Systemic transport of *Alfalfa mosaic virus* can be mediated by the movement proteins of several viruses assigned to five genera of the 30K family

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Summary words: 150

Main text words: 2500

Total Number of Figures: 3

Number of Colour Figures: 1

Abstract

We previously showed that the movement protein (MP) gene of *Alfalfa mosaic virus* (AMV) is functionally exchangeable for the cell-to-cell transport of the corresponding genes of *Tobacco mosaic virus*, *Brome mosaic virus*, *Prunus necrotic ringspot virus*, *Cucumber mosaic virus* and *Cowpea mosaic virus*. We have analyzed the capacity of the heterologous MPs to systemically transport the corresponding chimeric AMV genome. All MPs were competent for the systemic transport but required the fusion at their C-terminus of the coat protein-interacting C-terminal 44 amino acids (A44) of AMV MP. The A44 region was also required to reach vascular tissue. Except for the TMV MP, the presence of the hybrid virus in systemic leaves correlated with the capacity to move locally, suggesting a cell-to-cell threshold transport. These results suggest that all the MPs assigned to the 30K superfamily are exchangeable not only for the local virus movement but also for the systemic transport.

To establish a systemic infection, plant viruses must invade the adjacent cells via the cell wall connections known as plasmodesmata (PD), the so-called cell-to-cell transport (Fernandez-Calvino *et al.*, 2011), and reach distal parts of the plant through the vascular tissue, a process denominated systemic transport (Ueki and Citovsky, 2007; (Pallás *et al.*, 2011). For this purpose, part of the reduced viral genome is addressed to express one or a few movement protein(s) (MPs) to support virus transport which can determine host specificity (Waigmann et al., 2007) and, in some instances, can influence on viral pathogenicity (Pallás & García, 2011). Viral MPs facilitate the virus cell-to-cell transport by different mechanisms, permitting the transport of ribonucleoprotein complexes, between MP and viral RNA (e.g. *Tobacco mosaic virus*,TMV; Waigmann et al., 2007), plus the CP (*Cucumber mosaic virus* –CMV- or *Alfalfa mosaic virus* –AMV-) or virions particles (Ritzenthaler & Hofmann, 2007). In spite of the clear differences observed among the three transport mechanisms, a large number of these MPs has been assigned to the 30K superfamily, a group of MPs of viruses belonging to eighteen different genera that express a unique MP that are similar to the TMV MP of 30 kDa.

Systemic transport implies the entry into and the exit from the vascular tissue and, consequently, the infection of different cell types associated with it (see Ueki & Citovsky, 2007 and Pallás *et al.*, 2011, for recent reviews). In addition to the MP, the capacity of plant viruses to reach vascular tissue implies the use of other viral proteins that can be related to the inhibition of plant defenses (e.g. silencing suppressors), protein translation (e.g. VPg) (Rajamaki & Valkonen, 2002), viral RNA-dependent RNA replication –RdRp- (Traynor *et al.*, 1991) or the CP, probably through to the stabilization of virion complexes (Ueki & Citovsky, 2007; Bol, 2008). AMV is the type member of the genus *Alfamovirus* which virus particles are required for systemic transport (Herranz *et al.*, 2012; Sánchez-Navarro & Bol, 2001; Tenllado & Bol, 2000).

In addition, we reported that the MP of AMV is functionally exchangeable by different MPs assigned to the 30K superfamily, allowing the cell-to-cell transport of the corresponding chimera constructs (Sánchez-Navarro *et al.*, 2006; 2010; Sánchez-Navarro & Bol, 2001). Except for the TMV MP, the remaining heterologous MPs require the fusion at its C terminus of the C-terminal 44 amino acids of the AMV MP (A44), responsible to interact with the cognate CP (Sánchez-Navarro *et al.*, 2006). The present work analyzes the capacity of several MPs of the 30K superfamily to support the systemic transport of chimeric AMV RNA3, including representative members that were reported to transport virus particles (e.g. CPMV or *Brome mosaic virus*, BMV), ribonucleoprotein complexes with only the MP (e.g. TMV) or with both the MP and the CP (e.g. AMV or CMV).

First of all, we quantified the cell-to-cell transport of the AMV RNA3 chimera carrying the previously described heterologous MPs (Sánchez-Navarro *et al.*, 2006). To do this, T7 transcripts from the AMV RNA 3 chimera constructs carrying the green fluorescent gene and the corresponding MP gene of PNRSV (PNRSV:A44), CMV (CMV:A44), CPMV, (CPMV:A44), BMV (BMV:A44), BMV with the A44 fused before its C-terminal 48 amino acids (BMV:A44:B48) and the TMV with (TMV:A44) or without (TMV) the A44 fragment, were inoculated on transgenic tobacco plants constitutively expressing the AMV P1 and P2 protein (P12 plants; Taschner *et al.*, 1991). Figure 1 shows the size average of 50 infection foci at 2 days post inoculation, which is the best time when greater differences are observed between individual infection foci. The results grouped constructs into three clusters with an infection foci size of around 800 µm (AMV, CMV:A44, CPMV:A44 and TMV:A44), 600 µm (PNRSV:A44 and BMV:A44) and 400 µm (BMV:A44:B48 and TMV). Interestingly, the absence of the A44 fragment (TMV construct) or its location inside the heterologous

MP (BMV:A44:B48) negatively affects the cell-to-cell transport (compare TMV:A44 vs TMV in Figure 1).

In the next step, we analyzed the capacity of the heterologous MPs to support the systemic transport of the AMV RNA 3. For this purpose, we used a wild-type AMV RNA 3 since the RNA 3 derivatives carrying the GFP reporter gene do not move systemically in P12 tobacco plants (Sánchez-Navarro *et al.*, 2001). All the heterologous MPs were introduced into the AMV RNA 3 (plasmid pAL3NcoP3 in van der Vossen *et al.*, 1993) by exchanging the *Nco*I-*Pst*I fragment. RNA accumulation levels of the different AMV RNA 3 hybrids were first analyzed in P12 protoplasts as described previously (Sánchez-Navarro *et al.*, 2010). Chimeric RNA 3 and 4 accumulated at comparable levels of AMV wild-type RNA (lanes 2-6 in Figure 2b) except for the RNA 3 of the AMV constructs carrying the MP of TMV, either fused or not to the A44 fragment, which was significantly reduced (10%, lanes 7 and 8 in Figure 2b). The accumulation of all the RNA 3 derivatives was then analyzed in inoculated and systemic infected P12 plants leaves by tissue printing of petioles, as described previously (Mas & Pallás, 1995;Sánchez-Navarro *et al.*, 2010). The tissue printing results (Figure 2c) allow us to discern three different patterns according to the detection of a positive hybridization signal in: i) all the inoculated and systemic leaves (AMV, PNRSV:A44, CMV:A44 and CPMV:A44), ii) in the inoculated leaves and some systemic leaves (BMV:A44 and TMV:A44) and iii) only in the inoculated leaves (BMV:A44:B48 and TMV). The accumulation of viral RNAs in the petioles of inoculated (not shown) and systemic leaves showing positive hybridization signal by tissue printing was later confirmed by northern-blot analysis (Figure 2d). The results shown in Figure 2 revealed that all the analyzed MPs are able to support the systemic transport of the AMV RNA3. We also observed that the lack of the A44 fragment (TMV construct) or its location

inside the heterologous MP (BMV:A44:B48) completely abolished systemic transport. Except for the TMV construct, all the AMV RNA 3 chimeras showing large infection foci on the inoculated leaves were able to infect all the systemic leaves (CMV, CPMV and AMV). This result strongly suggests that reaching the vascular tissue at early time of the infection gives an advantage to the pathogen that could counteract the plant defense mechanisms (e.g. silencing). Indeed, in some well characterized plant-virus interactions, the capacity to reach systemic tissue has been associated with a successful blockage of the RNA silencing-mediated plant defense barriers (Cao *et al.*, 2010; Hamilton *et al.*, 2002; Schwach *et al.*, 2005; Wintermantel *et al.*, 1997; Yelina *et al.*, 2002). To date, no RNA silencing suppressor has been identified for AMV. This observation permits argue about the possibility that the virus would counteract the RNA silencing mechanism by moving faster than the putative systemic silencing RNA signal. However, it was not possible to apply this idea to the TMV:A44 construct since the infection foci, observed on the inoculated leaves, were similar to those AMV chimeras infecting all systemic leaves. This result clearly reveals that the MP of TMV is very efficient in supporting the cell-to-cell transport of the AMV RNA 3 chimera, but very inefficient in invading vascular tissue. The observation that the TMV construct is also competent for the cell-to-cell transport indicates that the MP transports viral RNA without any interaction with the AMV CP. In this scenario, it is tempting to speculate that the TMV:A44 MP mainly transports non-encapsidated viral RNA, which allows very efficient local transport, but most inefficient systemic movement for which AMV virus particles are critical. The group of AMV constructs showing medium infection foci on inoculated leaves (600 µm; BMV:A44 and PNRSV:A44) rendered two different systemic infection patterns which were differentiated in terms of their capacity to reach all the systemic leaves (PNRSV:A44; Figure 2c, line 6) or only part of them

(BMV:A44; Figure 2c, line 2). This result clearly indicates that AMV chimeras with reduced cell-to-cell transport are still able to infect all the systemic leaves. The differences observed between both constructs can be attributed to the greater compatibility between the PNRSV and AMV viruses. In this sense, PNRSV is phylogenetically more related to AMV than to BMV (Codoñer *et al.*, 2005;Sánchez-Navarro & Pallás, 1997), and its CP is fully exchangeable for the AMV CP for encapsidation, RNA replication and differential accumulation of positive viral RNAs (Aparicio *et al.*, 2003; Sánchez-Navarro *et al.*, 1997).

To further characterize the AMV constructs that are affected in the systemic transport we decided to perform a more precise tissue printing analysis by checking not only the petiole, but also the stem just above and below of the corresponding petiole (Figure 3). For the AMV wild-type, we observed positive hybridization signals in all the stem sections, covering the full ring and indicating the presence of viral RNA in all phloem tissue. However, the constructs that moved only to some of the systemic leaves (BMV:A44 and TMV:A44; lines 2 and 7) rendered a strong stem hybridization signal close to inoculated leaves that decreased at the upper part of the plant, where the hybridization signal was observed in only part of the cross-section (Figure 3b, the St between systemic leaves S3 and S4). This result indicates that both constructs are able to reach the vascular tissue but do so less efficiently than the AMV wild-type. Poorer efficiency would allow the virus to reach the uppermost leaves, which already underwent the sink-source transition, as shown in other virus-host interactions (Cheng *et al.*, 2000; Mas & Pallás, 1996). For the constructs that do not move systemically, we observe two different patterns on the stem sections. First, the BMV255:A44:B48 chimera shows a clear hybridization signal only in the stem sections around the inoculated leaf (Figure 3b, line 4) and second, the TMV construct with no hybridization

signal at all in the stem (Figure 3b, line 8). Regarding the hybridization signal observed with the BMV255:A44:B48 construct on the border of the stem section, we can conclude that this construct is competent enough to reach vascular tissue, but that it is quite likely that the delay involved in reaching it does not allow to establish a systemic infection. For TMV, we observed the opposite situation in which the lack of the A44 fragment compromises the accession of the virus to the phloem. In line with this, we have recently reported that virus particles and the A44 fragment are essential for the systemic transport of an AMV chimera carrying the MP of *Cauliflower mosaic virus* (Sánchez-Navarro *et al.*, 2010). Regarding the TMV construct it is tempting to speculate that the loading of the virus particles on the phloem is affected by the absence of the critical A44 region required for a compatible interaction with the virions.

In summary, we show that all the MPs analyzed in the present work are competent enough to systemically transport the AMV chimera constructs to the distal parts of the plant when the last 44 aa of the AMV MP were fused at their C-terminus. This result allow us to argue the idea that probably all the MPs of the 30K family are functionally exchangeable for both the local and systemic transports of AMV, irrespectively of the virus, the model described for the local transport (e.g., MP of TMV or CPMV) or the pathway used to reach the plasmodesmata (e.g., MP of TMV or *Grapevine fanleaf virus*;Sánchez-Navarro *et al.*, 2010). In addition, this work also shows that inefficient cell-to-cell transport compromises systemic invasion, permitting to postulate the idea of minimal cell-to-cell speediness being required to reach the upper part of the plant as formerly reported for other viruses (Deom *et al.*, 1994).

Figure 1

Analysis of the cell-to-cell transport of the hybrid AMV RNA3 in which its movement protein (MP) gene was exchanged by the corresponding genes of different movement protein. (a), Schematic representation shows the GFP/AMV/CP AMV RNA 3 derivative (1). Reading frames encoding the GFP, MP and coat protein (CP) are represented by green, red and yellow boxes, respectively. The MPs analyzed correspond to *Brome mosaic virus* (BMV)(2, 4), *Cucumber mosaic virus* (CMV)(3), *Cowpea mosaic virus* (CPMV)(5), *Prunus necrotic ringsport virus* (PNRSV)(6) and *Tobacco mosaic virus* (TMV)(7, 8). The C-terminal 44 and 48 amino acids of the AMV and BMV MP are indicated as 'A44' and 'B48', respectively. The numbers in the boxes represent the total amino acids residues of corresponding MP. The *Nco*I and *Nhe*I restriction sites used to exchange the MP gene are indicated. Images at the right of the scheme correspond to representative pictures of the size of infection foci observed in inoculated P12 leaves. (b), Graphics showing the average of the size of 50 independent infection foci developed by the inoculated transcripts originated from the constructs shown in (a). Fluorescence was monitored with a confocal laser scanning microscope 2 days post-inoculation of plants. Bar represents 200 μm.

Figure 2

Analysis of the replication and systemic transport of the AMV RNA 3 hybrids. (a), Schematic representation shows the AMV RNA 3 wild-type (1). Reading frames encoding the MP and CP are represented by red and yellow boxes, respectively. The MP genes exchanged in the AMV RNA 3 are as indicated in figure 1. (b), Northern blot analysis of the accumulation of the AMV RNA 3 and 4 chimeras in P12 protoplasts. (c) Tissue printing analysis of P12 plants inoculated with the AMV RNA 3 derivatives.

Plants were analyzed at 14 dpi by printing the transversal section of the corresponding petiole from inoculated (I) and systemic (S) leaves. The position of each leaf is indicated by numbers which correspond to the position of the leaves in the plant from the lower to the upper part. (d), Northern blot analysis of a mixture of total RNA extracted from the S2, S3 and S4 systemic leaves. M, mock inoculated plant. Numbers at the top of each membrane correspond to the constructs represented in (a). In all cases, the blots were hybridized with an AMV probe complementary to the 3'-untranslated region. The positions of the RNA3 and RNA4 chimeras are indicated in the left margin of the pictures b) and d).

Figure 3

Tissue printing analysis of AMV RNA 3 derivatives affected in the systemic transport. P12 plants were inoculated with transcripts of AMV RNA 3 wild-type (1) or hybrids carrying the MP gene of BMV (2 and 4) and TMV (7 and 8) represented in Figure 2 (a). (a) Schematic representation of the localization of all the analyzed leaves and the distribution of the transversal sections of petioles (P) and stems (St). (b) Tissue printing analysis of the P12 plants at 14 dpi by printing transversal sections of all petioles and the stem around them. 'I' and 'S' are referred to inoculated and systemic leaves, respectively. The hybridization was performed as described in Figure 2.

Acknowledgments

A. P. M. is the recipient of a JAE-Pre contract from the Consejo Superior de Investigaciones Científicas (CSIC). We thank L. Corachán for her excellent technical assistance. This work was supported by grant BIO2011-25018 from the Spanish

granting agency DGICYT and by grant PROMETEO 2011-003 from the Generalitat Valenciana.

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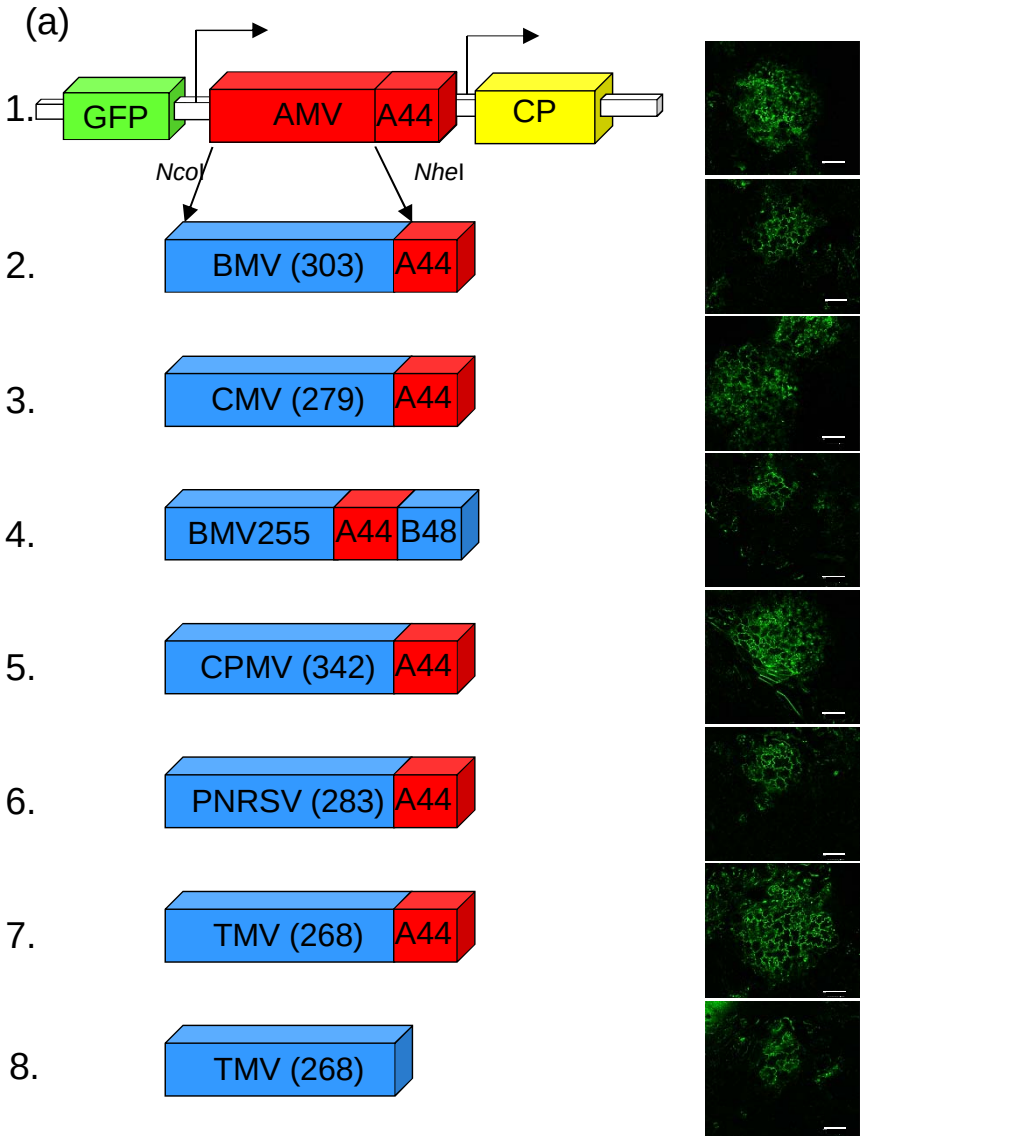
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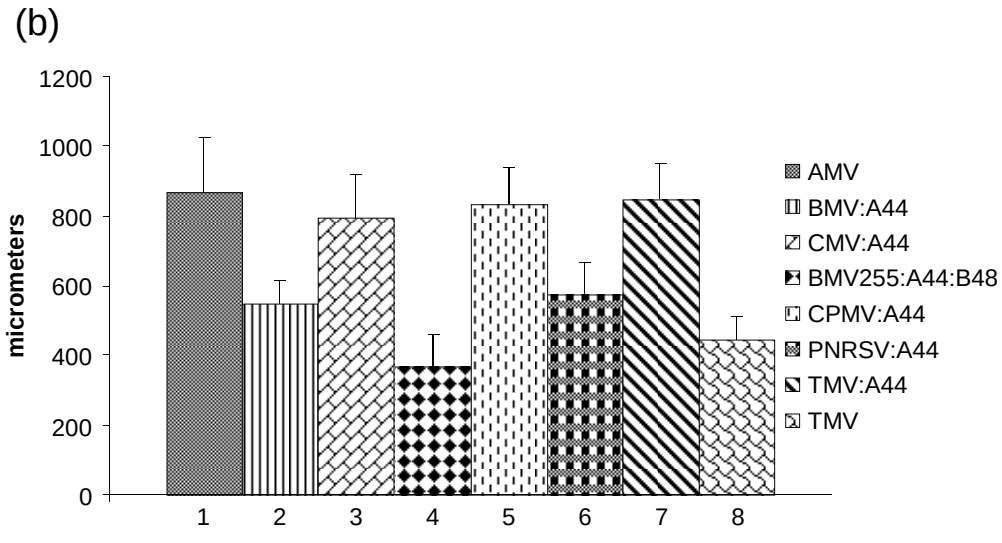
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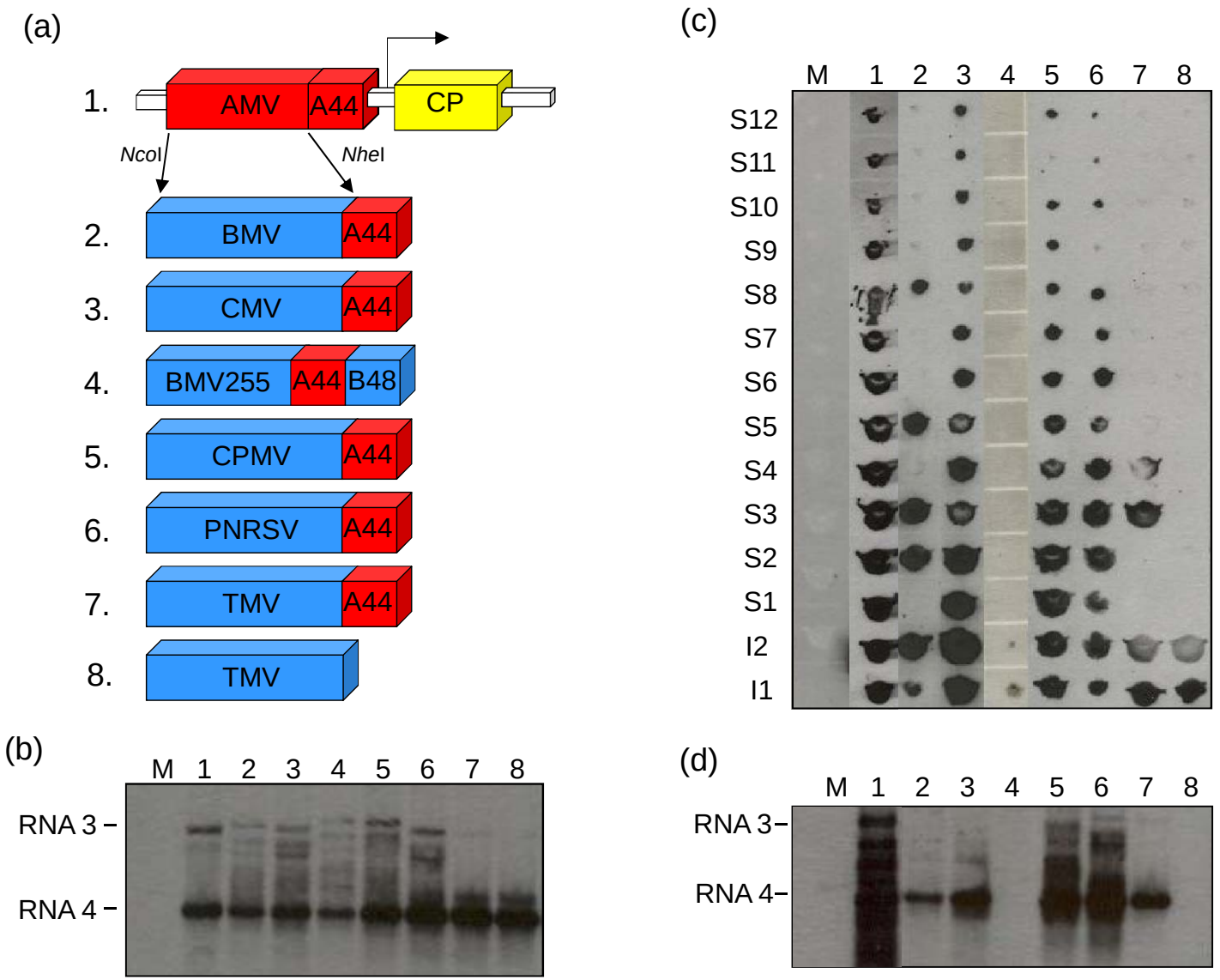
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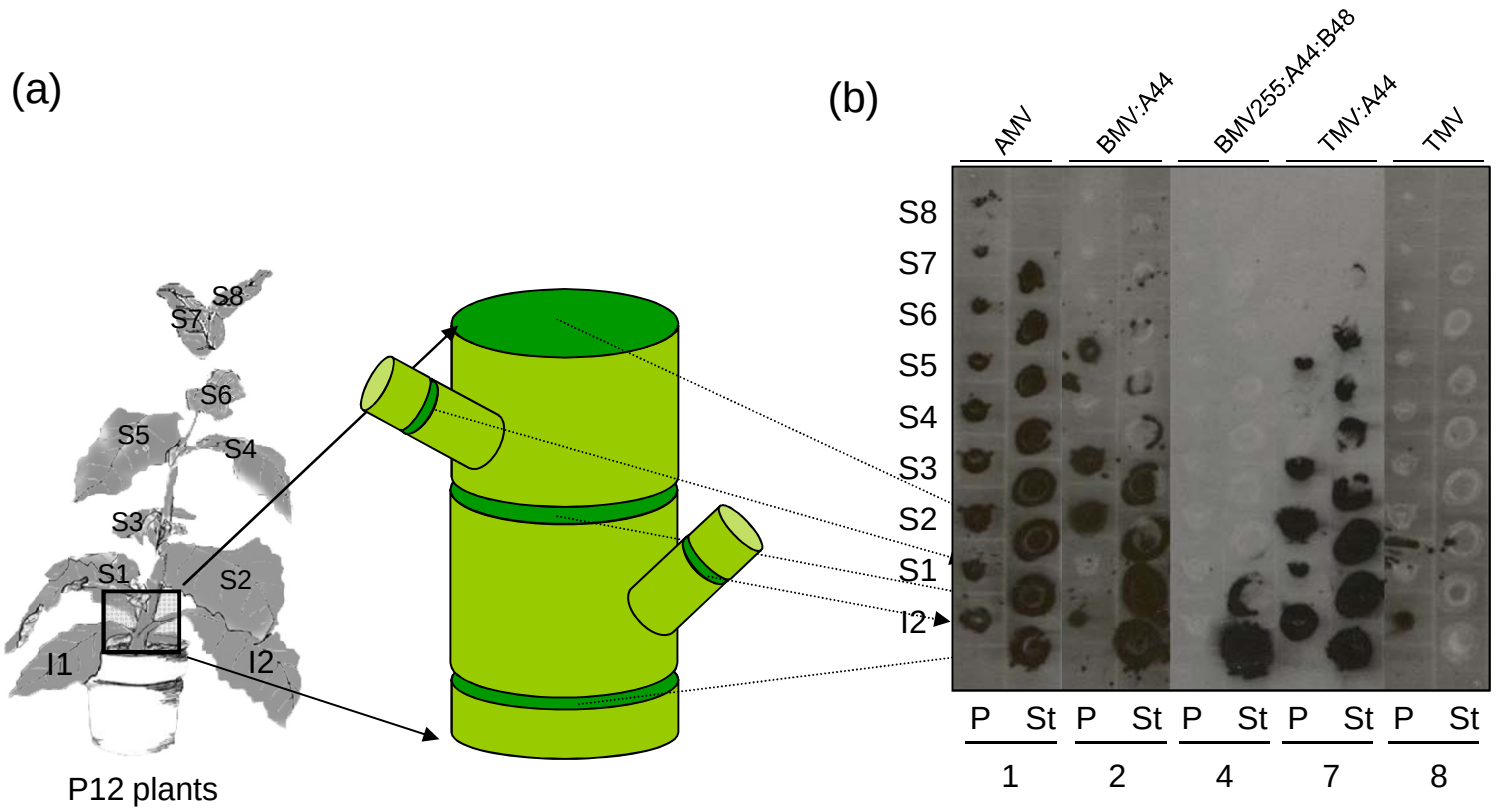
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