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Summary

Lettuce mosaic potyvirus (LMV) has been a destructive virus on lettuce crops and the emergence of new strains requires to find out new resistance genes. The coat protein mediated protection approach was shown previously to confer some protection to LMV. Additional transformed lettuce lines were analysed for resistance against LMV-0. The R_2 homozygous progenies of four R_1 resistant Jessy plants showed a protection against LMV similar to the one obtained in the R_2 Cocarde line (Cocarde 9a-91) initially described. Only few plants showed a complete resistance and a large majority of R_2 plants of each progeny showed a recovery phenotype. This LMV protection phenotype was transmitted after cross with untransformed lettuce. A new screening of transgenic lettuce plants was performed on R_1 plants and did not allow the characterization of highly resistant plants. Therefore, the protection conferred by the LMV-0 coat protein seems to be triggered only after a period 5 to 7 weeks after inoculation, period during which plants can not develop any resistance to virus infection.

Lactuca sativa, LMV, coat protein-mediated protection, resistant transformed lines

Introduction

Lettuce mosaic potyvirus (LMV) has been a destructive virus of lettuce crops throughout the world (4). The ability of LMV to be transmitted both through seed and by aphids in a non-persistent manner is highly favorable for the development of the disease. LMV is usually controlled by use of LMV-free seed and tolerant lettuce varieties. However, there are still severe outbreaks due to new emerging virulent strains of LMV (3, 8, 10).

Research of new LMV resistance in lettuce is an important goal for lettuce crop. Genetically engineered virus resistance constitutes an alternative for virus control. Coat protein-mediated protection (CPMP) has been reported for a number of plant RNA viruses (1, 2, 9), including potyviruses (7). Since suitable resistance gene against new LMV strains has been identified only in wild *Lactuca* difficult to cross with lettuce (Maisonneuve et al, in this EUCARPIA meeting), a program of CPMP for LMV has been developed. The LMV coat protein (CP) gene was introduced into three lettuce varieties and few resistant plants were found (6), showing phenomenon of recovery as well as complete resistance. In the present work, the protection was characterized in several other transformants, to determine whether such protection phenotypes were consistent in independent transgenic lines. Moreover, attempts to perform early screening for this CPMP protection were made in order to identify highly resistant lines at heading stage.

Material and methods

Transgenic plants and progeny

Three European lettuce cultivars susceptible to LMV were used, including two butterhead cultivars, Girelle (INRA) and Jessy (Caillard-France), and one leaf lettuce cultivar, Cocarde (Gautier-France). The production of transformants used in this study was described previously (6). The coat protein gene from LMV-0 controlled by an enhanced 35S promoter was introduced in the lettuce genome with the neomycin phosphotransferase marker gene (*nptII*). The integration of a full-length copy of the CP coding region was proved by the presence of a 834bp DNA fragment amplified from genomic DNA by PCR with LMV-specific primers (6). Primary transformants (R_0 generation) were self-pollinated and their derived progenies (R_1 and R_2) seeds were obtained. A cross between cv. Girelle and homozygous transgenic Cocarde 9a-91 line was generated, then the F_2 derived family was produced. The number of insertion loci of the *nptII* gene in R_0 plants was determined by segregation of kanamycin resistance of the R_1 progeny. Homozygous or hemizygous R_1 plants were characterized by segregation of kanamycin resistance of the R_2 progeny.

Protection against LMV

Kanamycin resistant seedlings were maintained in a growth chamber (22 °C /16 °C day/night, 16 hr photoperiod) for inoculation; then some plants were transferred 4-5 weeks after mechanical inoculation (4-5 wpi) to an insect-proof greenhouse for seed production. The inoculum was an extract from infected lettuce plants, cv. Trocadero (1 g grinded in 4 ml of extraction buffer). Plants were inoculated twice (day 0 and 2), at different stages of development: 5-6 leaves (4 weeks after sowing), 10-15 leaves (5-6 weeks after sowing) or 18-20 leaves (6-8 weeks after sowing). These conditions ensured 100% infection of untransformed plants.

The protection of the plants was evaluated according to two methods. On the one hand, to study R_2 progenies of resistant R_1 Jessy plants and the F_2 (Girelle x Cocarde 9a-91-46), DAS-ELISA (Double Antibody Sandwich-ELISA) was used according to the method used previously (6). On the other hand, to research an early protection in R_1 progeny of different transgenic Girelle and Jessy, only observations of symptom development were made within two weeks after inoculation.

Results and discussion

Resistance to LMV-0 in R_1 and R_2 progenies of two transformed Jessy after late inoculation

Two R_1 lettuce families, Jessy-1b and Jessy-4a, were inoculated at the stage 15-20 leaves, stage of development shown previously to give the best results of protection. For Jessy-1b, 5 out of 28 inoculated plants showed some level of protection, and for Jessy-4a, 4 plants out of 30 did. Resistant as well as recovery plants were observed (Table 1). The recovery phenotype corresponds to plants initially infected but which did not showed virus accumulation neither symptom in the upper leaves at later stages after inoculation (e.g. plant Jessy-4a-30 with OD = 1.4 at 9 dpi, 1.7 at 22 dpi and 0.1 in upper leaf at 34 dpi).

Table 1. Protection against LMV-0 in the kanamycin-resistant R_1 progenies of two R_0 Jessy transformed plant (evaluation of resistance by DAS-ELISA test)

	No. of LMV-0 resistant plants / No. of studied inoculated plants		
No. of days post inoculation	Jessy-1b	Jessy-4a	Jessy [a] (control)
9 dpi	nd	1 R / 30	0 R / 15
20 or 22 dpi	6 R / 28	1 R / 30	0 R / 15
34 dpi [b]	2 R / 4	4 R / 6 [c]	0 R / 5
60 dpi	5 R / 13[d]	nd	nd

[a] untransformed Jessy : 0.6<OD<1.5 at 9 dpi ; 1.7<OD<2.4 at 22 dpi ; 1.4<OD<2.3 at 34 dpi for upper leaves. nd: not determined;
[b] control of only few plants including plants –1b-8 and -4a-26, -28, -30;
[c] 3 R plants had not infected upper leaves (OD<0.1), infected lower leaves (OD>2.6); plant 28 had no virus (OD =0.0 in both level of leaves);
[d] upper and lower leaves of 4 plants (including plant 8) were not infected (OD<0.2); 1 plant with virus in lower leaves (OD = 0.1 for upper and 1.0 for lower leaves).

Table 2. Protection against LMV-0 in some homozygous R_2 progenies from two different transformed Jessy

R_0 plant	Jessy1b	Jessy4a			Jessy [a]
R_1 plant	8	28	26	30	(control)
Phenotype	R	R	recovery [b]	recovery [b]	
No. of days post inoculation	No. of resistant plants / No. of inoculated plants [c]				
16 dpi	1 R / 30	nd	nd	nd	0 R / 20
26 dpi	nd	0 R / 24	0 R / 24	1 R / 24	0 R / 15
30 dpi	1 R / 30	nd	nd	nd	0 R /20
34 dpi	nd	2 R / 24	13 R / 24	7 R / 24	0 R / 10
44 dpi	30 R / 30	nd	nd	nd	0 R / 20
47 dpi	nd	15 R / 24	20 R / 24	17 R / 24	0 R / 15
Treatment	Seeds production (av. weight / per plant in g) [d]				
Inoculated		7.7	8.4	10.9	4.3
Control		8.7	12.6	13.5	13.5

[a] 20 and 15 inoculated plants as control respectively of 1b-8 and 4a families;
[b] plant accumulating LMV-0 at 9 and 22 dpi; at 34 dpi, no detectable virus in upper leaves;
[c] plants were inoculated in a growth chamber at the 15-20 leaf stage, and assayed by DAS-ELISA with 2 levels of leaf (upper and lower) after 30 dpi (OD<0.06 in not inoculated plants, OD>0.5 in inoculated Jessy); transfer in greenhouse at 31 dpi (1b family in April) or 28 dpi (4a families in September);
[d] average of 24 inoculated and 4 not inoculated plants of Jessy-4a, average of 15 inoculated and 4 not inoculated plants of Jessy (control).

Homozygous R_1 plants were selected by segregation of kanamycin resistance of the R_2 progenies. The LMV resistance of four of these lines was extensively studied: two out of these progenies derived from LMV resistant plants (R_1 plants = 1b-8 and 4a-28) and two derived from recovery plants (R_1 plants = 4a-26 and 4a-30). Kanamycin resistance tests (germination on 100mg/l kanamycin) suggested that these plants were homozygous for the insertion (100% resistant seedlings on 139, 52, 47 and 67 germinated seeds). As observed for parental plants, total resistance as well as recovery was observed for each of these lines (Table 2) with 63% to 100% of plants without virus at 44-47 days after inoculation. The fertility of the plants was only slightly affected by virus infection in the transgenic CP plants compared to control plants (Table 2).

Expression of the transgenes, *nptII* and CP-LMV0, in F_2 (cv. Girelle x Cocarde-9a-91-46)

The segregation for kanamycin resistance (309 resistant : 86 susceptible) fit with the expected ratio (3 : 1) for one dominant gene ($\chi^2 = 1.6$). Test of F_2 with LMV-0 gave 11 recovery plants on 50 inoculated (Table 3). Considering that the ratio of homozygous to hemizygous plants could be 1 to 2, the number of recovery (11 plants), for 16.6 expected homozygous plants, was consistent with previous percentage of recovery plants in homozygous line (66% in F_2 vs 63 to 83% in Jessy-4a). This results suggest a good mendelian transmission of the both transgenes (*nptII* and CP) and a stable expression through crosses with untransgenic varieties.

Table 3. Protection against LMV-0 in the kanamycin resistant plants of the F_2 between a cultivar (Girelle) and the homozygous transgenic line (Cocarde-9a-91-46)

No. of days post inoculation	No. of resistant plants / No. of inoculated plants[a]		
	F_2 (cv Girelle x Cocarde-9a-91-46)	Control	
		cv. Girelle	cv. Cocarde
20 dpi	0 R / 50	0 R / 10	0 R / 10
36 dpi	10 R / 50	0 R / 10	0 R / 10
43 dpi	11 R / 50	0 R / 10	0 R / 10

[a] plants were inoculated in a growth chamber at the 15-20 leaf stage, and assayed by DAS-ELISA with 2 levels of leaf (upper and lower) at 36 and 43 dpi; transfer in greenhouse at 30 dpi.
OD<0.05 in not inoculated plants (14 plants); OD>0.9 in inoculated Girelle and Cocarde; OD>1.7 for 50 F_2 plants at 20 dpi; OD<0.1 for upper leaves of resistant plants

Early screening for LMV-0 resistance in different R_1 transgenic families

In previous transgenic lines, Cocarde-9a, Jessy-1a and Jessy-4a, LMV-0-CP gene did not give an immunity to LMV. Because this early protection is the most important thing for growers, a screen of the other available transgenic Jessy and Girelle progenies was performed. Fifteen to 45 kanamycin resistant seedlings per family were inoculated

with LMV-0 at 4-leaf stage. In six R_1 Girelle and eight R_1 Jessy (progenies of 10 different bud clusters), typical mosaic symptoms were observed on every plant within two weeks after inoculation in growth chamber. Similar results were obtained in 10 R_1 inoculated at later stage (10 to 15 leaf stage).

These results suggest that screening based on the routine procedure used in many genetic programs for virus resistance (early inoculation of seedlings and observation of symptoms expressed in 2-3 weeks after inoculation) does not lead to the identification of resistant CP-plants. It is also clear from these data that the level of protection against LMV after artificial inoculation in these transgenic plants is not sufficient. But in the natural infection by aphids, the level of protection could be higher because of the lower concentration of virus in inoculum. This has to be tested to know if this CPMP strategy could be interesting for lettuce production.

Conclusions

This work confirms previous data obtained on one transgenic Cocarde line. The LMV-0 coat protein gene, controlled by enhanced 35S promoter, confers homologous protection to LMV-0 in lettuce. The protection is characterized in all lines by a phenotype of recovery in majority of plants as well as total resistance in few plants. Genetic analysis of segregation of kanamycin marker associated to LMV-CP gene suggests that the resistance is expressed only in homozygous plants. However, the plant factors inducing recovery or total resistance are not known. This CPMP resistance as well as kanamycin resistance, which is controlled by a NOS promoter, is stable in progeny of hybrid F_1 between a lettuce variety and a homozygous transgenic line.

The consistency of the protection obtained in different lines suggests that the CP gene interferes only partially with the multiplication of LMV. Recovery phenotype could be interesting for seed production, but immunity or, at least resistance at heading stage, would be even more interesting. The moderate protection observed so far may result from insufficient level of expression of coat protein in transgenic plants. Coat protein accumulation was however detected in Cocarde-9a and Jessy-1b transformed plants (6). It will be interesting to compare other strong constitutive promoters to control the expression of the CP gene. A similar construct introduced into tobacco plants conferred immunity in cases of heterologous protection against PVY (5). Heterologous protection could therefore be more efficient than homologous protection against potyvirus; therefore a comparison in lettuce of homologous versus heterologous protection against LMV could also give some indication of the mechanism underlying this protection.

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