NTH201, a Novel Class II KNOTTED1-Like Protein, Facilitates the Cell-to-Cell Movement of *Tobacco mosaic virus* in Tobacco

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NTH201, a novel class II KNOTTED1-like protein gene, was cloned from tobacco (*Nicotiana tabacum* cv. Xanthi) and its role in *Tobacco mosaic virus* (TMV) infection was analyzed. Virus-induced gene silencing of NTH201 caused a delay in viral RNA accumulation as well as virus spread in infected tobacco plants. Overexpression of the gene in a transgenic tobacco plant (*N. tabacum* cv. Xanthi nc) infected by TMV showed larger local lesions than those of the nontransgenic plant. NTH201 exhibited no intercellular trafficking ability but did exhibit colocalization with movement protein (MP) at the plasmodesmata. When NTH201-overexpressing tobacco BY-2 cultured cells were infected with TMV, the accumulation of MP but not of viral genomic and subgenomic RNA clearly was accelerated compared with those in nontransgenic cells at an early infection period. The formation of virus replication complexes (VRC) also was accelerated in these transgenic cells. Conversely, NTH201-silenced cells showed less MP accumulations and fewer VRC formations than did nontransgenic cells. These results suggested that NTH201 might indirectly facilitate MP accumulation and VRC formation in TMV-infected cells, leading to rapid viral cell-to-cell movement in plants at an early infection stage.

Additional keywords: KNOX, transcription factor.

To establish a systemic infection, plant viruses move first from cell to cell via plasmodesmata (PD), channels in the cell wall that provide symplastic continuity between adjacent plant cells. Cell-to-cell movement requires virus-encoded movement proteins (MPs) and several kinds of host plant proteins. It has

been reported previously that viral MPs are essential in cell-to-cell movement, which themselves are able to move between cells, localize to PD, and increase the size-exclusion limit (SEL) (Boevink and Oparka 2005; Lucas 2006).

One of the most studied MPs is the 30-kDa protein of *Tobacco mosaic virus* (TMV). The 6.3-kb RNA genome of TMV encodes an RNA-dependent RNA polymerase (RdRp) (126 and 183 kDa), a 30-kDa MP, and a 17.5-kDa coat protein (CP). TMV MP can bind viral single-stranded RNA to form a viral ribonucleoprotein (vRNP) complex to deliver its own genome to neighboring cells (Citovsky et al. 1990, 1992). Although TMV CP is not necessary for cell-to-cell movement (Saito et al. 1989, 1990), RdRp has an important role in vRNP movement (Hirashima and Watanabe 2001, 2003; Tamai and Meshi 2001). Recent studies have indicated that virus replication complexes (VRC), which include viral RNA, MP, RdRp, and some host proteins, are formed on the endoplasmic reticulum (ER) and might move to the adjacent cells (Asurmendi et al. 2004; Kawakami et al. 2004). It is important to identify which cellular factors interact with MP and to determine their functions in cell-to-cell movement. TMV MP has been reported to interact with cell-wall-associated pectin methylesterase (Chen and Citovsky 2003; Chen et al. 2000; Dorokhov et al. 1999) and calreticulin (Chen et al. 2005) as well as cytoskeletal elements such as F-actin (McLean et al. 1995), microtubules (Ashby et al. 2006; Heinlein et al. 1995; McLean et al. 1995), and microtubule-associated protein MPB2C (Kragler et al. 2003; Winter et al. 2007). Also, non-cell autonomous pathway protein 1 (NCAPP1) and PD-associated protein kinase (PAPK) that interacts with non-cell autonomous proteins (NCAP) were shown to affect TMV cell-to-cell movement (Lee et al. 2003, 2005). However, in vivo functions of these host factors in virus movement are not well understood.

As with viral MPs, some plant-encoded transcription factors are known to be able to traffic between cells and serve as signals to determine cell fate through cell-to-cell communication (Gallagher and Benfey 2005). Maize KN1 was the first characterized endogenous plant MP among KNOTTED1-like homeobox (KNOX) proteins (Lucas et al. 1995). KNOX proteins are divided into two classes depending on the homeobox (HOX) sequence (Bharathan et al. 1999; Deshpande et al. 1999; Hake et al. 2004; Kerstetter et al. 1994; Reiser et al. 2000). KN1, a class I KNOX protein, can increase the SEL and traffic itself and its own mRNA between cells, and normally is expressed in the shoot apical meristem (SAM), where it function in meristem development and maintenance (Kim et al. 2002, 2003; Lucas et al. 1995). Also, the other class I KNOX proteins, *Arabidopsis*

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KNAT1 and STM, and tomato LeT6 proteins that are closely related to KN1, can pass between cells (Kim et al. 2003, 2005). However, *Arabidopsis* KNAT2 and KNAT6, which are class I KNOX proteins but less closely related to KN1, have no trafficking ability (Kim et al. 2005).

On the other hand, class II KNOX proteins are expressed universally in most tissues but have no trafficking ability (Janssen et al. 1998; Kerstetter et al. 1994; Kim et al. 2005). However, the authentic functions of class II KNOX proteins in the plant cell are not clearly understood thus far.

Here, we show that NTH201, a novel class II KNOX protein of tobacco plant, positively controls the cell-to-cell move-

ment of TMV by regulating the accumulation of MP and the formation of VRC, although it has no trafficking ability of its own.

RESULTS

Isolation of *NTH201*, a novel class II KNOX gene of tobacco.

To isolate a KNOTTED1-like HOX gene, a fragment of KNOX cDNA was amplified from *Nicotiana tabacum* total RNA by reverse transcription with a primer set based on the conserved sequences of three class II KNOX genes followed by polymerase chain reaction (PCR). We cloned a full-length

Fig. 1. Distinction of NTH201 as a novel class II KNOTTED1-like homeobox (KNOX) protein of tobacco. **A**, Deduced structure of NTH201 protein. The conserved domains of KNOX proteins (i.e., KNOX1, KNOX2, ELK, and homeobox [HOX]) are indicated with the amino acid numbers. Arrowheads are the corresponding positions of a set of polymerase chain reaction (PCR) primers. **B**, Amino acid sequences of HOX domains in several KNOX proteins. The HOX domains of tobacco NTH201 (this study; AB333840), NTH20 (AB025714), NTH22 (AB025715), and NTH23 (AB004797); *Arabidopsis* KNAT1 (U14174), KNAT2 (U14175), KNAT3 (X92392), and KNAT4 (X92393); tomato LeT12 (AF000142); and maize KN1 (Knotted1; X61308) are aligned using the CLUSTALW (version 1.83) and edited using BOXSHADE 3.21. Amino acids conserved among four or more of the proteins are highlighted in boldface, and similar residues are in gray. I = class I KNOX proteins and II = class II KNOX proteins. **C**, Phylogenetic tree of the same KNOX proteins as those in B based on their total amino acid sequences. *Arabidopsis* BEL1 (U39944), a member of another homeodomain protein group, also was added for comparison. Numbers on branches indicate bootstrap values (1,000 repetitions) compatible with the 50% majority rule. Only values >50% are shown. CLUSTALW and NJPlot programs were used to generate the neighbor-joining tree.

class II KNOX cDNA with a size of 1,551 bp by 5'- and 3'-rapid amplification of cDNA ends (RACE) methods with gene-specific primers. Sequence analysis of the full-length cDNA revealed 1,176 bp of open reading frame (ORF) encoding 391 amino acids with a predicted molecular weight of 43.6 kDa, designated as NTH201 (*N. tabacum* HOX protein 201; DDBJ accession number AB333840) following the previous naming convention of tobacco KNOX proteins (Sentoku et al. 1998). NTH201 belongs typically to the KNOX family proteins, with an N-terminal MEINOX region including the KNOX1 and KNOX2 domain, an ELK domain, and a highly conserved C-terminal HOX domain (Fig. 1A) (Bharathan et al. 1999; Kerstetter et al. 1994). Comparison of the HOX domain amino acid sequence of NTH201 with those of class I KNOX proteins (tobacco NTH20 and NTH22, *Arabidopsis* KNAT1 and KNAT2, and maize KN1) and class II KNOX proteins (tobacco NTH23, tomato LeT12, and *Arabidopsis* KNAT3 and KNAT4) revealed that NTH201 is highly homologous to class II KNOX proteins (Fig. 1B). This also was supported by the phylogenetic analysis of the same KNOX proteins based on their total amino acid sequences (Fig. 1C). The total amino acid sequence homology between NTH201 and NTH23 was 65%, which indicates that NTH201 is a novel class II KNOX protein of tobacco.

Silencing of *NTH201* impaired TMV infection.

First, to examine whether NTH201 is involved in TMV infection, the endogenous *NTH201* gene was silenced using the virus-induced gene silencing (VIGS) method (Burton et al. 2000; Ruiz et al. 1998). A partial sequence in the 3'-untranslated region (UTR) of the *NTH201* gene was cloned into a *Potato virus X* (PVX) cDNA vector to construct pPVX-NTH201, while the empty vector (pPVX) was used as a negative control. Approximately 3 weeks after bombarding of pPVX-NTH201 or pPVX vectors into young *N. benthamiana* leaves, the upper leaves showing very mild symptoms by the vectors were secondarily inoculated with TMV-green fluorescent protein (GFP) that is capable of expressing GFP together with RdRp, MP, and heterologous CP (Shivprasad et al. 1999). Approximately 1 week after the second inoculation, the inoculated leaves were visualized under UV irradiation to detect GFP-expressing infection foci formed by the virus spread.

Results of silencing experiments are shown in Figure 2. Silencing led to approximately 50% reduction of *NTH201* mRNA in NTH201-silenced plants compared with the control plants infected with pPVX vector alone, as indicated by real-time reverse-transcription (RT)-PCR analysis (Fig. 2A). These NTH201-silenced plants showed no major morphological differences from the control pPVX-infected plant. In contrast to

Fig. 2. Effects of NTH201 silencing in *Tobacco mosaic virus* (TMV)-infected leaves. **A**, Efficiency of NTH201 silencing quantified by real-time reverse-transcription polymerase chain reaction. The content of *NTH201* mRNAs in the upper leaves of *Nicotiana benthamiana* plants infected with p-*Potato virus X* (pPVX)-NTH201 was compared with that of the control infected with pPVX vector alone at 3 weeks postinoculation. Data represent average values of three plants with indicated standard deviation values. **B**, Fluorescent images of the leaves secondarily inoculated with TMV-green fluorescent protein (GFP). The upper leaves of pPVX-infected (left) or pPVX-NTH201-infected (right) *N. benthamiana* plants were secondarily inoculated with TMV-GFP after 3 weeks of the first inoculation and the GFP fluorescent loci were observed after 1 week of the second inoculation. **C**, Averaged areas of GFP foci with standard deviation bars. The areas of the GFP foci shown in B were quantified using the National Institute of Health image software. **D**, Accumulation of viral RNA in TMV-GFP-inoculated leaves of NTH201-silencing and nonsilencing plants after 1 week of the second inoculation, analyzed by Northern hybridization using a digoxigenin-labeled RNA probe to detect GFP region. vRNA = TMV-GFP genomic RNA and MP-sgRNA = movement protein-subgenomic RNA.

Fig. 3. Spread of the local lesions on the NTH201-overexpressing Xanthi nc tobacco leaves inoculated with *Tobacco mosaic virus* (TMV) and the colocalization of NTH201 and movement protein (MP) in *Nicotiana benthamiana* epidermal cells. **A**, Overexpression of NTH201 in three transgenic lines of Xanthi nc tobacco shown by Northern hybridization. WT = wild type; lines 7, 14, and 32 = transgenic T₁ lines of Xanthi nc tobacco. **B**, Spread of the local lesions on the leaves of WT and transgenic Xanthi nc tobacco plants (line 32) after 9 days of TMV inoculation. **C**, Diameters of local lesions produced on the leaves of WT and three lines of transgenic Xanthi nc tobacco plants after 3 to 9 days of TMV inoculation. **D**, Colocalization of NTH201 and MP in *N. benthamiana* epidermal cells by a particle bombardment and observed under confocal laser scanning microscopy at 24 h after bombardment. a and b, Green fluorescence by GFP:NTH201; c, red fluorescence by MP:DsRed; and d, merged b and c. N = nucleus and arrowhead = colocalized signal in the plasmodesmata.

the nonsilenced plants, cell-to-cell spread of TMV-GFP obviously was delayed in NTH201-silenced plants (Fig. 2B). The areas of these GFP foci were quantified using National Institute of Health (NIH) image software and averaged. As a result, in NTH201-silenced plants, GFP foci were approximately half the size of those of the nonsilenced plants (Fig. 2C). The accumulation of viral RNA in TMV-inoculated leaves of NTH201-silenced plants revealed a marked reduction compared with that of the nonsilenced plants (Fig. 2D). Moreover, the decrease in the size of GFP foci in NTH201-silenced plants also was observed in another VIGS system based on *Tobacco rattle virus* (TRV) vector (data not shown). Therefore, it is conceivable that the NTH201 protein is involved in some stages of the TMV infection cycle.

NTH201 facilitated TMV cell-to-cell movement.

To further clarify the functions of NTH201 in TMV infection, we generated transgenic tobacco Xanthi nc plants overexpressing the full-length *NTH201*. Xanthi nc tobacco has the *N* gene for resistance to TMV. These plants produce local necrotic lesions in response to TMV infection. Three independent lines of transgenic T₁ plants overexpressing *NTH201* were confirmed by Northern hybridization (Fig. 3A). Ectopic expression of class I KNOX genes has been reported previously to cause dramatic morphological changes while that of class II KNOX genes results in no morphological change in tobacco (Nishimura et al. 2000; Sakamoto et al. 1999). The latter was confirmed with

NTH201-overexpressing plants in this experiment. The spread of the local lesions was measured on the inoculated leaves at 3 to 9 days post-TMV inoculation. At 2 to 3 days postinoculation, the local lesions became visible for both wild-type (WT) and NTH201-overexpressing plants; however, their sizes and rates of spread on the NTH201-transgenic plants were significantly larger and more rapid than those on WT plants. At 9 days postinoculation, expansion of the lesions in all lines of NTH201-transgenic plants was prominently larger than those of WT plants (Fig. 3B and C). Similar results were obtained in the experiments where *Wasabi mottle virus* (WMoV), another member of the genus *Tobamovirus*, was inoculated on these transgenic plants. The diameter of local lesions on the leaves of transgenic plants was approximately 1 mm larger than that of WT plants after 10 days of WMoV inoculation (Supplementary Fig. 1). However, there was no significant difference between WT and NTH201-transgenic plants when PVX, a type virus of the genus *Potexvirus*, was inoculated (Supplementary Fig. 2). These results suggested that NTH201 might specifically facilitate the virus spread of the genus *Tobamovirus*.

NTH201 had no ability to self-traffic or to transport GFP between cells.

There is a possibility that NTH201 may be able to directly enhance the trafficking of TMV MP to the adjacent cells. To investigate this possibility, we checked whether NTH201 could traffic itself or transport GFP between cells in *N. benthamiana* leaves. The epidermis of detached leaves was bombarded with an expression vector containing the complete coding region of *NTH201* fused to *GFP* (pUC18-GFP:NTH201 or pUC18-NTH201:GFP) and combinations of the expression vectors for GFP (pUC18-GFP), MP:DsRed (pUC18-MP:DsRed; *MP* fused to *DsRed*), or NTH201 (pUC18-NTH201).

GFP:NTH201 or NTH201:GFP, derived from pUC18-GFP:NTH201 or pUC18-NTH201:GFP, respectively, was found to reside only in the nucleus of the bombarded cells (Fig. 3D-a) at 24 h after bombardment, whereas free GFP signal derived from pUC18-GFP showed diffused localization in the nucleus and the cytoplasm, and MP:DsRed derived from pUC18-

Table 1. Assay of ability of NTH201 to transport green fluorescent protein (GFP) via plasmodesmata

Coexpressed protein ^a	Cell-to-cell movement of GFP (%) ^b	Observed cells (experiments)
GFP + MP:DsRed	44.0	93 (2)
GFP + MP:DsRed + NTH201	32.0	72 (4)
GFP + NTH201	0	64 (2)

^a MP:DsRed = fusion protein of movement protein (MP) and DsRed.

^b The percentage of events where the fluorescent cells were detected around the first bombarded cells at 24 h after bombardment.

Fig. 4. Assay of NTH201 expression in several transgenic BY-2 cell lines. **A**, Western blot analysis of NTH201 in several NTH201-transgenic BY-2 cell lines. WT = nontransformed wild-type cell line. **B**, Real-time reverse-transcription polymerase chain reaction assay of *NTH201* mRNA in several Δ NTH201-transgenic BY-2 cell lines. CK = control DsRed transgenic cell line.

MP:DsRed was observed to spread intercellularly (data not shown). Nuclear localization of the GFP:NTH201 was confirmed by overlapping signal with 4',6-diamidino-2-phenylindole stain (data not shown).

GFP was found only in single epidermal cells when coexpressed with NTH201 whereas GFP moved to the adjacent cells when coexpressed with MP:DsRed at the rate of 44.0% at 24 h after bombardment (Table 1). Under these experimental conditions, NTH201 exhibited no accelerating effect on GFP transport by MP. These data indicated that NTH201 lacks the abilities to self-traffic between cells as well as to transport GFP to the neighboring cells.

NTH201 colocalized with MP when coexpressed together.

Next, we observed the subcellular localization of GFP:NTH201 when coexpressed with DsRed-tagged MP (MP:DsRed) in the bombarded epidermal cells of *N. benthamiana*. In contrast to GFP:NTH201 sole expression where it existed only in the nucleus (Fig. 3D-a), GFP:NTH201 coexpressed with MP:DsRed was detected not only in the nucleus but also in the cytoplasm and PD at 24 h after bombardment (Fig. 3D-b), although MP:DsRed was observed in the cytoplasm and especially in the PD but not in the nucleus (Fig. 3D-c). GFP signals of NTH201 were well merged with DsRed signals of MP except in the nucleus (Fig. 3D-d). These results suggested that MP shifted NTH201 localization from the nucleus to the cytoplasm and PD following colocalization of both proteins.

NTH201 increased the accumulation of MP in TMV-infected protoplasts.

To examine the effects of NTH201 on the viral RNA replication and protein synthesis in protoplasts, we generated the transgenic tobacco BY-2 cultured cell lines overexpressing NTH201, with silenced NTH201 or overexpressing DsRed as a negative control. Two cell lines overexpressing NTH201 (NTH201 lines 3 and 5) were selected by Western blot assays (Fig. 4A). Also, two cell lines with silenced NTH201 (Δ NTH201 line 2 and 10; approximately 50 to 70% reduction relative to the control) were selected by real-time RT-PCR (Fig. 4B). To gain insight into the effects of NTH201 on viral RNA replication, the protoplasts were isolated from these transgenic BY-2 cells and infected with TMV RNA by electroporation. The amounts of viral genomic RNA and subgenomic RNA (sgRNA) in the infected protoplasts were quantified by real-time RT-PCR analysis at regular intervals after virus inoculation. In the protoplasts overexpressing NTH201 (NTH201 line) or silenced NTH201 (Δ NTH201 line), the accumulation patterns of TMV genomic RNA (Fig. 5A) as well as TMV genomic RNA plus MP-sgRNA (Fig. 5B) were almost similar to those in the protoplasts overexpressing DsRed as a control. Next, the kinetics of accumulation of MPs and CPs in these infected protoplasts were examined. NTH201-overexpressing protoplasts indicated similar CP accumulation to the control protoplasts, whereas Δ NTH201-protoplasts showed less CP accumulation (Fig. 5C).

The accumulation kinetics of MP in the control protoplasts were similar to those reported previously in the TMV-infected protoplasts (Reichel and Beachy 2000). However, in the NTH201-overexpressing protoplasts, the increase in MP accumulation was noticeably higher compared with that of the control, whereas it was markedly lower in Δ NTH201 protoplasts. Actinomycin D (ActD) is known to increase TMV MP accumulation (Blum et al. 1989). When ActD was added to the protoplasts after inoculation of TMV RNA, MP accumulation in NTH201-overexpressing protoplasts was significantly higher compared with that of the control. These results were repro-

ducible in all three independent experiments using different transgenic cell lines. The above experimental results suggested that NTH201 might facilitate specifically MP accumulation but not viral RNA replication in TMV-infected protoplasts.

Fig. 5. Accumulation of Tobacco mosaic virus (TMV) RNA, movement protein (MP), and coat protein (CP) in the infected protoplasts of NTH201- and Δ NTH201-transgenic BY-2 cell lines. **A** and **B**, Time course of the relative accumulation rate of **A**, TMV genomic RNA or **B**, TMV genomic RNA plus MP-subgenomic RNA in the infected protoplasts quantified by real-time reverse-transcription polymerase chain reaction. Relative TMV RNA levels represent the relative fluorescence ratios of TMV RNA/ubiquitin mRNA. **C**, Time course of the accumulation of TMV MP and CP in the infected protoplasts with (+) or without (-) actinomycin D (ActD) exhibited by Western blot analysis using anti-MP antibody or anti-CP antibody; hpi = hours postinoculation.

NTH201 enhanced the formation of VRC in TMV-infected protoplasts.

In TMV-infected plant cells, viral RdRp, MP, and host proteins formed VRC to replicate the viral genome and to move between cells (Asurmendi et al. 2004; Kawakami et al. 2004). To determine whether NTH201 affects the formation of VRC in transgenic BY-2 protoplasts, the infectious TMV-MP:GFP RNA expressing GFP fused to MP (MP:GFP) was electroporated into the protoplasts overexpressing NTH201 (NTH201) or silenced NTH201 (Δ NTH201). The specific localization of MP:GFP—in small punctate structures at early stage of the infection, small irregular bodies, and large irregular bodies at mid to late stages—was found in WT protoplasts (Fig. 6A through D) at 12 to 24 h after inoculation as reported previously (Heinlein et al. 1998; Mas and Beachy 1999). Small and large irregular bodies were considered as VRC (Asurmendi et al. 2004; Kawakami et al. 2004). In NTH201-overexpressing protoplasts, the localization patterns of MP:GFP (Fig. 6E through H) were the same as those in WT protoplasts, but VRC appeared earlier and in greater numbers and sizes than those in WT protoplasts. In Δ NTH201-protoplasts, punctate structures and irregular bodies were few and MP:GFP was diffused in cytoplasm at 16 to 22 h after inoculation (Fig. 6I and J). Although it was unclear whether VRC contained NTH201 or not, these data demonstrated that NTH201 is an indispensable factor for the accumulation of TMV MP and for the formation of VRC.

DISCUSSION

In the present study, the overexpression of NTH201, a novel class II KNOX tobacco protein, resulted in upregulation of MP accumulation, VRC formation, and virus cell-to-cell movement in TMV-infected cells and plants. In contrast, suppression of NTH201 resulted in downregulation of all of these phenomena.

Other class II KNOX proteins have several homologues and it is possible that NTH201 could have homologues in addition to NTH23 (Sentoku et al. 1998; Serikawa et al. 1996). Thus, it is conceivable that the silencing effects of NTH201 may have affected other unknown homologues. However, this is highly unlikely because we used the vectors containing 3'-UTR specific for NTH201 (Burch-Smith et al., 2004). The overexpressing effects of NTH201 were undoubtedly specific for itself.

It also was shown that NTH201 had no self-trafficking or GFP-transporting ability and colocalized with MP as seen in the transient expression experiments. Class II KNOX proteins such as tobacco NTH23 and *Arabidopsis* KNAT3 also have no trafficking ability (Kim et al. 2005; Sentoku et al. 1998). NTH23 was reported to express at the basal part of leaf blade primordial (Sentoku et al. 1998). KNAT3 was expressed in several tissues and at various times during plant development, indicating that it plays several different roles in plant (Serikawa et al. 1997).

Although the authentic physiological function and expression pattern of NTH201 is unknown at present, what is the role of NTH201 in the TMV-infected cells? NTH201 was observed to migrate from the nucleus to the cytoplasm and PD followed by colocalization with MP when expressed together with MP. It has been reported that P22 MP of *Tomato bushy stunt virus* (TBSV) interacted with homeodomain leucine-zipper protein (Desvoyes et al. 2002); however, no direct binding between NTH201 and TMV MP was detected by the yeast two-hybrid assay (data not shown). Therefore, it is possible that NTH201 might bind indirectly to MP via another host protein that could affect, solely or together with NTH201, some activities of MP.

NTH201 had no ability to self-traffic or transport GFP into neighboring cells but apparently could facilitate TMV cell-to-cell movement in the NTH201-overexpressing plants. Such a facilitating effect was not detected in the cells where NTH201 was transiently expressed. Consequently, the preceding expres-

Fig. 6. Effects of the overexpression or silencing of NTH201 on the virus replication complex (VRC) formation during *Tobacco mosaic virus* (TMV) infection in tobacco BY-2 protoplasts. NTH201-overexpressing or -silenced protoplasts were inoculated with TMV-movement protein:green fluorescent protein RNA and observed under confocal laser scanning microscopy at regular time intervals after inoculation. **A** through **D**, wild type protoplasts; **E** through **H**, NTH201-overexpressing protoplasts; **I** and **J**, NTH201-silenced protoplasts. Time intervals: **A** and **E**, 12 h postinoculation (hpi); **B**, **F**, and **I**, 16 hpi; **C**, **G** and **J**, 22 hpi; **D** and **H**, 24 hpi; and arrowhead = VRC.

sion of NTH201 might be necessary before the virus infection to confer this effect. In the NTH201-overexpressing cells infected with TMV, the increased accumulation of MPs but not of viral genomic RNAs or MP-sgRNAs and the accelerated formation of VRC were demonstrated. This shows that the increased accumulation of MPs was not dependent on that of newly synthesized MP-mRNAs but was likely dependent on the increased stability of MP. MP was known to be expressed transiently and rapidly degraded by the 26S proteasome at an early infection stage in the cells (Reichel and Beachy 2000; Watanabe et al. 1984), whereas ActD increased TMV MP accumulation (Blum et al. 1989), which also was confirmed in the present study. The MP accumulation level in NTH201-overexpressing protoplasts without ActD was almost the same as that in nontransgenic protoplasts with ActD. Hence, it is possible that NTH201 might stabilize the active MP molecules indirectly by assisting MPs to fold their tertiary structure or by protecting their degradation by the 26S proteasome. Because it is conceivable that NTH201 may be a transcription factor like other KNOX proteins, the products of NTH201 target genes might directly regulate these actions.

VRC of TMV were reported to include MP, RdRp, and some host proteins as well as viral RNA and suggested to traffic to the adjacent cells (Asurmendi et al. 2004; Kawakami et al. 2004). Therefore, it seems reasonable to suppose a possibility that the increase in active MP molecules mediated indirectly by NTH201 could facilitate VRC formation and its intercellular traffic as well as virus multiplication in the infected cells, which results in rapid virus cell-to-cell movement in the tissues.

MATERIALS AND METHODS

Plant materials.

N. benthamiana was grown in a temperature-controlled greenhouse and 4- to 5-week-old plants were used for the silencing experiments. Nontransgenic *N. tabacum* cv. Xanthi plants and transgenic *N. tabacum* cv. Xanthi nc plants overexpressing NTH201 were grown in a growth chamber with a cycle of 16 h of light and 8 h of darkness at 25°C.

Cloning of NTH201 gene.

Total RNA was prepared from tobacco leaves (*N. tabacum* cv. Xanthi) using RNeasy midi kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions, and their cDNAs were synthesized using SuperScript III reverse transcriptase (Invitrogen Corp., Carlsbad, CA, U.S.A.) and oligo(dT)₂₀ according to the manufacturer's instructions. A partial NTH201 cDNA was amplified with Phusion high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA, U.S.A.) from them using a set of degenerate primers, 5'-CG(C/T)GT(C/T)CATGCAATGGAAGC-3' and 5'-TGGATTGCT(A/G)TGCCA(A/G)TTCCT-3', designed for the conserved sequences of class II KNOX genes, *Arabidopsis* KNAT3 and KNAT4 (Serikawa et al. 1997) and tomato *LeT12* (Janssen et al. 1998). The remaining 5' and 3' ends of the complete NTH201 cDNA were obtained from tobacco total RNA pool by RNA ligase-mediated oligo-capping 5'- and 3'-RACE methods using GeneRacer Kit (Invitrogen) and Phusion High-Fidelity DNA Polymerase with appropriate gene-specific primers according to the manufacturer's instructions. Then, a full-length NTH201 cDNA clone was sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). DNA and predicted protein sequence analyses were performed with the BLAST algorithm at the National Center for Biotechnology Information (NCBI, Bethesda, MA, U.S.A.).

Multiple alignment of several KNOX proteins was performed based on CLUSTAL W and the phylogenetic tree was

calculated on their total amino acid sequences using CLUSTAL W and NJPlot programs.

VIGS of NTH201.

For gene silencing of endogenous NTH201, we used a PVX-based expression vector. Briefly, the Gateway reading frame cassette A (Invitrogen) was inserted into the *Sma*I site of pCXV, to generate pPVX as a gateway destination vector. D. Baulcombe gave us plasmid pCXV (Baulcombe et al. 1995), in which PVX cDNA was modified to express an exogenous gene inserted between the CaMV 35S promoter and the nopaline synthase gene terminator. Then, the partial 3'-UTR sequence of the NTH201 gene was cloned into the multicloning site of the pENTR 1A (Invitrogen) and designated pENTR-NTH201 as an entry vector. The 3'-UTR of NTH201 was amplified using the following primers: primer 5' NTH201-UTR including a *Bam*HI site, 5'-TCGGGATCCATAGCAATCCATCGACATCG-3'; and primer 3' NTH201-UTR including a *Kpn*I site, 5'-TCCGGTACCAACAGGGGAAATCGTTCATACC-3'. The gene-silencing vector pPVX-NTH201 was generated by Gateway LR Clonase (Invitrogen) recombination reaction between the pENTR-NTH201 and the pPVX according to the manufacturer's instructions.

The plasmid DNAs, pPVX-NTH201 or pPVX as a control, were inoculated directly into the leaves of *N. benthamiana* plants using a hand-held particle delivery system (Helios Gene Gun system; Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's instructions. At 3 weeks postinoculation, the upper leaves became systemically infected with the recombinant pPVXs. These upper leaves were secondarily inoculated with TMV-GFP, a recombinant TMV (U1 strain) expressing GFP together with RdRp, MP, and heterologous CP of *Tobacco mild green mosaic virus* (U5 variant). To prepare the inoculum TMV-GFP, the in vitro transcripts of p30B-GFP (Shivprasad et al. 1999), a gift from W. O. Dawson, were inoculated on *N. benthamiana* plants and the propagated virus particles were partially purified as previously described (Canto and Palukaitis 2002).

At 1 week postinoculation of TMV-GFP on the silenced leaves, GFP-derived fluorescent images on the infected plants were examined nondestructively by visualization or photography with a hand-held long-wave UV lamp (366 nm) and digital camera CAMEDIA C-4040 ZOOM (Olympus, Tokyo). The areas of these GFP foci were quantified using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA, U.S.A.) and NIH image 1.63 (NIH, Bethesda, MD, U.S.A.) software and averaged using Microsoft Excel (Microsoft, Redmond, WA, U.S.A.).

To verify the reduction of NTH201 mRNA, a set of primers, 5'-ATGGCTTGTGGGAGCTAGA-3' and 5'-TCTGCCTGGT CATCATC-3', were used for the real-time RT-PCR analysis. These primers anneal to the sequences of outside region of NTH201 genes targeted for silencing. The primers described below were used for ubiquitin (UBI) mRNA internal control; UBI forward primer: 5'-CGGCATGCTTAACACATGCA-3', and UBI reverse primer: 5'-AGCCGTTTCCAGCTGTTGTT C-3'. Total RNA was extracted from post-inoculated leaves, and cDNA was synthesized from 1 µg of total RNA by using the High-Capacity cDNA Archive Kit (Applied Biosystems). Subsequently, the NTH201 gene was amplified using SYBER Green PCR Master Mix (Applied Biosystems) and analyzed by using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions.

The accumulation of TMV-GFP RNA was analyzed by Northern hybridization using a digoxigenin-labeled RNA probe to detect the GFP region.

NTH201-overexpressing transgenic plants and TMV infection.

To overexpress NTH201 in tobacco plants, full-length of *NTH201* was inserted into the multicloning site of pBI121 vector (Clontech, Mountain View, CA, U.S.A.). Transgenic lines of *N. tabaccum* cv. Xanthi nc that constitutively overexpress NTH201 were generated via *Agrobacterium*-mediated leaf-disk transformation as described (An 1987; Horsch et al. 1985). Total RNA from T₁ transgenic plants was purified, and 10 µg of total RNA was analyzed by Northern hybridization to confirm the overexpression of *NTH201*. For inoculation tests, 6-week-old transgenic T₁ plants were mechanically inoculated with TMV (OM strain) (Watanabe et al. 1999) using Carborundum. At 3 to 9 days after inoculation, the sizes of necrotic local lesions were measured.

Assay of NTH201 traffic ability and detection of NTH201 localization.

To assay the NTH201 self-traffic ability, a full coding region of *NTH201* was fused with *GFP* at the N-terminal side or C-terminal side and the fusion gene was inserted into pUC18-based 35S-driven GFP-expression vector to construct pUC18-GFP:NTH201 or pUC18-NTH201:GFP following a bombardment into *N. benthamiana* leaves using a PDS1000/He system (Bio-Rad). In these vectors, a synthetic GFP S65T mutant derived from p35S-sGFP (gift from Y. Niwa) was used instead of native GFP and the fusion proteins were spaced by two to four glycine linkers. To assay the NTH201 ability to transport GFP via PD, pUC18-based 35S-driven plasmids which express GFP (pUC18-GFP), MP fused to a red fluorescent protein (DsRed2) (Clontech) (pUC18-MP:DsRed), or NTH201 (pUC18-NTH201) were constructed and the combinations of the three plasmids were cobombarded as follows. Briefly, 1 µg of plasmid DNA was combined with 10 µl of 1.0-micron prepared gold particles (Bio-Rad). The coated gold particles were introduced to the abaxial side of the leaf with a PDS-1000/He system with a 900-psi rupture disk (Bio-Rad). Following bombardment, leaf disks were incubated in the dark in a petri dish with Murashige and Skoog-agar medium.

Fluorescence microscopy.

Confocal laser scanning microscopy (CLSM) was conducted with a Zeiss LSM 5 PASCAL (Carl Zeiss, Oberkochen, Germany), according to the manufacturer's instructions. Data of GFP signals were acquired using a 488-nm laser line of an argon laser (Carl Zeiss) with a 505- to 530-nm emission filter. DsRed signals were acquired using a 543-nm laser line of a helium neon laser (Carl Zeiss) with a 560- to 615-nm emission filter.

Transformation of suspension-cultured tobacco BY-2 cells.

The full-length coding region of *NTH201* and the inverted construct of 3'-UTR of *NTH201* or *DsRed2* gene (Clontech) as a control were inserted into the multicloning sites of binary vector pBI121 to construct pBI-NTH201, pBI-ΔNTH201, or pBI-DsRed, respectively. For silencing of the endogenous genes, an intron containing inverted construct was known to efficiently silence the target genes (Smith et al. 2000; Wesley et al. 2001). For pBI-Δ NTH201, we amplified the second intron of tobacco actin gene (X63603), and the 3' UTR corresponding to nucleotides 1,269 to 1,551 of *NTH201* was ligated to 5' and 3' arms of this intron to be transcribed as a hairpin RNA containing an inverted repeat.

These constructs were transferred to *Agrobacterium tumefaciens* LBA4404 by electroporation as described (Nagel et al. 1990), and the transformant bacteria were used to transform the suspension-cultured tobacco BY-2 cells, essentially as de-

scribed by An (1987). The transgenic tobacco BY-2 cells were selected on BY-2 agar medium (Murashige and Skoog salts at 4.3 g/liter, sucrose at 30 g/liter, KH₂PO₄ at 180 mg/liter, myo-inositol at 100 mg/liter, thiamine HCl at 1 mg/liter, 2,4-D at 0.2 mg/liter, and Gelrite at 4 g/liter) (Nagata et al. 1981) containing kanamycin at 50 mg/liter and claforan at 500 mg/liter. Then, several transgenic BY-2 cell lines were checked by PCR, selected by Western blot or real-time RT-PCR analyses, and maintained in liquid culture at 28°C in the dark on a rotary shaker at 120 rpm. Every 7 days, 2 ml of culture was transferred to a new flask containing BY-2 medium.

Quantification of viral RNAs and proteins in TMV-infected protoplasts.

Tobacco protoplasts were prepared from BY-2 suspension cell cultures as described previously (Watanabe et al. 1987) and 2 × 10⁶ protoplasts suspended in an electroporation buffer (5 mM morpholineethanesulfonic acid, 70 mM KCl, and 0.4 M D-mannitol) were inoculated with 2.5 µg of purified RNA of TMV (OM strain) via electroporation using a Gene Pulser II (Bio-Rad) at 750 V/cm and 100 µF according to the manufacturer's instructions. After the inoculation, protoplasts were resuspended in 10 ml of BY-2 suspension medium containing 0.4 M D-mannitol, and 1-ml aliquots in 35-mm petri dishes were cultured in the dark from 8 to 24 h at 28°C at 60 rpm. ActD (30 µg/ml), which increases the accumulation of TMV-MP (Blum et al. 1989), was added to the protoplast after the inoculation from a stock solution prepared with dimethyl sulfoxide as a solvent.

To quantify the amounts of viral genomic RNA and MP-sgRNA in inoculated protoplasts, total RNA from 2 × 10⁵ protoplasts was extracted using 100 µl of ISOGEN (Nippon Gene, Tokyo) according to the manufacturer's instructions. We analyzed the accumulation of TMV genomic RNA by using real-time RT-PCR with forward primer 126K/1293 (5'-ACCAGGC GAAAGCTCTTACA-3') and reverse primer 126K/1440 (5'-TGCAGGTAAACGTCATGGA-3'). These 126K primers amplify only the RNA-dependent RNA polymerase coding region of TMV genomic RNA. For MP-sgRNA, forward primer MP/5147 (5'-CAATTGCAGAGGAGGTGTGA-3') and reverse primer MP/5251 (5'-GGGAACGACCTTGAAGTAA-3') were used which amplify both TMV genomic RNA and MP-sgRNA. UBI mRNA was used as an internal control as mentioned above.

To quantify the amounts of viral MP and CP proteins in infected protoplasts, 2 × 10⁵ protoplasts were mixed, boiled, and solubilized with 100 µl of 2× sodium dodecyl sulfate (SDS) sample buffer (Tris-HCl/SDS, pH 6.8; 20% glycerol; 4% SDS; 2-mercaptoethanol; and 0.001% bromophenol blue). Then, 1/10 vol of the solution was loaded in the SDS-12.5% polyacrylamide gel electrophoresis, and the accumulation of viral proteins was analyzed by Western blot using anti-MP polyclonal antibody or anti-CP monoclonal antibody (each 1/5,000 dilution). Western blot analysis was conducted using ECL Plus Western blotting detection reagents (GE Healthcare, Buckinghamshire, U.K.), and signal detection was acquired using an LAS-1000 Imaging System (Fujifilm, Tokyo) according to the manufacturer's instructions.

Effect of NTH201 on the MP accumulation and VRC formation in TMV-infected protoplasts.

We constructed TMV cDNA clones (pTMV-MP:GFP) expressing GFP fused to MP based on pT7-TMVom, a T7 promoter driven expression vector for an infectious in vitro transcript of TMV (OM strain). For this, we amplified an MP gene fragment from pT7-TMVom using the following primers: forward primer, including *NcoI* site, 5'-TCGTCATATGGCTCTA

GTTGTTAAAGGAAAAG-3'; and reverse primer, including *AvrII* site, 5'-AAAGGGCCCTGCAGCCTAGGAAACGAATC CGATTCGGCGA-3'. Then, an *AvrII*-*KpnI* fragment encoding the GFP S65T region amplified from p35S-sGFP was fused to the *NcoI*-*AvrII* MP fragment to be translated as a fusion protein. This fused fragment was cloned between *NcoI* and *KpnI* sites of pT7-TMVom, and the resultant clone lacking the CP gene was designated as pTMV-MP:GFP. For virus inoculation to the protoplasts, *KpnI*-linearized pTMV-MP:GFP was transcribed in vitro using a MEGAscript T7 kit (Ambion, Austin, TX, U.S.A.), including cap analogue (10:1 in GTP ratio). Capped transcripts were introduced into BY-2 protoplasts by electroporation. The accumulation of MP:GFP and formation of VRC in infected protoplasts were observed under a CLSM at regular culture time intervals.

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