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Fate of hairpin transcript components during RNA silencing and its suppression in transgenic virus-resistant tobacco

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Abstract

Transgenic tobacco plants, carrying a Potato virus Y (PVY)-NIa hairpin sequence separated by a unique unrelated spacer sequence were specifically silenced and highly resistant to PVY infection. In such plants neither PVY-NIa nor spacer transgene transcripts were detectable by specific quantitative real time reverse transcriptase PCR (RT-qPCR) assays of similar relative efficiencies developed for direct comparative analysis. However, small interfering RNAs (siRNAs) specific for the PVY sequence of the transgene and none specific for the LNYV spacer sequence were detected. Following infection with Cucumber mosaic virus (CMV), which suppresses dsRNA-induced RNA silencing, transcript levels of PVY-NIa as well as spacer sequence increased manifold with the same time course. The cellular abundance of the single-stranded (ss) spacer sequence was consistently higher than that of PVY dsRNA in all cases. The results show that during RNA silencing and its suppression of a hairpin transcript in transgenic tobacco, the ssRNA spacer sequence is affected differently than the dsRNA. In PVY-silenced plants, the spacer is efficiently degraded by a mechanism not involving the accumulation of siRNAs, while following suppression of RNA silencing by CMV, the spacer appears protected from degradation.

Keywords: Post-transcriptional gene silencing; Real time RT-PCR; RNA silencing suppressor; Double stranded RNA

1. Introduction

Double-stranded (ds) RNA has been shown to induce sequence-specific gene silencing in plants and many other eukaryotic organisms (Fire et al., 1998; Waterhouse et al., 1998; Zamore et al., 2000). The mechanism of RNA silencing involves an initial processing of the dsRNA into small interfering (si) RNAs of 21–25 nucleotides, which correspond to both sense and antisense strands of the target gene (Hamilton and Baulcombe, 1999). Dicer, an ATP-dependent RNase III-like ribonuclease, specifically cleaves dsRNA into siRNAs (Bernstein et al., 2001). RNA silencing can be induced to a greater degree by dsRNA-encoding

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inverted repeat constructs as compared to sense or antisense constructs (Waterhouse et al., 1998; Wesley et al., 2001). The generation of dsRNA-encoding gene constructs in bacterial plasmids requires insertion of a spacer sequence to separate the sense and antisense target sequences forming the dsRNA. The spacer sequence can be a splicable or non-splicable intron or any other unrelated sequence (Smith et al., 2000; Wesley et al., 2001; Kalantidis et al., 2002; Lacomme et al., 2003). When such constructs are expressed in transgenic plants, introns will be spliced out and degraded in the nucleus (Moore, 2002), while transcripts of hairpin loop constructs with a non-splicable spacer sequence will contain a dsRNA stem and a single-stranded (ss) RNA loop.

Viruses encode proteins, which can suppress RNA silencing, a mechanism that may have evolved as part of a counter-defensive strategy to overcome silencing-mediated intrinsic defence systems of plants (Marathe et al., 2000; Carrington et al., 2001; Lecellier and Voinnet, 2004). The 2b protein of *Cucumber mosaic virus* (CMV) suppresses the signal-mediated spread of RNA silencing into new tissues (Brigneti et al., 1998; Guo and Ding, 2002). Infection by CMV has been shown to transiently suppress dsRNA-induced transgene silencing and to break the associated viral immunity to *Potato virus Y* (PVY) in transgenic tobacco (Mitter et al., 2003). Since RNA silencing leads to post-transcriptional degradation of the transgene transcripts, accurate quantitative measurements of transcript levels in silenced plants and any changes induced as a result of viral suppression of RNA silencing will assist us to understand the underlying mechanisms.

Since we have shown previously that CMV breaks down resistance to PVY in transgenic tobacco (Mitter et al., 2001, 2003), we were interested in determining if we can use the unique spacer sequence as an indicator of silencing in the presence of homologous viral sequences in plants showing breakdown of resistance. In this context, we were interested in the fate of the ssRNA spacer: would it induce RNA silencing like an aberrant RNA or be degraded like RNA not channelled into translation? To address these questions, we have developed RT-qPCR assays targeting either sequence and used them to measure the accumulation of both the dsRNA trigger and ssRNA spacer transcript sequences in transgenic plants during RNA silencing and its suppression. Furthermore, we used RNA blotting to determine transcript sizes and to detect siRNAs specific for both sequences.

2. Materials and methods

2.1. Transgenic plants

Transgenic tobacco line #16 (Mitter et al., 2001) carried a single copy PVY hairpin loop construct comprising an inverted repeat fragment of 735 nt from the PVY-NIa protease gene separated by a 350 nt spacer sequence, derived from a fragment of the *Lettuce necrotic yellows virus* (LNYV) 4b gene, under the control of a *Cauliflower mosaic virus* 35S promoter (Fig. 1A).

2.2. Virus inoculations and detection

Plants at the four-leaf stage were mechanically inoculated on leaf 0 with CMV isolate 207 (subgroup 1A) as described previously (Mitter et al., 2003). Leaf 1 and in some cases leaf 2 were present on the plant at the time of CMV inoculation, whereas leaves 3 and onwards emerged after CMV inoculation (Fig. 3F). Presence of CMV in inoculated plants was confirmed by visual symptoms and enzyme linked immuno-sorbent assay using a commercial kit (Sanofi Diagnostics Pasteur, Chaska, NM, USA) 6 weeks after inoculation.

2.3. In vitro transcripts

The phagemid pBC SK+ (Stratagene, LaJolla, CA, USA) carrying the PVY hairpin loop construct was transcribed using the MAXIscript T3 in vitro transcription kit (Ambion, Austin, TX, USA). Plasmid DNA was removed by treatment with RNase-free DNase I (Roche Biochemicals, Australia) for 30 min at 37 °C. DNase was inactivated by adding 0.5 mM EDTA and heating at 65 °C for 15 min. Purified transcripts obtained after two phenol–chloroform extractions and ethanol precipitation were quantified using a spectrophotometer and the size was confirmed by agarose gel electrophoresis.

2.4. RNA extraction and reverse transcription-quantitative PCR

Total plant RNA was extracted from 100 mg samples of leaves using the RNeasy Plant Mini kit (Qiagen,