplified fragment was inserted downstream of the TMV 3′ leader, known to increase the translation efficiency by a factor of two to ten (Gallie et al. 1987). The coding capacity of the hybrid gene was checked in vitro using rabbit reticulocyte lysate (Promega). This hybrid gene was then cloned between the strong constitutive CaMV 35S promoter with its enhancer region duplicated (70S, Kay et al. 1987) and the nosyn gene, terminator (NOS) of plasmid pCa2Nos (a gift from Dr. M. Tepfer, INRA Versailles, France). The expression cassette containing the hybrid gene was finally inserted into the HindIII site of the binary pBItC8 vector, obtained in our laboratory and derived from pBin19 vector (Bevan 1984), between the kanamycin resistance gene and the GUS gene (Fig. 1), to give plasmid pBiCPC8.

**Plant transformation**

Tobacco plants were transformed using the leaf disk procedure as described by Draper et al. (1988). After transformation with the T-DNA sequence containing the CPm gene of pBiCPC8, transgenic plants (CPm+) were obtained. Control plants (TC8) were also obtained after transformation with the empty T-DNA sequence of the pBItC8 vector. Non-transgenic (NT) tobacco plants were also used as controls in inoculation experiments.

Analysis of transgenic plants

The expression of the GUS reporter gene in the primary transformants was checked during the regeneration process. The expression of the viral transgene was checked later at two levels: expression of the CPm gene messenger RNAs and expression of the CPm protein. Total RNAs were extracted as described by Verwoerd et al. (1989). After electrophoresis through a 1% formaldehyde agarose denaturing gel, the RNAs were blotted to Hybond-C extra membrane and hybridized with a 32P-labelled specific riboprobe (Melton et al. 1984). The expression of the CPm protein was checked by DAS-ELISA and by Western blotting as previously described by Brault et al. (1993) using a rabbit antiserum raised against purified TBRV-ED(–) particles.

**Transgenic plants protection assay**

The protection of the transgenic CPm+ plants against viral infection was analyzed by inoculation with purified viral suspensions and comparison with control NT and TC8 plants. All inoculations were carried out on greenhouse-grown, 4-week-old plants. The

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**Fig. 1A–C** Schematic representation of the T-DNA part of pBItC8 and of the hybrid gene driving the synthesis of the mutated coat protein of TBRV-ED. A Structure of TBRV-ED RNA2 and position of the CP coding region (hatched). The position of the frameshift mutation (asterisk, deletion of T₃₉₆) is indicated. B Structure of the expression cassette containing the mutated TBRV-ED CP gene. C Structure of the T-DNA of pBItC8. The expression cassette was cloned into the unique HindIII site of pBItC8 to give pBItCPC8, and the correct orientation of the cassette was checked by restriction mapping. ATG Translation initiation codon, CPm mutated CP sequence, GUS uidA gene, Init optimal translation initiation context, Km’ NPTII kanamycin resistance gene, LB left border for T-DNA transfer, NosP promoter of the nopaline synthase gene, NosT terminator of the nopaline synthase gene, RB right border for T-DNA transfer, Stop 4239 stop codon at position 4239 due to the frameshift mutation, 3′’ 3′’ leader, known to increase the translation efficiency by a factor of two to ten (Gallie et al. 1987). The coding capacity of the hybrid gene was checked in vitro using rabbit reticulocyte lysate (Promega). This hybrid gene was then cloned between the strong constitutive CaMV 35S promoter with its enhancer region duplicated (70S, Kay et al. 1987). The expression cassette containing the hybrid gene was finally inserted into the HindIII site of the binary pBItC8 vector, obtained in our laboratory and derived from pBin19 vector (Bevan 1984), between the kanamycin resistance gene and the GUS gene (Fig. 1), to give plasmid pBItCPC8.