Research Note

Mutants of *Tobacco mosaic virus* with Temperature-Sensitive Coat Proteins Induce Heat Shock Response in Tobacco Leaves

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We analyzed, with respect to heat shock proteins (HSPs), systemically reacting tobacco leaves inoculated with *Tobacco mosaic virus* (TMV), wild-type *vulgare*, and temperature-sensitive coat protein (CP) mutants *Ni 118* (P20L) and *flavum* (D19A), kept at 23 or 30°C. HSP18 and HSP70 mRNAs and proteins were induced with temperature-sensitive CP mutants after 1 to 2 days at 30°C. After 4 to 6 days, HSP70 was also induced at 23°C. The induction of HSPs paralleled the amount of insoluble TMV CP in leaf extracts, indicating that denatured TMV CP by itself induces a heat-shock response.

As the result of single amino acid replacements, the coat proteins (CPs) of a number of *Tobacco mosaic virus* (TMV) mutants are extremely temperature labile, i.e., at moderately elevated temperatures, they misfold and form insoluble aggregates rather than virus particles (Jockusch 1964; Jockusch 1966a; Jockusch 1966c; Jockusch 1968b). Yet under such “nonpermissive” conditions, temperature-sensitive CP mutants spread in the plant similarly to the wild-type, temperature-resistant TMV, which indicates that their replication and motility functions are not impaired (Jockusch 1966b; Jockusch 1968a). In model experiments on *Xenopus laevis* oocytes, microinjected, denatured bovine serum albumin elicited a heat shock response, whereas native albumin did not (Ananthan et al. 1986). We therefore tested whether denatured CPs of temperature-sensitive TMV mutants would elicit a heat shock response (Neumann et al. 1989; Vierling 1991) in infected leaves at moderately elevated temperatures.

We used wild-type TMV temperature-resistant *vulgare* and two temperature-sensitive CP mutants, *Ni 118*, with the amino acid replacement P20L, and *flavum* (Melchers 1942), with D19A (Wittmann 1962; Wittmann 1964; Wittmann and Wittmann-Liebold 1966), as confirmed by sequencing the reverse transcription-polymerase chain reaction (RT-PCR) products of CP genes. Within positions 5,600 to 6,190 (Goelet et al. 1982), no additional base changes were found, indicating a rather low level of as yet undetected mutations (cf. Zimmern and Hunter 1983).

Mature leaves of systemically reacting tobacco plants (*Nicotiana tabacum* cv. *Samsun n/n*) were dusted with carborundum and inoculated with 0.5 mg of purified TMV per ml. After 1 day at 23°C, half of the plants were shifted to 30°C. At 1 to 6 days later, leaves were harvested and shock frozen in liquid N₂.

To analyze for proteins, 500 mg of frozen leaves were homogenized in 1,000 µl of 100 mM Na phosphate, 5 mM ascorbate, 10 mM EDTA, and 5 mM dithioerythrol. The homogenate was centrifuged at 10,000 g for 30 min to 80 µl of supernatant 20 µl of sodium dodecyl sulfate (SDS) containing dissociation buffer were added. The sediment was washed twice with 1,000 µl of extraction buffer and taken up in SDS dissociation buffer in order to adjust the concentration (in reference to fresh weight) to that of the supernatant. Proteins were separated on SDS polyacrylamide gels, which were fixed and stained with Coomassie brilliant blue or blotted onto Protran nitrocellulose filters (Schleicher and Schuell, Dassel, Germany). TMV CP was identified by a monoclonal antibody against intact *vulgare* virus, which also reacted with SDS denatured TMV CP.

At 23°C, soluble TMV CP comprising native CP and virions was in the supernatant of the nondenatured extracts. Under nonpermissive conditions, e.g., with temperature-sensitive mutants at elevated temperatures, most of the CP was in the pellet as a result of denaturation and aggregation (Jockusch 1966a; Jockusch 1968a; Jockusch 1968b). At 30°C, however, some wild-type CP denatures, whereas a sizable proportion of the CP of temperature-sensitive mutants already denature at 23°C (Fig. 1).

To detect HSP18 (Mansfield and Key 1987), we used a polyclonal anti-*Arabidopsis* HSP18 that reacted only with nondenatured HSP18 of tobacco on dot blots. There was a positive reaction with anti-HSP18 1 day after temperature shift in all samples, including mock inoculation. One day later, there were stronger responses, as indicated by antibody staining at higher dilutions, with *Ni 118* and *flavum* at 30°C (Fig. 2A).

A monoclonal antibody against bovine brain uncoating AT-Pase (Höning et al. 1994) was used to detect HSP70 (Aranda et al. 1996) on immunoblots after SDS electrophoresis (Fig. 2B). Initially, HSP70 was detected after inoculation, even in mock-inoculated leaves. On day 2, signals from *Ni 118* and *flavum*-inoculated leaves were by far the highest, exceeding the 48°C positive control. At approximately day 4, the tem-

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perature-sensitive mutants gave signals, even at 23°C, and on day 6, the signals were still specific for the temperature-sensitive mutants, although not different for 23 and 30°C. Additionally, there were no signals above background upon incubation at 30°C alone.

Total RNA was extracted from frozen leaves with the plant RNA version of the RNase Kit (Qiagen, Hilden, Germany). Four days after the shift, there were intense 6-kb TMV RNA bands in *vulgare*-infected plants, at 23 and 30°C, in ethidium bromide-stained RNA gels. With *Ni 118* and *flavum*, bands were weaker and seen only at the lower, permissive temperature (not shown). Most likely, these bands reflect TMV RNA packaged in virions, a function defective with temperature-sensitive mutants at 30°C and already impaired at the permissive temperature. The primers used to sequence the CP gene and detect the CP message of TMV were based on Goellet et al. (1982). TMV-CPs: GTAGTAATGATCGGTCAGTGC; TMV-CPas1: CGCTCCTTATGGCCACCG; TMV-CPas2: T-ATTATGCATCTTGACTACC; for HSP18, HSP18s: GGAG-CAATATTTTCGACCCG; HSP18as: CCCAAAATATCCAA-CACATCC (accession no. X70688); for HSP70, HSP70s: GG-TGAAGAGAAACAGTTTGCTC; HSP70as: CTCCTCAGA-GCTCTAGCATTG (accession no. X63106); for actin, Atact-S: GAGAAGATGACTCAGATC; and Atact-A: ATCCTTCC-TGATATCGAC (accession no. U37281).

Leaf RNAs were analyzed by RT-PCR. One day after temperature adjustments, the 507-bp amplificate of CP sequence (representing the CP message) was present in high amounts with *vulgare* and the temperature-sensitive mutants at both temperatures (Fig. 3A). All samples, except the uninfected control at 23°C, showed HSP18 amplificate. Next intense to the positive-control, uninfected leaves heat shocked at 48°C, were the bands of temperature-sensitive, mutant-infected leaves at 30°C, followed by those at 23°C and the *vulgare*-infected samples (Fig. 3B). Thus, the levels of HSP18 mRNA amplificate 1 day after temperature shift were roughly equivalent to the corresponding protein levels 2 days after the shift.

With HSP70 RT-PCR, signal intensities were F 30°C ~ *Ni 118*, 30°C > F 23°C > *Ni 118*, 23°C ~ *vulgare*, 30°C > *vulgare*; 23°C (Fig. 3C). Whereas with HSP18 there was a "background" response to the 30°C incubation alone, the HSP70 reaction was highly specific to TMV infection.

The difference between temperature-resistant and temperature-sensitive CPs is not all or nothing but is reflective of shifts in the "melting temperatures" of tertiary structures (Jockusch 1968b). Even the CP of *vulgare*, with a melting temperature of 36°C, is relatively labile, and some denatured CP is produced by TMV *vulgare* at 30°C. Moreover, with *Ni 118* CP, with a melting temperature of 27°C, denatured CP and

Fig. 1. Native and denatured *Tobacco mosaic virus* (TMV) coat proteins (CPs) in leaf tissue inoculated with the wild-strain *vulgare* temperature-resistant and temperature-sensitive mutants, *Ni 118* and *flavum*, respectively, kept at 23°C (23) or shifted to 30°C (30) and harvested 6 days after the shift. Supernatants (S) and washed pellets (P) of extracts corresponding to 3 mg of fresh weight per lane were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% polyacrylamide, 90 min × 10 V per cm) and **A**, stained with Coomassie blue or **B**, immunoblotted with anti-TMV or X-ray film exposed to ECL-developed filter. Mock: Leaves rubbed with phosphate buffer without TMV. +V: 1 mg of TMV added to 500 mg of mock-inoculated leaf material prior to homogenization; V': 1 µg of oxidized virus preparation. Lower band: CP monomer, 17.5 KDa. Upper band: CP dimer, 35 KDa. When measured by dye elution, TMV-infected leaves contained 6–8 mg of TMV CP per g of tissue. Fractions soluble-total CP: V 23°, < 0.01; V 30°, ≤ 0.02; 118 23°, 0.67; 118 30°, > 0.95; F 23°, 0.55; F 30°, > 0.95.

Fig. 2. Levels of HSP18 and HSP70 as shown by antibody detection in soluble leaf extracts (second antibody is horseradish peroxidase [HRP] conjugated). Positive-control (48) leaf tissue heat shocked for 2 h at 48°C. **A**, Dot blot stained with polyclonal anti-HSP18. Incubation period of plants after shift to experimental temperature (left) and relative concentrations of extracts (right). HRP-chloronaphtol development. **B**, Immunoblot after sodium dodecyl sulfate electrophoresis (10% polyacrylamide, approximately 3 mg of fresh weight per lane) stained with monoclonal anti-HSP70. Co 48: Positive control as in **A**. X-ray film exposed to HRP-ECL-developed filter.

reduced virus yields are also found at 23°C. Denatured TMV CPs accumulate in the insoluble fraction but also may be degraded preferentially by proteolysis. The low level of unspecific mutations in *Ni 118* and *flavum* support the view that the temperature-sensitive CP mutations are indeed responsible for the heat-shock responses described. Thus, the notion that denatured proteins can induce a heat-shock response, as shown in an animal cell model (Ananthan et al. 1986), seems to also hold for the tobacco-TMV system.

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Fig. 3. Tobacco mosaic virus (TMV) coat protein, HSP18, and HSP70 mRNA levels as estimated by reverse transcription-polymerase chain reaction. **A**, Gels after ethidium bromide (EtBr) staining, UV images. Bars to the left: 500 bp. **B and C**, EtBr signals relative to the β -actin signal. Combined results from two independent experiments, with individual results indicated by $\bullet$ and $\blacktriangle$. Contr. 23: Untreated tobacco leaves grown at 23°C. Contr. 48: Leaf tissue heat shocked for 2 h at 48°C **B**, HSP18 mRNA; **C**, HSP70 mRNA.

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