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Field resistance against potato virus Y infection using natural and genetically engineered resistance genes

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Abstract

Commercial tobacco cultivars BB16 (burley) and PBD6 (dark air cured) were transformed with the coat protein gene of lettuce mosaic potyvirus (LMV). Transgenic BB16 plants showed resistance to potato virus Y (PVY) infection, against the necrotic strain PVY-N Versailles, as well as the resistance breaking necrotic strain PVY-N 107. Transgenic PBD6, which carries the recessive 'va' gene conferring resistance to PVY, was also protected against PVY-N 107. In the progenies of most BB16 and PBD6 transformants, 45% to 100% of the inoculated plants were immune to PVY infection. The rest of the plants were tolerant, with atypical attenuated non necrotic symptoms and reduced virus accumulation. No recovery was observed in the tolerant plants, which stably expressed detectable level of LMV coat protein. This suggested a protein-mediated mechanism of heterologous protection.

Since the initial report in 1986 (Powell-Abel et al., 1986), many examples of pathogen-derived resistance were obtained against plant viruses (Lomonossoff, 1995; Baulcombe, 1996; Beachy, 1997). Discrepancies were reported in the degrees of protection obtained with similar virus-derived constructs, raising the hypothesis that the plant genotype influences the efficiency of the protection obtained in transgenic plants and making difficult to predict the efficiency of the protection conferred by any virus-derived transgene. Among strategies designed to obtain virus-derived resistance, the most successful approach for positive-strand RNA viruses was the use of coat protein (CP). Among different mechanisms described to date (Baulcombe, 1996), RNA-mediated protection was usually strain specific, while several cases of protein-mediated protections were broad-spectrum, such as the heterologous protection described for potyviruses (Stark and Beachy, 1989; Ling et al., 1991; Dinant et al., 1993). We previously showed that the expression of the CP gene of lettuce mosaic potyvirus (LMV) conferred a heterologous protection in tobacco Xanthi against several strains of potato virus Y (PVY) (Dinant et al., 1993) as well as protection to other potyviruses (Blaise et al., 1995). The protection observed against PVY was characterised in some lines as a complete resistance against distinct strains of PVY, responsible on the Xanthi plants of systemic mosaic or of systemic veinal necrosis. In this work, we demonstrate that the high level of protection conferred by the LMV CP gene can be transferred to commercial cultivars and can act additionally with natural resistance gene.

The LMV CP gene, including the coding region and the 3′ untranslated region, was modified by addition of a translation initiation codon and the TMV Ω′ translation enhancer (Dinant et al., 1993) and transferred in the binary vector pKYLMVCP (Dinant et al., 1997). It was introduced into commercial tobacco cultivars BB16 (Burley) and PBD6 (air cured Paraguay) by *Agrobacterium tumefaciens* co-culture of leaf explants (Dinant et al., 1997, with 1 mg/ml BA and

Table 1. Protection against PVY in transgenic Burley BB16 R_1 progeny plants expressing the LMV CP gene

R_1 plants name[a]	PVY-N Versailles				PVY-N 107			
	#[b]	Immune	Tolerant	Susceptible	#[b]	Immune	Tolerant	Susceptible
control	20	0	0	20	20	0	0	20
BB16-2	23	15	8	0	nd	nd	nd	nd
BB16-3	18	11	7	0	nd	nd	nd	nd
BB16-4	19	14	5	0	nd	nd	nd	nd
BB16-5	45	22	23	0	15	11	4	0
BB16-7	66	33	33	0	22	18	4	0
BB16-10	42	21	21	0	15	11	4	0
BB16-11	56	25	31	0	15	7	8	0
BB16-12	61	34	27	0	15	6	9	0
BB16-14	46	26	20	0	nd	nd	nd	nd

Plants were mechanically inoculated with PVY-N Versailles and PVY-N 107 infected sap extract, and the PVY infection was assessed by symptom notation and DAS-ELISA, three months after inoculation. a: name of the R_0 parent; b: number of plants tested per line. nd: not determined.

0.5 mg/ml IAA in culture media). PBD6 inherited from the cultivar P48 the recessive 'va' gene (Gupton and Burk, 1973; Ano et al., 1995) which confers resistance to most PVY strains. The plants were challenged with PVY-N Versailles (Robaglia et al., 1989, strain PVY-NV in Dinant et al., 1993) or with PVY-N 107 (Vam-B strain: Gooding and Tolin, 1973, Pathotype 1-2: Blancard et al., 1995). Typically, BB16 tobacco plants, such as Xanthi plants (Dinant et al., 1993), infected with PVY-N Versailles and PVY-N 107 showed systemic symptoms of vein clearing 2–3 weeks post-inoculation, followed by veinal necrosis in systemic leaves as well as inoculated leaves 5–6 weeks post-inoculation. These symptoms caused by PVY-N 107 were usually more severe, with necrotic lesions on the veins as well as on the leaf laminas. Similar symptoms were observed on PBD6 tobacco plants infected by PVY-N 107, which overcomes the va gene as well as the VR gene (Blancard et al., 1995). Nine primary transformants (R_0 plants) for BB16 and four for PBD6 were obtained. The inheritance pattern of kanamycin resistance in these lines indicated that the gene was inserted at a single locus for all BB16 transformants. This situation is similar to the observation in Xanthi transformants previously described (Dinant et al., 1993), and for three PBD6 transformants (PBD6-5, PBD6-8 and PBD6-9). Kanamycin resistant progenies obtained after self-pollination of R_0 plants (R_1 progeny) or R_1 plants (R_2 progeny) were assayed for resistance to PVY-N Versailles and PVY-N 107, by mechanical inoculation using PVY infected plant extracts. For BB16 R_1 progenies (Table 1), 40% to 82% of the plants were immune to PVY infection (no viral multiplication, as assessed by ELISA and back inoculation on BB16 control plants). The non-immune plants showed an atypical systemic infection: the plants did not show any vein clearing, nor veinal necrosis, but diffuse chlorotic lesions on the lower leaves and the inoculated ones, while the top of the plants remained symptomless. These plants had limited growth reduction as opposed to control plants whose growth was seriously reduced. Tolerant plants also exhibited an atypical distribution of PVY accumulation, with maximum accumulation occurring in the lower leaves, which correlated with symptom expression (Figure 1). No PVY accumulation was observed in the upper leaves of tolerant plants. This distribution was maintained during the plant growth: upper leaves were constantly virus free, while lower leaves became progressively invaded by PVY, with no recovery. By contrast, non transformed BB16 plants always showed PVY accumulation in the young emerging leaves. Similar results were observed against a reference resistance breaking strain, PVY-N 107, in transgenic LMV CP Paraguay PBD6 lines (Table 2).

The protection was tested in the field under natural aphid transmission of PVY. R_2 progenies of three individual BB16 plants were naturally infected by aphids, which disseminated PVY with a moderate frequency (Figure 2-D). In the control group of 222 non transformed BB16 plants, 27 (12.1%) showed PVY symptoms, confirmed by ELISA. In the three transgenic progenies, 2/217, 3/217, and 5/218 plants (1.5 $\pm$ 0.6%), respectively for BB16-5-10, -5-17 and -5-22, were infected by PVY. Thus, the susceptibility to

Table 2. Protection against PVY-N 107 in transgenic Paraguay PBD6 R_2 progeny plants expressing the LMV CP gene

PBD6 Name[a]	PVY-N 107 #[b]	Immune	Tolerant	Susceptible
Control	20	0	0	20
PBD6-2-6	20	20	0	0
PBD6-2-9	22	22	0	0
PBD6-5-2	19	19	0	0
PBD6-8-5	22	6	16	0
PBD6-9-3	20	1	19	0

Plants were mechanically inoculated with PVY-N 107 infected sap extract and the PVY infection was assessed by symptom notation and DAS-ELISA two months after inoculation a: name of the R_1 parent; b: number of plants tested per line.

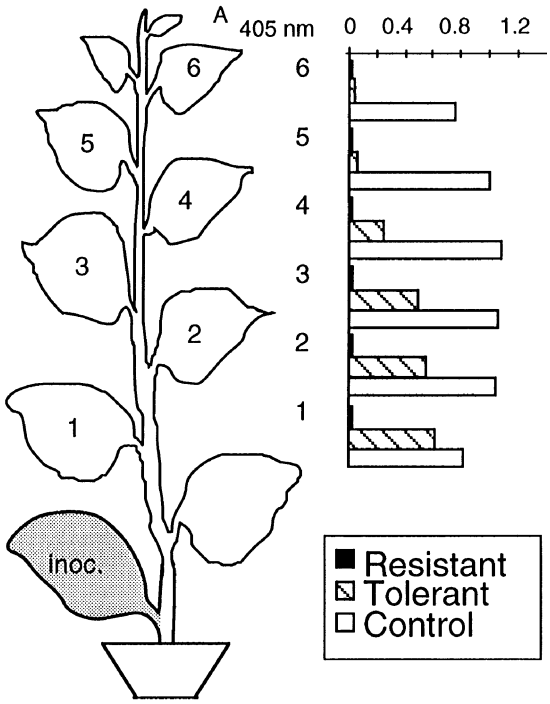

Figure 1. Reduction of PVY accumulation in infected tolerant transformed BB16 plants. PVY accumulation was quantified by DAS-ELISA (double antibody sandwich-ELISA, as described in Dinant et al., 1993), on leaf samples taken at different positions on the plant. The inoculated leaf (inoc.) is indicated in the figure. Usually, the first fully expanded leaf directly above the inoculated one was not infected. Values represent average absorbance at 405 nm (A_{405nm}) obtained from R_1 progeny of one transformant (line BB16-12), inoculated at an early stage (four fully developed true leaves), and separated in two groups of immune (19/28) and tolerant (9/28) plants.

PVY of the transformed BB16 plants was significantly reduced. In transformed BB16 lines, the quality of the plants, as determined from leaf number, yield, and quality rate of tobacco leaves after curing, was not affected by the expression of the transgene (Figure 2A, B, C).

Expression of the LMV CP gene was determined by ACP-ELISA (antibody coated plate-ELISA, Lommel et al., 1982) in R_0 plants (data not shown) and confirmed by Western blot in R_1 progenies (Figure 3). All the BB16 and PBD6 lines showed detectable levels of LMV CP accumulation. This LMV CP expression was consistent with the level driven by the same construct in lettuce (Dinant et al., 1997) or in tobacco (Dinant et al., 1993). There was no clear correlation between the level of LMV CP accumulation and the level of protection. Since the phenotype of resistance against PVY was similar in all the lines, this observation suggests that the accumulation of LMV CP was probably not a limiting factor in the extent of the protection. Homozygosity of each individual R_1 or R_2 plants was assessed by kanamycin resistance tests on their progeny. There was no correlation between homozygosity and the level of protection in the BB16 or PBD6 lines, i.e. homozygous as well as hemizygous plants showed immunity or tolerance in each line. Therefore, in these lines, which all showed resistance, the heterologous protection phenotype against PVY was not influenced by zygosity, but was associated with a detectable level of LMV CP accumulation.

This case of heterologous protection is different from the cases of RNA-mediated protection against potyviruses (Lindbo and Dougherty, 1992; van der Vlugt et al., 1992; Ravelonandro et al., 1993; Lindbo et al., 1993): these last types of resistance were associated with a recovery phenotype and a low steady state RNA accumulation. The viruses used in this experiment, PVY and LMV, share 58% nucleotide identity in their CP genes, which is incompatible with a similar RNA-mediated mechanism. Moreover, the accumulation of the LMV CP was rather constant during the growth of the plant and did not decline after PVY inoculation. These observations suggested a protein-mediated protection mechanism. Since the potyvirus coat protein is implicated in encapsidation as well as in virus movement (Dolja et al., 1994, 1995), the coat protein might interfere with the uncoating of the virus as well as with virus translocation, acting in this case as a non adapted movement protein (tobacco is non host for LMV). Several other cases of protection against PVY were described in tobacco, using either homologous or heterologous potyvirus coat protein genes (Stark and Beachy, 1989; Ling et al.,

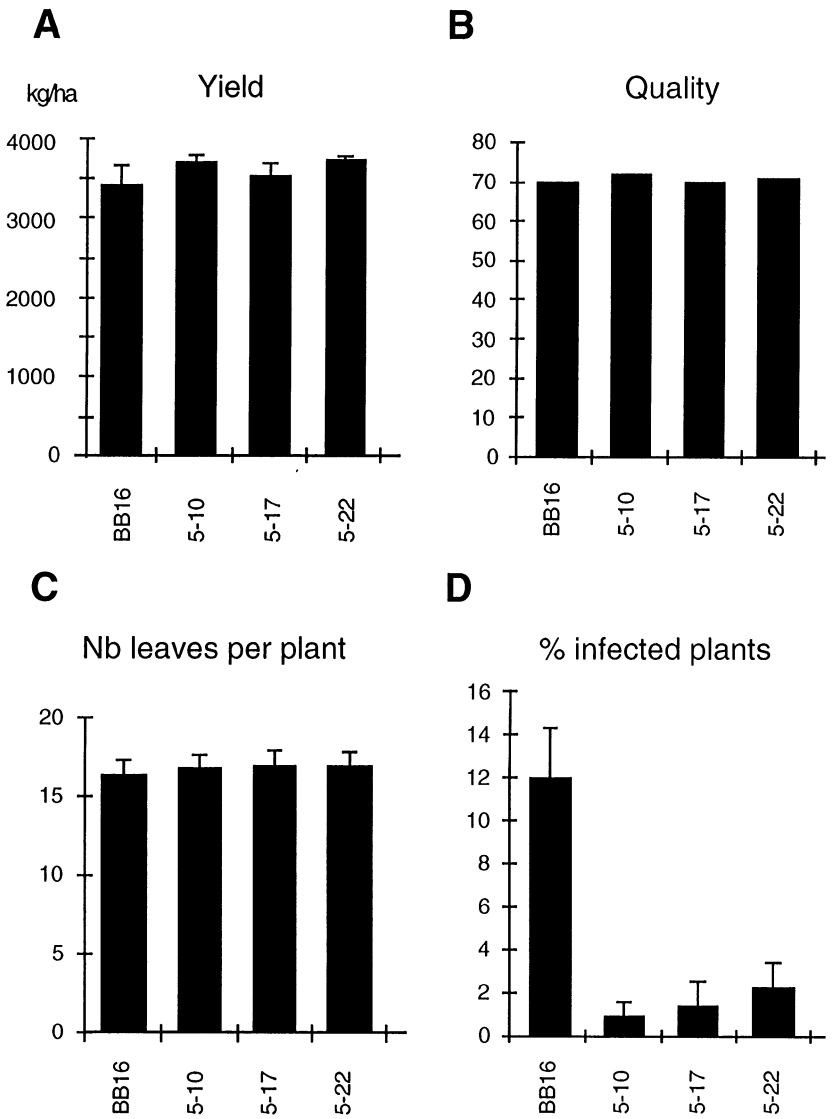

Figure 2. Agronomic quality of the transformants. The quality of the transformant lines was assessed on transgenic BB16 R_2 progeny plants, obtained by self-pollination of three R_1 homozygous plants, either resistant to PVY (BB16-5-10), or tolerant to PVY (BB16-5-17, and BB16-5-22), obtained from the primary transformant BB16-5. A: Total leaf yield (kg/ha), at 25% humidity, evaluated from four repetitions of 55 plants each. B: Quality rate: Index derived from the evaluation of the industrial quality of the leaves in three categories (100 for the best quality, 70, and 40). C: Average number of harvestable leaves per plants. D: Percentage of plants infected by PVY under natural field infection, assessed by symptom notation and ELISA, using a PVY polyclonal antibody (SANOFI Diagnostic PASTEUR).

1991; Farinelli and Malnöe, 1993; van der Vlugt and Goldbach, 1993; Dinant et al., 1993; Kollar et al., 1993; Vardi et al., 1993; Sudarsono et al., 1995). This work reports a detailed study of the modification of PVY distribution in tolerant plants, which shows for the first time an atypical PVY distribution in tolerant plants, while an high percentage of immune plants per line was obtained. This emphasises the variability of the protection obtained by engineered CP genes and the unpredictability of the resultant protection. In addition, the mechanism underlying homologous or heterologous protection may be quite different.

We have shown that transgenic cultivars BB16 and PBD6 commercially deployed tobaccos, expressing the LMV CP gene, were protected against PVY-N Versailles and PVY-N 107, which was confirmed under field conditions in BB16 lines. In PBD6, this protection resulted from an additive effect of the LMV CP

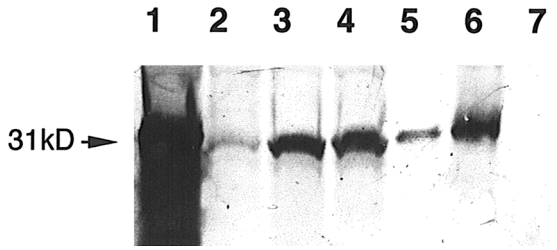

Figure 3. Detection of LMV CP in R_1 transformants by western blot analysis. Total soluble proteins were isolated from leaf samples and separated by 10% SDS-PAGE before transfer to a Hybond C membrane, and immunodetection with a LMV antiserum (H. Lot, Montfavet). The LMV CP is ca. 31 kDa. A polypeptide of the expected size was observed in R_1transformants (lanes 3-6). Lane 1: LMV CP from lettuce infected with LMV. Lane 2: transgenic Xanthi tobacco plant expressing the LMV CP gene (line 69, Dinant et al., 1993). Lane 3-6: transgenic BB16 tobacco plants expressing the LMV CP gene (lines 14, 12, 11 & 10). Lane 7: non transformed BB16 tobacco plant.

gene and the 'va' resistance gene. Since PVY is one of the most prevalent viruses in tobacco in France (Blancard et al., 1994), such new resistance traits could be useful in commercial tobacco cultivars.

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