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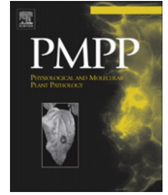

Interference with initial and short-distance cell-to-cell movement of *Tomato mosaic virus* in transgenic tobacco plants with high expression of BcKELP, a virus movement protein interactor from *Brassica campestris*

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ABSTRACT

A putative transcriptional coactivator KELP of *Arabidopsis thaliana* and its homolog of *Brassica campestris* (BcKELP) can bind the movement protein (MP) of *Tomato mosaic virus* (ToMV) *in vitro* and, when transiently over-expressed, interfere with the cell-to-cell movement of ToMV. In this study, we generated and selected transgenic *Nicotiana tabacum* lines expressing BcKELP constitutively and examined their resistance to ToMV. We also investigated changes in MP localization in initially infected *Nicotiana benthamiana* cells expressing BcKELP transiently. Our results indicate that a high expression of BcKELP can reduce viral initial and short-distance movement probably through disturbance of plasmodesmal targeting of MP.

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1. Introduction

Tomato mosaic virus (ToMV), formerly referred to as *Tobacco mosaic virus* (TMV) L strain, belongs to the genus *Tobamovirus* and contains a single-stranded, messenger sense genomic RNA, which encodes four proteins; 126-kDa, 180-kDa, and 30-kDa nonstructural proteins, and a 19-kDa structural coat protein (CP) [1]. The 126-kDa and 180-kDa proteins are both involved in virus replication. The 30-kDa protein, which is regarded as a movement protein (MP), is indispensable for virus cell-to-cell movement from an initially infected cell to adjacent cells (initial movement) and subsequently to surrounding cells (short-distance movement) through plasmodesmata, cytoplasmic channels connecting adjacent cells. The CP involved in encapsidation is required for virus long-distance spread throughout a whole plant but not for initial or short-distance cell-to-cell movement within a leaf [2]. This virus is thought to move in the form of ribonucleoprotein complexes rather than virus particles. The 30-kDa proteins of ToMV and TMV are the best-studied MPs and shown to have several biochemical and biological properties relating to movement functions. These MPs are reported to bind single-stranded nucleic acid *in vitro* [3], accumulate at plasmodesmata [4–6], facilitate macromolecular trafficking by increasing the size exclusion limit of plasmodesmata [7,8] and be closely associated with the endoplasmic reticulum and the cytoskeleton such as actin filaments and microtubules [9–13]. A recent study has suggested that TMV MP as well as *Cucumber mosaic virus* (CMV) MP has the ability to sever actin filaments, which are the cytoskeletal and plasmodesmal components involved in virus intracellular and intercellular movement [14]. Although the 30-kDa MP is undoubtedly a key factor for tobamovirus cell-to-cell movement, a couple of studies have reported that the 126-kDa/180-kDa replicase proteins, which form virus replication complexes (VRCs) transiently with MP [15,16], also play an important role in virus cell-to-cell movement [17–19]. So far, however, little is known about how the replicase proteins are involved in cell-to-cell movement processes.

In the processes of virus cell-to-cell movement, MP is thought to interact with many host proteins [20]. To date, the MPs of ToMV and TMV are shown to bind to diverse host proteins, which may be implicated in facilitating or disturbing virus movement [21]. These proteins include cell-wall-associated pectin methylesterase and calreticulin [22–25], cytoskeletal elements such as actin and tubulin [9,10,14,26], microtubule-associated MPB2C and EB1a [27,28], protein kinases such as RIO kinase and CK2 [29,30], transcription-related factors MBF1 and NTH201 [31,32]. Although the number of the reports on MP-interacting host proteins is increasing, *in vivo* functions of these proteins in virus cell-to-cell movement are not fully understood.

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Previously, to identify plant proteins interacting *in vitro* with ToMV, we performed far-western screening by using a cDNA library of *Brassica campestris*, a nonhost for ToMV. One of the MP-interacting proteins is highly homologous to a putative transcriptional cofactor called KELP of *Arabidopsis thaliana* (AtKELP) [33] and accordingly referred to as BcKELP [34]. AtKELP could also bind to the MPs of *Wasabi mottle virus* (WMV; a wasabi strain of crucifer tobamovirus) and CMV [34]. In our recent study, transient over-expression of AtKELP in *Nicotiana benthamiana* cells resulted in remarkable inhibition of the initial cell-to-cell movement of a ToMV mutant encoding a GFP reporter [35]. Similar results were also obtained from transient expression assays using BcKELP and other KELP homologs of *Nicotiana tabacum*, *Solanum lycopersicum*, and *Oryza sativa* [36]. In intracellular localization analysis by using fluorescently labeled AtKELP and ToMV MP, transiently expressed AtKELP was found predominantly in the nucleus and the cytoplasm in the absence of MP [35]. On the other hand, when MP was expressed transiently in a replication-independent manner, AtKELP was co-localized together with MP at the nucleus, cytoplasmic aggregations and filamentous structures. In accordance with this, the characteristic localization of MP to plasmodesmata was significantly decreased [35]. These results have suggested that KELP proteins can function as an inhibitory factor to prevent the cell-to-cell movement of ToMV through an interaction with MP at least when over-expressed transiently.

In this study, we generated and selected transgenic tobacco lines expressing BcKELP constitutively at high levels and investigated whether they can block the cell-to-cell movement of ToMV effectively. We also observed a time course of changes in intracellular localization of MP at initially infected cells transiently expressing BcKELP.

2. Materials and methods

2.1. Plant materials and growth conditions

N. benthamiana, *N. tabacum* cv SR1 and transgenic tobacco plants for the expression of BcKELP-HA were grown as described previously [35]. Leaves of 5- to 7-week old *N. benthamiana* and 6- to 9-week old *N. tabacum* were used for experiments. For segregation analysis, seeds were placed on selection medium containing 0.33% Gamborg's B5 Medium Salt Mixture (Wako, Osaka, Japan), 250 μg/mL kanamycin and 1.5% agar and grown in a growth chamber at 25 °C with a 16-h light/8-h dark photoperiod.

2.2. Plasmid construction

pART27-Bc2-HA used for BcKELP expression was constructed as follows. A cDNA for HA-tagged BcKELP was amplified by PCR with pGEX-Bc2 [34] as a template and primers pGEX (5′-CACGTTTGGTGGTGGCGACC-3′) and Bc2R04HA (5′-GCTCTAGA-CTAAGCATAATCAGGAACATCATAAGGATAGACACGCGATTCCATTTT-3′; HA complementary sequence underlined) and ligated into a TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA) to introduce the *BcKELP* ORF in the same orientation as the *nptII* gene. Following digestion of the intermediate pCR2.1-based plasmid with *Eco*RV and *Bam*HI, 0.6-kb fragment containing the *BcKELP* cDNA was inserted between *Eco*RV and *Bam*HI sites in pART7, and subsequently to a *Not*I site in pART27 by using the pART7/pART27 binary vector system [37].

piL.G3-MPGFP was constructed as follows. First PCR was carried out by using piL.G3 [38] as a template and a set of primers attB1/MP/F01 (5′-AAAAAGCAGGCTACCATGGCTCTAGTTGTTAAA-3′) and attB/MPG3GFP/R01 (5′-TTCTTCTCCTTTACTATACGAATCAGAATC-3′) or primers att/MPG3GFP/F01 (5′-GATTCTGATTCGTATAGTAAAGGA-GAAGAA-3′) and attB/G3GFP/R01 (5′-AGAAAGCTGGGTTTTATTTG-TATAGTTC-3′). Those two PCR products were purified and mixed as templates together with attB adapter primers [35] in second PCR. The resultant PCR product was purified and introduced to an entry vector pDONR221 (Invitrogen) through a BP reaction. Following digestion of the pDONR-based entry plasmid with *Aat*II and *Hpa*I, the 0.5-kb fragment was replaced by the 10.5-kb fragment of piL.G3 located between *Aat*II and *Hpa*I.

The coding sequences derived from the PCR fragments in pART27-Bc2-HA and piL.G3-MPGFP were determined to confirm no mutations were present.

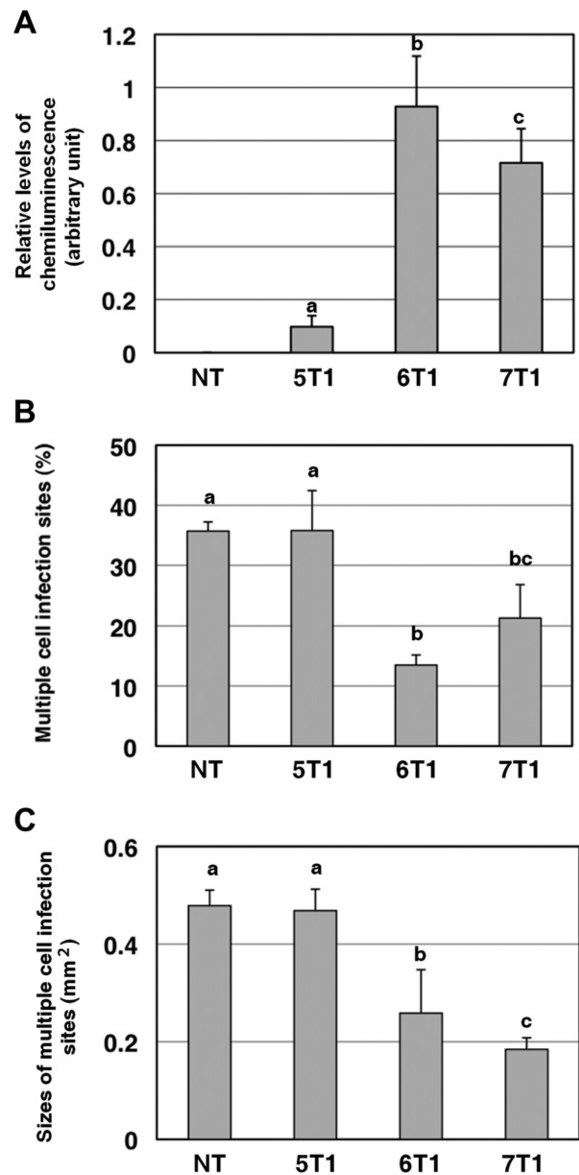

Fig. 1. Effects of stable expression of BcKELP-HA on virus cell-to-cell movement. (A) Relative chemiluminescence levels of BcKELP-HA were measured for non-transformed (NT) tobacco and T1 progeny lines of transgenic tobacco (5T1, 6T1, and 7T1). BcKELP-HA was detected with an anti-HA antibody. 5 ng of GST-fused recombinant BcKELP-HA protein from *Escherichia coli* was used as a signal standard which level was set as 1.0 (data not shown). (B, C) Virions of TocJ/GFP were inoculated to non-transformed and transgenic tobacco plants. Infection sites with GFP fluorescence were observed under an epifluorescence microscope at 48 h post-inoculation. The percentage of multiple-cell infection sites in total infection sites is shown in B. Sizes of multiple-cell infection sites in non-transformed and transgenic plants are shown in C. All transgenic plants used in these experiments contained the transgene of BcKELP-HA. Bar graphs illustrate average values with standard errors from experiments using eight different plants. Different letters (a, b, and c) above the bars indicate significant difference at $p < 0.05$ by Tukey's test.

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