

Characterization of epitopes on zucchini yellow mosaic potyvirus coat protein permits studies on the interactions between strains

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Monoclonal antibodies (MAbs) raised against the coat protein of zucchini yellow mosaic potyvirus (ZYMV) were characterized by epitope mapping using synthetic oligopeptides. Two mutant viruses with a mutation in the amino acid sequence important for epitope recognition *in vitro* were obtained by site-directed mutagenesis of a full-length cDNA of ZYMV. Two MAbs, CC11 and DD2, could distinguish specifically between these mutants in mixed infections, or after sequential inoculations of muskmelons. Sequential inoculations of the mutants and analysis with MAbs CC11 and DD2 revealed that cross-protection was established between these quasi-isogenic strains within 48 h.

Potyvirus present an important variability in their biological and serological properties, probably related to the frequent generation of mutants due to the high error rate of viral RNA polymerases (Domingo & Holland, 1994). Understanding factors involved in the competition between virus strains, and in plant virus evolution, is the first step towards developing durable control strategies, specially through breeding for resistance or cross-protection. Nucleotide or amino acid sequencing of parts of the viral genome provides direct information on the level of variation (Ward *et al.*, 1992), but it is not always convenient when studying large numbers of strains. Barbara *et al.* (1995) could distinguish isolates of zucchini yellow mosaic potyvirus (ZYMV) in mixed infections by PCR amplification and restriction fragment length polymorphism analysis, but this method is difficult to use for quantitative estimations. Serological tests, particularly using monoclonal antibodies (MAbs), can reveal virus variability more easily, but a precise determination of the epitopes recognized by the MAbs is required to localize the variable domains.

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One of the most accurate current techniques for epitope analysis is the use of synthetic overlapping peptides (Geysen *et al.*, 1984). The contribution of individual residues to the formation of linear epitopes recognized by MAbs can be determined (Shukla *et al.*, 1989; Andreeva *et al.*, 1994).

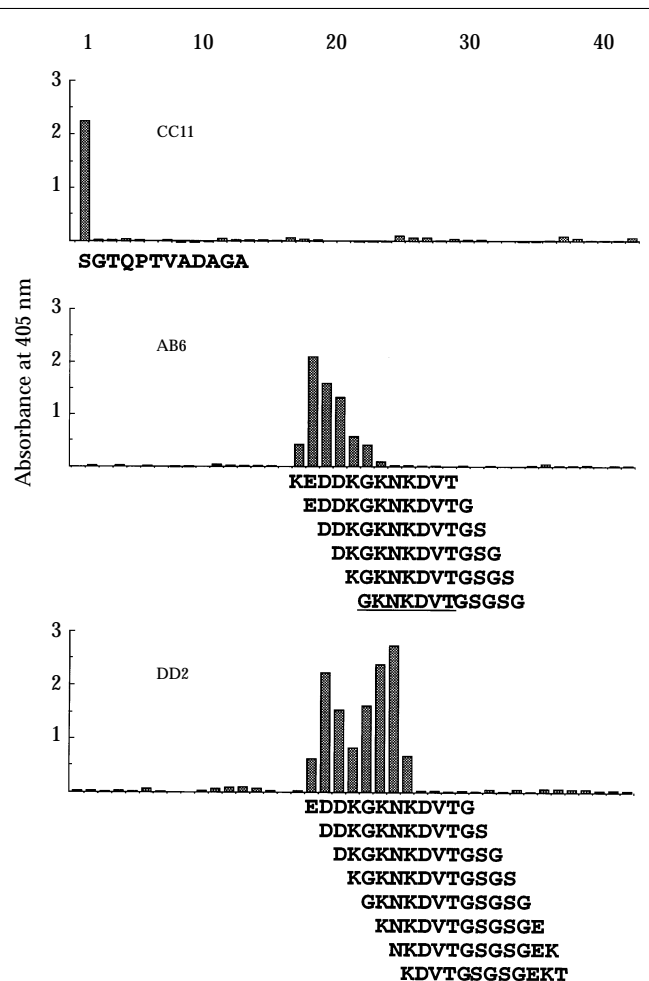


Fig. 1. Reactivity of MAbs CC11, AB6 and DD2 with overlapping synthetic dodecapeptides representing the N-terminal part of ZYMV CP. Underlined amino acids indicate the linear epitopes recognized by the antibodies.

(a)	Amino acid sequence			Recognition by MAbs		
				CC11	AB6	DD2
MT2	Q / <u>SGTQPTVADT</u>	GATKKDKEDD	KGKNKDVTGS	+++	+++	+++
MT9	./ D	+	+++	+++
MT9.C3	./ D .. S	0	+++	+++
MT1	./..... A ..	+++	+	0
 (b)						
NAT	Q / <u>SGTQPTVADT</u>	GATKKDKEDD	KGKNKDVTGS	+++	+++	+++
Mutant A	./..... S	0	+++	+++
Mutant B	./..... A ..	+++	+	0

Fig. 2. Mutations in the N-terminal part of ZYMV CP and serological reactivity in TAS-ELISA tests. (a) Amino acid sequence of the N-terminal part of the CP of ZYMV isolates from Martinique (MT isolates), and their serological reactivity in TAS-ELISA tests with MAbs CC11, AB6 and DD2. The Q/S sequence identifies the cleavage site between the putative polymerase and the CP. Absorbance at 405 nm (A) 3 h after substrate incubation: + + +, $A > 1.5$; + +, $0.5 < A < 1.5$; +, $0.05 < A < 0.5$; 0, $A < 0.05$. (b) Amino acid sequence of the N-terminal part of the CP of ZYMV mutants A and B obtained by site-directed mutagenesis and the NAT strain, and their serological reactivity in TAS-ELISA with MAbs CC11, AB6 and DD2.

However, this method is not suitable for analysis of the conformational epitopes present only in intact virions.

MAbs to ZYMV coat protein (CP) were obtained as previously described (Desbiez *et al.*, 1996). The amino acids involved in the formation of epitopes recognized by these MAbs were mapped and analysed using synthetic overlapping peptides according to Geysen *et al.* (1984). Forty-one dodecapeptides with a slippage of one amino acid were used to analyse the N-terminal part of the CP, while 45 dodecapeptides with a slippage of 5 amino acids were used to study the core and C-terminal part of the CP of 238 amino acids. The peptides were synthesized by Chiron Mimotope Peptide System (CMPS; Clayton VIC 3169, Australia) on polyethylene rods. The choice of peptides allowed a precise epitope characterization for the N-terminal part of the CP, which is known to be highly variable, surface-located and to contain most of the potyvirus-specific epitopes (Shukla *et al.*, 1988). Two additional peptides, PLAQGGGG and GLAQGGGG, were synthesized and used as negative controls. Peptide recognition by the MAbs was tested as described by Geysen *et al.* (1984), except that blocking solution, antibody solution and conjugate were incubated in batches instead of on ELISA plates. Each MAb was tested twice, using the same peptide set and protocol.

Epitope mapping using overlapping peptides revealed that MAbs AB6, CC11 and DD2 recognized linear epitopes, defined by oligopeptides from the N-terminal part of the ZYMV CP (Fig. 1). No reaction was observed with oligopeptides from the core or C-terminal part of the CP (data not shown). MAb CC11 recognized the N-terminal extremity of the CP. The precise size of the epitope was not determined, but the first amino acid of the CP was required for reactivity. The sequences GKNKDVT and KDVTG, which share four amino acids (underlined), play an important role in the formation of epitopes recognized by AB6 and DD2, respectively. These results agree with data obtained for field isolates of ZYMV that showed a partial correlation between recognition by DD2 and AB6 (C. Desbiez, unpublished).

A preliminary study on ZYMV isolates from Martinique revealed some serological variability with MAbs CC11, DD2 and AB6, without any apparent correlation with the biological properties of the isolates (Desbiez *et al.*, 1996). Sequencing of the N-terminal part of the CP coding region revealed point mutations (Fig. 2a) which fall within the domains defined by epitope mapping (Fig. 1).

The mutations present in natural ZYMV populations from Martinique were introduced into an infectious cDNA of ZYMV (Fig. 2b) to confirm the importance of these amino acids for *in vivo* epitope formation, and to obtain markers for the specific detection of quasi-isogenic ZYMV variants. An infectious cDNA of the NAT strain of ZYMV was cloned (Gal-On *et al.*, 1991). Site-directed mutagenesis was performed according to the method of Kunkel (1985) on a 5.6 kbp *Pst*I₃₈₂₅-*Eco*RV₉₄₅₀ fragment subcloned in pBlueScript II KS+ (Stratagene). Oligonucleotides A (GCACTCAG-AGTACTGTG) and B (GGATGTTGCCGGCTCCG) were used to obtain mutants A and B, respectively. Bold letters indicate mutated nucleotides and underlined letters indicate restriction sites (for *Sca*I and *Nae*I, respectively) introduced during mutagenesis. Each mutation was introduced in full-length ZYMV cDNA under a cauliflower mosaic virus 35S promoter by replacement of the 2.2 kbp *Sph*I₆₅₁₈-*Mlu*I₈₇₄₅ fragment. Mutated viral cDNA was inoculated onto cotyledons of zucchini squash or cucumber plantlets by particle bombardment, a technique that has been shown to be more efficient than mechanical inoculation of cDNA or transcripts (Gal-On *et al.*, 1995). The presence of the mutations in the infectious viruses was checked by sequencing RNA from systemically infected leaves of inoculated plants (Sanger *et al.*, 1977). Subsequent mechanical inoculations to propagate the mutant strains followed standard methods (Lecoq *et al.*, 1991). Tests for aphid transmission and the host range of the mutants were as described previously (Desbiez *et al.*, 1996).

Mutant and wild-type ZYMV strains were quantified by double-antibody sandwich (DAS)-ELISA with polyclonal anti-

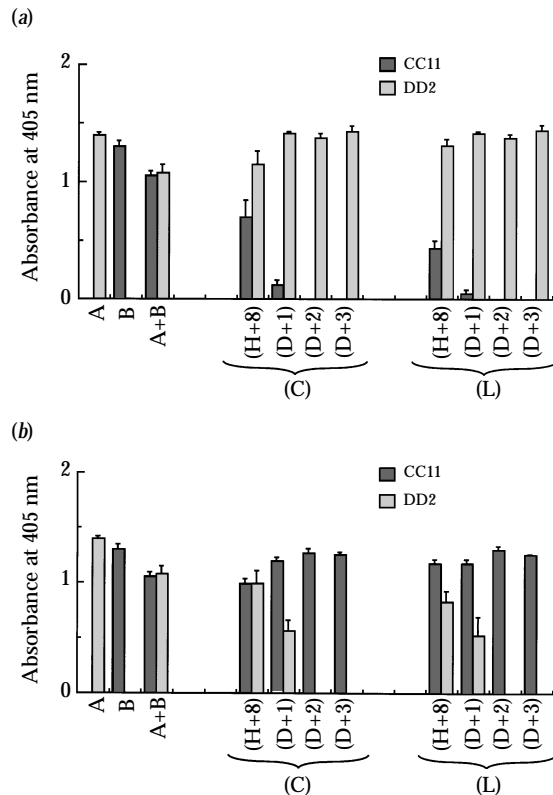


Fig. 3. Specific detection of ZYMV mutants A or B in TAS-ELISA using MAbs CC11 and DD2. The challenging strain was inoculated at intervals from 8 h (H+8) to 3 days (D+3) to melon Védraçais, either on the cotyledons already inoculated with the protecting strain (lanes 'C') or on the non-inoculated first true leaf (lanes 'L'). Results are expressed as the mean of two independent experiments, using four plants for each assay, with the standard error indicated. Mutant viruses were inoculated alone (lanes A, B) or co-inoculated at the same time (lane A+B). (a) Mutant A is the protecting strain, and mutant B the challenging strain. (b) Mutant B is the protecting strain, and mutant A the challenging strain.

body raised against the ZYMV E9 strain, and an adapted triple-antibody sandwich (TAS)-ELISA for characterization with the MAbs (Desbiez *et al.*, 1996).

Plants inoculated with the two mutated cDNAs showed symptoms within 1 week. Symptoms on zucchini, melon Védraçais and cucumber were identical to those induced by wild-type virus: vein clearing on the young leaves followed by a systemic mosaic, yellowing, stunting and leaf deformations. Mutants A and B had the same pathology as wild-type ZYMV NAT strain in melon: they induced systemic necrotic spots in melon PI414723 possessing the *Zym* resistance gene to ZYMV, and they did not induce a rapid, lethal wilting in melon Doublon expressing the *Fn* gene (Lecoq & Pitrat, 1984). The mutants, like the NAT strain, had a 'DTG' sequence instead of the 'DAG' present in the N-terminal part of the CP of aphid-transmissible potyviruses (Atreya *et al.*, 1990; Gal-On *et al.*, 1992), and they were aphid non-transmissible (data not shown).

The results of three independent ELISA tests from crude leaf extracts ground 1:10 (w/v) in 0.03 M Na₂HPO₄ pH 9,

containing 0.2% sodium diethyldithiocarbamate trihydrate (DIECA) are indicated in Fig. 2(b). Mutation A totally abolished recognition by MAb CC11, and had no effect on recognition by AB6 and DD2. Mutation B had no effect on recognition by CC11, but abolished recognition by DD2 and reduced recognition by AB6 by up to 90%. Recognition of the mutants by polyclonal antiserum was unaffected. Similar results were obtained with purified virus diluted in phosphate with DIECA, or PBS pH 7.4 to a final concentration of 10 µg/ml. This indicates that the results were not due to a lowered multiplication of some mutants. A series of twofold dilutions of purified virus, ranging from 160 µg/ml to 0.3 µg/ml, was tested with MAbs CC11 and DD2, and revealed that the antibody signal strength for different quantities of viral antigen was similar for both MAbs (data not shown).

The differential reactivity of mutants A and B using CC11 and DD2 was exploited to investigate cross-protection between these two quasi-isogenic ZYMV strains. Mutants A and B were mechanically inoculated, alone or together, on the cotyledons of four melons Védraçais at the one-leaf stage. The singly infected plants were subsequently inoculated either on the cotyledons, or on the first leaf, with the reciprocal mutant, at intervals of 8 h (H+8), 1 day (D+1), 2 days (D+2) and 3 days (D+3) after the first inoculation. The presence of each strain in the third true leaf of each plantlet was checked 2 weeks later by TAS-ELISA using the specific MAbs CC11 and DD2. Fig. 3 shows the mean absorbance obtained for eight plants from two independent repeats at each inoculation interval. The results were similar when the fifth true leaves were tested 4 weeks after inoculation (data not shown).

The use of MAbs CC11 and DD2 to detect mutants A and B independently in mixed infections revealed a competition between strains that was readily established in less than 48 h (Fig. 3). Infection by the challenging strain could occur when the interval between inoculations was up to 24 h, but detection of the challenging strain was lowered, suggesting a reduced virus multiplication or migration under these conditions. Similar protection was observed whether the challenging strain was inoculated on the cotyledons (already inoculated with the protecting strain) or on the first non-inoculated leaf. The use of mutants A or B as protecting or challenging strain had symmetrical effects, suggesting that the protection was not related to the lower fitness of one of the mutants.

The protection obtained in this study was established in a very short time compared to previous cross-protection studies. Walkey *et al.* (1992) showed that a 2 week incubation of zucchini squash plants inoculated with the mild strain ZYMV WK (Lecoq *et al.*, 1991) was necessary for efficient protection (estimated by symptom severity) against challenge with severe ZYMV isolates; no protection was observed when the interval between inoculations was 2 to 8 days. Cross-protection is known to be more efficient when the protecting and challenging strains are closely related (Wang *et al.*, 1991). The mild and severe strains used by Walkey *et al.* (1992) originated from

different hosts and geographical regions, and probably presented a lower homology than the quasi-isogenic mutants used in this paper. However, our results measured actual amount of virus antigen, whereas Walkey *et al.* (1992) depended on symptom expression, which may not be directly correlated to virus replication and accumulation.

In this study, we characterized the ZYMV CP epitopes recognized by three MAbs. The results confirmed the importance of the N-terminal part of the CP in epitope formation (Shukla *et al.*, 1988, 1989), and correlated well with serological and sequence data available for ZYMV isolates (Desbiez *et al.*, 1996). Site-directed mutagenesis of a full-length cDNA identified some of the amino acids important for epitope formation *in planta* with whole virus, which confirmed the results obtained *in vitro* with overlapping peptides. The point mutations introduced had no obvious effects on symptomatology on the hosts examined, or on viral multiplication, as estimated by ELISA tests. These mutations could be used as neutral markers for the study of interactions between ZYMV variants in mixed infections.

Biologically neutral point mutations specifically detected by MAbs, introduced in ZYMV variants, may also be useful for quantitative analysis of the competition between strains presenting biological differences, and may contribute to our understanding of the evolution of plant virus populations through competition between variants with differential fitness.

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