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Inhibition of long-distance movement of RNA silencing signals in *Nicotiana benthamiana by Apple chlorotic leaf spot virus* 50 kDa movement protein

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ABSTRACT

Apple chlorotic leaf spot virus 50 kDa movement protein (P50) acts as a suppressor of systemic silencing in *Nicotiana benthamiana*. Here, we investigate the mode of action of P50 suppressor. An agroinfiltration assay in GFP-expressing *N. benthamiana* line16c (GFP-plant) showed that P50 could not prevent the short-distance spread of silencing. In grafting experiments, the systemic silencing was inhibited in GFP-plants (scion) grafted on P50-expressing *N. benthamiana* (P50-plant; rootstock) when GFP silencing was induced in rootstock. In double-grafted plants, GFP-plant (scion)/P50-plant (interstock)/GFP-plant (rootstock), the systemic silencing in scion was inhibited when GFP silencing was induced in rootstock. Analysis of P50 deletion mutants indicated that the N-terminal region (amino acids 1–284) is important for its suppressor activity. In gel mobility shift assay, P50 lacks binding ability with siRNAs. These results indicated that P50 has a unique suppressor activity that specifically inhibits the long-distance movement of silencing signals.
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Introduction

RNA silencing is widely conserved across eukaryotic organisms including fungi, animals, and plants (Cogoni, 2001; Hannon, 2002; Zamore, 2002), and it acts as an adaptive immune system against invading nucleic acids such as viruses, viroids, transposons and transgenes in plants (Vance and Vaucheret, 2001; Baulcombe, 2004; Ding et al., 2004; Voinnet, 2005a; Wang and Metzlaff, 2005). This mechanism involves initial processing of double-strand RNA (dsRNA) into 21- to 25-nucleotide (nt) small interfering RNAs (siRNA) having 2-nt 3′ overhangs, and 5′ phosphate by an RNaseIII-like enzyme called DICER [it is called DICER-LIKE (DCL) in plants]. These molecules are incorporated into a protein complex called RNA-induced silencing complex (RISC) that operates the sequence-specific degradation of target RNAs (Hamillton and Baulcombe, 1999; Hammond et al., 2000). In several organism including plants, fungi and worms, siRNAs are also used as primers by RNA-dependent RNA polymerase (RdRp) to convert single-strand RNA (ssRNA) into dsRNA (Herr, 2005). In plants, when silencing is induced in cells of a leaf (referred to as local silencing), it can spread from an initially silenced cell to a neighboring cell via the plasmodesmata, and silencing can spread over a long distance to different parts of the plant via the phloem, mimicking patterns of plant viral movement through the plant (Palauqui et al., 1997; Voinnet and Baulcombe, 1997). The spread of silencing throughout a plant (referred to as systemic silencing) is due to the movement of silencing signals containing a nucleic acid component, probably RNA (Mlotshwa et al., 2002; Voinnet, 2005b).

In plants infected with RNA virus, accumulation of dsRNA replication intermediate or certain virus ssRNA with high secondary structure can trigger RNA silencing. Local silencing may inhibit viral propagation in infected cells. On the other hand, systemic silencing may confer sequence-specific resistance to uninfected neighboring cells and distant tissues by the spreading of silencing signals ahead of viral movement (Voinnet et al., 2000; Voinnet, 2005a, 2005b). To counteract RNA silencing, many viruses have evolved RNA silencing suppressors. Over 30 viral suppressors have been identified among plant, animal and insect viruses (Voinnet, 2005a). Because of no obvious sequence similarity among these suppressors, it has been thought that they interfere with the RNA silencing pathway at different points (Roth et al., 2004; Voinnet, 2005a). Most plant viral suppressors, e.g., tombusviruses P19, potyviruses HC-Pro, cucumoviruses 2b, and closteroviruses P21, can interfere with local silencing (Guo and Ding, 2002; Lakatos et al., 2004, 2006; Zhang et al., 2006; Goto et al., 2007; Shiboleth et al., 2007). On the other hand, only three suppressors, a coat protein (CP) of *Citrus tristeza virus* (CTV), P50 of ACLSV, and Vp20 of *Apple latent spherical virus* (ALSV), were reported to inhibit systemic silencing without interfering with local silencing (Lu et al., 2004; Yaegashi et al., 2007a, 2007b). However, how and where these proteins act as suppressors have yet to be understood.

ACLSV is classified into the type species of *Trichovirus* genus, *Flexiviridae* family (Fauquet et al. 2005; Martelli et al., 2007). ACLSV has a flexuous filamentous particle (740 to 760 nm in length and

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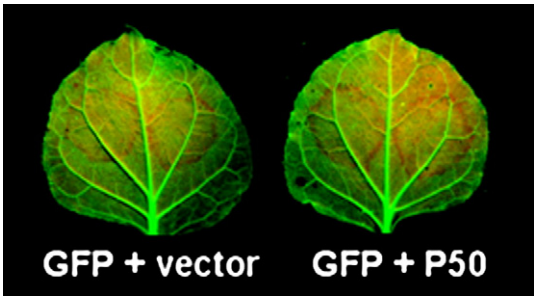

Fig. 1. P50 does not inhibit the short-distance spread of silencing. The leaves of GFP-plant were infiltrated with a mixture of agrobacteria carrying pBI-GFP and the pBE2113-P35T (vector), or pBE2113-P50 (P50), and photographed with a yellow filter at 7 dpif under a long-wave UV lamp.

12 nm in width) and contains a single RNA species and a single coat protein (Yoshikawa and Takahashi 1988). The ACLSV genome contains three open reading frames that encode a replication-associated protein, a movement protein (P50), and a CP, respectively (German et al., 1990; Sato et al., 1993). The P50 localizes to plasmodesmata of infected cells and in cells expressing P50 from a transgene and also accumulates in the parietal layer of sieve elements and on sieve plates (Yoshikawa et al., 1999, 2006). The protein can spread from cells that initially produce it into neighboring cells and enables cell-to-cell trafficking of GFP when P50 and GFP are co-expressed in leaf epidermis (Satoh et al., 2000). The P50 also has two independently active, single-stranded nucleic acid-binding domains (Isogai and Yoshikawa, 2005). In addition to these functions, our recent study revealed that P50 functions as a silencing suppressor and inhibits systemic silencing without interfering with local silencing in *N. benthamiana* (Yaegashi et al., 2007a). Based on these findings, we have hypothesized that P50 would interfere with the cell-to-cell movement through plasmodesmata and/or the systemic movement through phloem of the silencing signals.

In this study, we investigated whether P50 interferes with the cell-to-cell and/or long-distance movement of silencing signals by an agroinfiltration assay and grafting experiments using GFP-expressing *N. benthamiana* line 16c (GFP-plant) and P50-expressing transgenic *N. benthamiana* (P50-plant). The results indicated that P50 is a unique suppressor that specifically inhibits the long-distance movement of silencing signals through phloem.

Results

P50 suppresses long-distance but not short-distance spread of silencing

It has been reported that RNA silencing can spread from initially silenced cells to neighboring cells (Voinnet and Baulcombe, 1997). In GFP-plants, this cell-to-cell spreading provokes shutting down of GFP expression in neighboring cells, which is manifested by a narrow red border around the initially silenced spot (Himber et al., 2003). To examine whether the expression of P50 could suppress the cell-to-cell movement of silencing signals, an agroinfiltration assay using GFP-plants was carried out. The developed leaves of GFP-plants were infiltrated with a mixture of agrobacteria carrying pBI-GFP (GFP) plus

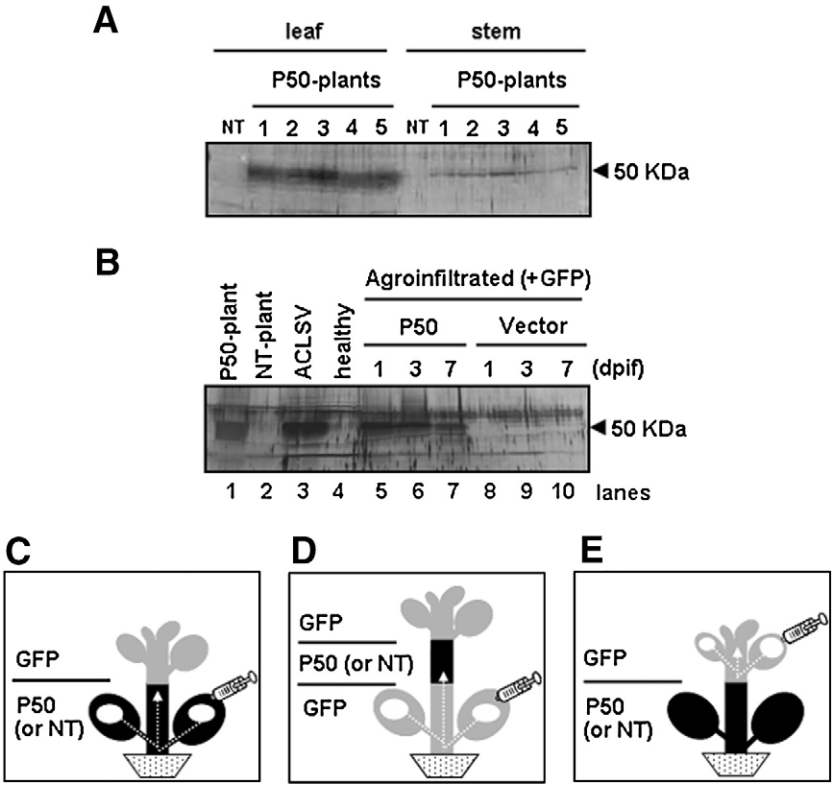

Fig. 2. Schematic description of three types of grafting experiments using P50-expressing transgenic *Nicotiana benthamiana*. (A) Immunoblot analysis of the accumulation of P50 protein in leaf and stem tissue of P50 expressing transgenic *Nicotiana benthamiana* (P50-plants) plants using P50 antiserum. The number in each lane indicates the total protein samples extracted from leaf and stem tissues of five P50-plants (lanes 1–5). Total protein samples from leaf and stem tissue of nontransgenic *N. benthamiana* (NT-plants) were used as negative controls (lane NT). (B) Immunoblot analysis of P50 accumulation level in P50-plants, ACLSV-infected plants, and transient expression using P50 antiserum. Total protein samples were extracted from leaf tissue of P50- or NT-plants (lane 1, 2), ACLSV inoculated or healthy *Chenopodium quinoa* leaves at 4 days post inoculation (lane 3, 4), and agroinfiltrated leaves at 1, 3, and 7 dpif (lanes 5–7; GFP+P50, lanes 8–10; GFP+vector). (C) Single-grafted plants with agroinfiltrated leaves in rootstock. (D) Double-grafted plants with agroinfiltrated leaves in rootstock. (E) Single-grafted plants with agroinfiltrated leaves in scion. The gray parts of the illustration indicate GFP-plants (GFP), and black parts indicate P50-plants (P50) or NT-plants (NT). The white area shows silenced tissue by infiltration with a mixture of agrobacteria carrying pBI-GFP plus pBI-dsGFP, the white arrows indicate the direction of spread of silencing signals.

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