Identification of a movement protein of Mirafiori lettuce big-vein ophiovirus

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Mirafiori lettuce big-vein virus (MiLBVV) is a member of the genus Ophiovirus, which is a segmented negative-stranded RNA virus. In microprojectile bombardment experiments to identify a movement protein (MP) gene of ophioviruses that can trans-complement intercellular movement of an MP-deficient heterologous virus, a plasmid containing an infectious clone of a tomato mosaic virus (ToMV) derivative expressing the GFP was co-bombarded with plasmids containing one of three genes from MiLBVV RNAs 1, 2 and 4 onto Nicotiana benthamiana. Intercellular movement of the movement-defective ToMV was restored by co-expression of the 55 kDa protein gene, but not with the two other genes. Transient expression in epidermal cells of N. benthamiana and onion showed that the 55 kDa protein with GFP was localized on the plasmodesmata. The 55 kDa protein encoded in the MiLBVV RNA2 can function as an MP of the virus. This report is the first to describe an ophiovirus MP.

Big-vein disease of lettuce (Lactuca sativa L.), first reported in California (Jagger & Chandler, 1934), occurs in major lettuce-growing areas in cool to temperate and occasionally subtropical regions and is a serious, economically important problem for lettuce production during cooler periods of the year (Barcala Tabarrozzi et al., 2010; Huijberts et al., 1990; Roggero et al., 2000; Vetten et al., 1987). Infected lettuce plants develop vein bandings and ruffled, distorted leaves, reducing market value.

A rod-shaped virus, named Lettuce big-vein associated virus (LBVaV; genus Varicosavirus), was initially thought to be the causal agent of big-vein disease for some time (Kuwata et al., 1983; Walsh & Verbeek, 2011), until a filamentous virus, named Mirafiori lettuce big-vein virus (MiLBVV), was isolated from big-vein-affected lettuce plants (Roggero et al., 2000). A number of studies have since confirmed that MiLBVV, but not LBVaV, induces big-vein symptoms in lettuce (Lot et al., 2002; Roggero et al., 2000; Sasaya et al., 2008).

MiLBVV is a member of the genus Ophiovirus and is transmitted to lettuce plants by motile zoospores of an obligate root-inhabiting fungus, Olpidium virulentus (Sasaya & Koganezawa, 2006; Vaira et al., 2011). MiLBVV is a segmented negative-stranded RNA virus, but virus particles contain nearly equimolar amounts of RNA molecules of both polarities; this virus uses negative and possibly ambisense coding strategies for replication and infection in plants (van der Wilk et al., 2002). The genome consists of four ssRNAs containing seven ORFs (Vaira et al., 2011). RNA1 is negative-sense and encodes two putative proteins with a molecular mass of approximately 263 and 25 kDa. The 263 kDa protein contains the core polymerase module with the five conserved motifs of RNA-dependent RNA polymerase and may function as an RNA-dependent RNA polymerase (Naum-Ongania et al., 2003). RNA2 is proposed to be ambisense and to encode two putative proteins with a molecular mass of approximately 10 and 55 kDa in the 5’-proximal region of the virion-sense strand and the virion-complementary sense-strand, respectively. RNA3 is negative-sense and encodes the 48.5 kDa protein, which is a nucleocapsid protein (NP) and a major component of thin filamentous particles. RNA4 is negative-sense and comprises two overlapping segments.
ORFs in different reading frames. The first ORF encodes a putative protein with a molecular mass of approximately 37 kDa. The second ORF has a coding capacity of 10.6 kDa, but lacks an initiation codon. This putative protein can be expressed potentially by a +1 translational frameshift, which has not been proven experimentally. The complexity of the unusual genome organization and replication strategy of MiLBVV has so far prevented the development of an infectious clone system for the virus. Lack of a reverse genetics system for MiLBVV has made it impossible to conduct functional studies on virus-encoded proteins using standard mutagenesis methods. Thus, except for the 263 and 48.5 kDa proteins, the functions of the other five putative proteins are still unknown.

Cell-to-cell movement is a primary requirement for the systemic spread of plant viruses in susceptible hosts. Due to the presence of the rigid cell wall in plants, plant viruses can only spread to neighbouring cells through cytoplasmic channels, called plasmodesmata (PD) that pass through the cell walls. The diameter of the PD is too small for most plant viruses to pass through. Therefore, plant viruses, in contrast to animal-infecting viruses, have evolutionally acquired a gene that encodes a cell-to-cell movement protein (MP) (Lucas, 2006; Taliansky et al., 2008).

The requirement of a viral component for cell-to-cell movement was first demonstrated in studies on a non-structural, 30 kDa protein of tobacco mosaic virus (TMV) (Atabekov & Dorokhov YuL, 1984). TMV was considered to move as a viral RNA–MP complex that facilitates movement of the virus through PD to adjacent neighbouring cells (Lucas, 2006; Oparka et al., 1997; Taliansky et al., 2008). A different movement strategy by cowpea mosaic virus was shown in studies on the 48 kDa protein (van Lent et al., 1991; Wellink et al., 1993), which forms tubular structures that penetrate the cell wall and protrude into the cytoplasm of neighbouring cells. Similar tubule-guided, cell-to-cell movement systems have been proposed for viruses of various genera including Caulimovirus, Nepovirus, Bromovirus and Tospovirus (Kasteel et al., 1997; Perbal et al., 1993; Storms et al., 1995; Wieczorek & Sanfaçon, 1993). Many other non-structural proteins, such as the 3a proteins of cucumoviruses and the products of a triple gene block in viruses of the families Alphaflexiviridae, Betaflexiviridae and Virgaviridae, have been identified as MPs (Morozov & Solovyev, 2003; Palukaitis & Garcia-Arenal, 2003; Scholthof, 2005; Taliansky et al., 2008). However, no MP gene has yet been identified in the ophiovirus genome and, consequently, there is no information also on the molecular mechanism of the cell-to-cell movement of ophioviruses.

Our attempts to engineer RNA interference (RNAi)-mediated resistance to several rice viruses have revealed that identifying the viral ‘Achilles heel gene’ is important for choosing an appropriate target for the RNAi attack when engineering plants that are strongly resistant to virus infection (Shimizu et al., 2009, 2011a, b). Transgenic studies on segmented negative-stranded RNA viruses, rice stripe tenuivirus and rice grassy stunt tenuivirus, indicated that transgenic rice plants that harboured the RNAi construct specific for the viral MP gene had complete resistance against viral infections (Shimizu et al., 2011a, 2012). The gene for the MP is thus a good candidate for RNAi targeting to confer resistance against segmented negative-stranded RNA viruses. Compared with the viral NP genes reported as potential targets for RNAi-mediated resistance in plants (Bucher et al., 2006; Kawazu et al., 2009; Reyes et al., 2011), the viral gene for the MP has been used far less as an RNAi target to control segmented negative-stranded RNA viruses. To reveal whether inhibiting MP expression can also effectively block infection by other segmented negative-stranded RNA viruses and to develop transgenic lettuce plants that are completely resistant to MiLBVV infection, we attempted to identify an MP gene of MiLBVV by trans-complementation experiments with a movement-defective tomato mosaic virus (ToMV).

Total RNA was isolated from lettuce leaves showing typical big-vein symptoms (Kawazu et al., 2003) and was reverse transcribed by SuperScript III reverse transcriptase (Invitrogen). Three ORFs for 25, 55 and 37 kDa proteins were amplified individually from the transcripts by PCR using KOD DNA polymerase (TOYOBO) and appropriate primer sets (Table S1, available in JGV Online). The PCR products were inserted into the plant expression plasmid pE7133-GW using the Gateway gene introduction system (Hiraguri et al., 2012, 2011). The recombinant plasmids for expression of 25, 55 and 37 kDa proteins were designated pE7133-MiL25K, pE7133-MiL55K and pE7133-MiL37K, respectively. As a positive control, the ToMV MP cDNA was cloned into pE7133-GW using the Gateway gene introduction system (pE7133-ToMV). All plasmids including those described below contain the cauliflower mosaic virus (CaMV) 35S RNA promoter for strong expression. The plasmid (piLMRD.ERG3) carrying an infectious cDNA of a movement-defective ToMV tagged with the endoplasmic reticulum-targeting green fluorescent protein (erGFP), was used for complementation experiments (Hiraguri et al., 2011, 2012). The plasmid lacks the start codon (from ATG to ACG) and a large part (18–557 nt) of the MP gene and contains the erGFP gene instead of the ToMV CP gene and generates an erGFP fluorescence replication-competent but MP- and CP-defective ToMV (L strain) mutant under the control of the CaMV 35S RNA promoter. Infection and spread of the ToMV mutant derived from piLMRD.ERG3 can thus be monitored by observing the distribution of GFP fluorescence. These plasmids were introduced into epidermal cells of Nicotiana benthamiana via particle bombardment using the PDS-1000/He system (Bio-Rad Laboratories) as described previously (Hiraguri et al., 2012). Mature leaves (6–9 cm long) of N. benthamiana (4–7 weeks old) were cut and placed on an empty plate at a target distance of 6 cm. Gold particles (1 μm diameter, 1.5 mg) were coated with
2 μg of a mixture of two plasmids (1 μg each), and the resultant particle suspension was divided into three equal aliquots, each of which was used to bombard leaves with a rupture disk of 900 lb/in². Bombarded leaves were incubated at 25 °C in the dark for 1–3 days. GFP signals were observed with an LSM510 confocal laser microscope (Carl Zeiss). An excitation wavelength of 488 nm produced by the argon laser and an emission filter of 505–530 nm allowed the detection of GFP-specific fluorescence.

When piLMRd.erG3 was used alone to bombard leaves of *N. benthamiana*, the green fluorescence from GFP was restricted to single epidermal cells at 100, 100 and 99% of the transfected sites at 1, 2 and 3 days post-bombardment, respectively [Fig. 1a(i) and Table 1]. Leaves bombarded with piLMRd.erG3 also displayed small foci of green fluorescence that were very difficult to detect with the Lumino image analyser (Fujifilm) [Fig. 1b(i)]. These results indicated that the ToMV mutant was able to replicate to express GFP in initially bombarded cells, but was defective in cell-to-cell movement due to the lack of viral MP. When piLMRd.erG3 was co-bombarded with pE7133-MiL55K, which expressed the MiLBVV 55 kDa protein, GFP fluorescence was seen in clusters of four or more cells, and the percentage of these fluorescent clusters increased over time [Fig. 1a(iii) and Table 1]. Similar results were obtained when piLMRd.erG3 was co-bombarded with pE7133-ToMVMP as a positive control [Fig. 1a(v) and Table 1]. The leaves co-bombarded with pE7133-MiL55K displayed green fluorescent foci of nearly the same size as the foci seen after co-bombardment with pE7133-ToMV [compare Fig. 1b(iii) and (v)]. The multicellular distribution of the expressed GFP indicated that the movement-defective virus had spread from initially infected cells through two or more cell layers because the GFP itself cannot spread to neighbouring cells. In contrast, leaves co-bombarded with pE7133-MiL25K or pE7133-MiL37K showed similar results; in almost all cases, the green fluorescence was restricted to single epidermal cells of *N. benthamiana* [Fig. 1a(ii) and (iv)], which was not observable at lower magnification [Fig. 1b(ii) and (iv)]. No cell clusters of more than three GFP fluorescent cells were observed at 1, 2 and 3 days post-bombardment (Table 1). These results suggested that the cell-to-cell movement of the MP-deficient ToMV mutant was trans-complemented with the 55 kDa protein, but not with the 25 or 37 kDa proteins.

To determine the subcellular localization of the MiLBVV 55 kDa protein in plant cells, we constructed pE7133-MiL55K–GFP, an expression plasmid for the 55K protein fused with GFP (55K–GFP). The 55K cDNA lacking the stop codon was amplified by PCR with pE7133-MiL55K as a template and an appropriate primer set (Table S1) and was cloned into pE7133-GW GFP, which was constructed by modifying pE7133-GW to express GFP fused to the C terminus of an arbitrary protein (Hiraguri *et al.*, 2011). The co-localization experiment of the 55K–GFP with a PD

![Fig. 1. Trans-complementation of a movement-defective ToMV by the 55 kDa protein of MiLBVV.](http://vir.sgmjournals.org)
marker protein was conducted using the expression plasmid (pB7WG2.0.PDCB1–mCherry) for the plasmodesmata callose binding protein 1 fused with mCherry (PDCB1–mCherry) (Simpson et al., 2009). After the two plasmids were used to co-bombard epidermal cells of N. benthamiana and onion, the co-bombarded leaves of N. benthamiana and scales of onion were incubated at 25 °C in the dark for 24 h. The fluorescent signals from GFP and mCherry were observed with an LSM510 confocal laser microscope. An excitation wavelength of 543 nm produced by the HeNe laser and an emission filter of 585–615 nm allowed the detection of mCherry-specific fluorescence. No images were taken of a Z stack of sections. In the epidermal cells of N. benthamiana, green fluorescence from 55K–GFP was mainly observed at the periphery of the cells, notably as punctate patterns [Fig. 2a(i)], and importantly, these GFP signals co-localized with the mCherry signals from the PD marker protein, PDCB1–mCherry [Fig. 2a(iii)]. Similar results were obtained when 55K–GFP and PDCB1–mCherry were co-expressed in the epidermis of onion scales (Fig. 2b). The co-localization of the two proteins in the punctate structures indicated that the 55K–GFP localizes at the PD.

Plant viruses encode one or more proteins that are required for intercellular movement in plants. In general, viral MPs are highly variable in their amino acid sequences, and accordingly they have diverse cell-to-cell movement strategies. However, the MP of a certain virus can often complement the cell-to-cell movement of other distantly related or even unrelated viruses (Morozov & Solovyev, 2003; Taliansky et al., 2008). Movement functions for several virally encoded proteins have been demonstrated by trans-complementation experiments with a movement-defective virus and viral proteins of interest. This approach is efficient and holds promise for identifying MPs of viruses, in particular for those without reverse genetics systems (Morozov & Solovyev, 2003; Wu et al., 2010; Xiong et al., 2008). We have recently demonstrated by the trans-complement experiment the

Table 1. Frequency of infection sites with one or more cells containing movement-defective ToMV from 1 to 3 days after bombardment (Dpb) with the MiLBVV gene

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Dpb</th>
<th>Cell–cell boundaries (frequency)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 cell</td>
<td>2 cells</td>
</tr>
<tr>
<td>pilMRd.erG3</td>
<td>1</td>
<td>75 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>67 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>138 (99)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>pilMRd.erG3 + pE7133-MiL25K</td>
<td>1</td>
<td>116 (98)</td>
<td>2 (2)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>124 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>211 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>pilMRd.erG3 + pE7133-MiL55K</td>
<td>1</td>
<td>160 (57)</td>
<td>36 (13)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>114 (50)</td>
<td>26 (11)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>112 (39)</td>
<td>28 (10)</td>
</tr>
<tr>
<td>pilMRd.erG3 + pE7133-MiL37K</td>
<td>1</td>
<td>134 (99)</td>
<td>1 (1)</td>
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<td></td>
<td>2</td>
<td>218 (99)</td>
<td>1 (1)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>228 (98)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>pilMRd.erG3 + pE7133-ToMVMP</td>
<td>1</td>
<td>49 (23)</td>
<td>26 (12)</td>
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<td></td>
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<td>3</td>
<td>23 (11)</td>
<td>20 (9)</td>
</tr>
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</table>

Fig. 2. Subcellular localization of the MiLBVV 55 kDa protein. (a) Co-localization of the MiLBVV 55 kDa protein fused with GFP (55K–GFP) and PDCB1 fused with mCherry (PDCB1–mCherry) in epidermal cells of N. benthamiana (a) and onion (b) at 24 h post-bombardment. Images were taken with a confocal laser microscope with appropriate emission wavelengths and filters to detect GFP-specific (i) or mCherry-specific fluorescence (ii). Merged images are shown in (iii). Bars, 20 μm.
abilities of the pC6 protein of rice grassy stunt tenuivirus and the P3 protein of rice transitory yellowing nucleorhabdovirus to traffic a heterologous viral genome between cells (Hiraguri et al., 2011, 2012). Our results presented here also demonstrated that the 55 kDa protein encoded in the virion-complementary sense-strand of MiLBVV RNA2 could trans-complement cell-to-cell movement of a movement-deficient mutant of ToMV and be associated with PD, representing the first experimental evidence that the 55 kDa protein functions as an MP in viral cell-to-cell movement.

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