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Short communication

Cocksfoot mottle sobemovirus establishes infection through the phloem

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ABSTRACT

Cocksfoot mottle virus (CfMV) localization in oat plants was analyzed during three weeks post infection by immunohistochemical staining to follow its spread through different tissues. In early stages of infection, the virus was first detectable in phloem parenchyma and bundle sheath cells of inoculated leaves. Bundle sheath and phloem parenchyma were also the cell types where the virus was first detected in stems and systemic leaves of infected plants. In later stages of infection, CfMV spread also into the mesophyll surrounding vascular bundles and was seldom detected in xylem parenchyma of inoculated leaves. In systemic leaves, CfMV was not detected from xylem. Moreover, sometimes it was found from phloem only. In straw and roots, CfMV was detected both from phloem and xylem. According to our observations, CfMV predominantly moves through phloem, which makes the systemic movement of CfMV different from that of another monocot-infecting sobemovirus, *Rice yellow mottle virus* (RYMV).

Cocksfoot mottle virus (CfMV) belongs taxonomically to the genus *Sobemovirus*. Sobemoviruses are plant RNA viruses with icosahedral virions containing a positive-sense single-stranded RNA genome with covalently bound viral genome-linked protein (VPg) at the 5′ terminus. The viral RNA is approximately 4 kb in size and contains open reading frames (ORFs) for RNA silencing suppressor, polyprotein (processed to protease, VPg, P10, P8 and RNA-dependent RNA polymerase) and coat protein (CP), respectively (Truve and Fargette, 2012).

Whereas most plant viruses are transported from the initial site of infection to other parts of the plant through the phloem in parallel with the transport of photosynthesis products (Oparka et al., 1996; Haywood et al., 2002) sobemoviruses are thought to be an exception. Their particles have been found more often in xylem than in phloem. This is well documented for *Rice yellow mottle virus*, for which the virus particles were found in both xylem parenchyma and xylem vessels while only very few particles were found in the phloem (Opalka et al., 1998; Brugidou et al., 2002). It is speculated that RYMV migrates through xylem where movement is mediated passively with the rising flow of water (Opalka et al., 1998).

Similarly to RYMV, other sobemoviruses like *Blueberry shoestring virus* (BSSV; Hartmann et al., 1973; Urban et al., 1989), *Sowbane mosaic virus* (Lombardo et al., 1971) and *Snake melon asteroid mosaic virus* (Lecoq et al., 2011) have been observed rather in xylem than in phloem. However, the fact that systemic spread of these sobemoviruses has been associated with xylem does not necessarily mean that other sobemovirus use the same route. Indeed, *Southern bean mosaic virus* (SBMV) and *Southern cowpea mosaic virus* (SCPMV) particles have been found mostly in phloem (Schneider and Worley, 1959a; Weintraub and Ragetli, 1970; Morales et al., 1995). In the case of CfMV, we are aware of only one intracellular localization study reporting the possible location of CfMV in leaf mesophyll and phloem companion cells (Chamberlain and Catherall, 1976). Hereby, we describe the dynamics of the tissue distribution of CfMV in oat plants during three weeks post inoculation (p.i.) by immunohistochemical staining of viral CP.

To obtain the infection, 108 plants in two-leaf stage were mechanically inoculated by rubbing the leaves at both sides with the sap obtained by homogenization of infected oat leaves with strong CfMV (Norwegian isolate; Mäkinen et al., 1995) symptoms in 0.1 M sodium phosphate buffer (pH 7.0) mixed with 2.5% Celite (1:1). Samples of leaves were collected from 3 to 4 plants at 1–23 days p.i. with one day interval. In addition, the samples of stems and roots were collected since the beginning of the second week of infection. It is impossible to collect all the samples from one plant at all time points. Therefore, independent new plants were seeded for every time point. Samples were always taken from the youngest part of both inoculated and systemically infected leaves, i.e. from the area of the leaf blade, which is closest to the stem. Also, the mock-inoculated plant samples were collected from 1 to 2 plants at every time-point. Half of each sample was used for total RNA extraction (according to Logemann et al., 1987) to perform Northern blot analysis and the other half was fixed, dehydrated and embedded into polyester wax (Steedman's wax) according to a

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protocol adapted from Vitha et al. (2000). Thin tissue sections were prepared on a microtome. Sections were attached to glass slides, dewaxed, rehydrated, washed in PBS and incubated with antibodies. Rabbit polyclonal antibodies against CfMV coat protein (Tamm et al., 1999) was used as a primary antibody (diluted 1:100 in PBS). A goat anti-rabbit IgG (H + L) antibody (diluted 1:100 in PBS) labeled with Alexa Fluor® 488 (Molecular Probes) was used as a secondary antibody. For specificity control of primary antibodies, the sections were incubated with preserum collected before immunization as well. Prepared slides were mounted in Mowiol (Calbiochem). Slides were imaged with the fluorescence microscopes and GFP filters to detect Alexa Fluor® 488 signal (shown in green in the figures). DAPI filters were used to visualize the autofluorescence of cell walls (shown in blue). The obtained images were merged. In addition, leaf, stem and root transverse sections were stained with 0.1% toluidine blue and imaged by bright-field microscopy to visualize tissue organization in detail.

Northern blot tests verified CfMV infection after 2 days p.i. in inoculated leaves and after 5 days p.i. in the first systemically infected leaf (data not shown). The samples proven to be infected with CfMV were analyzed further by immunohistochemical staining. In mock-inoculated plants, neither viral RNA nor CP was detected (data not shown).

During the first week, samples were collected only from leaves. In inoculated leaves, the virus was first detected in phloem parenchyma and/or phloem-side bundle sheath cells (mestome) (Fig. 1a). As the leaf samples were always collected from the youngest growing part (near the stem and apart from the actual inoculation site), it is most probably the reason why we detected CfMV in vascular bundle instead of mesophyll at first. A similar picture recurred in systemic leaves (Fig. 1b) indicating a use of the phloem transport pathway in the very beginning of infection. During the first week of infection, we did not see any CP expression outside the vascular bundles.

The bundle sheath cells are considered as a boundary that a virus has to cross to get in or out of plant vasculature (Waigmann et al., 2004). In case of sobemoviruses, a delayed egress of RYMV from vascular tissues (xylem and phloem parenchyma) to the inner layer of bundle sheath (mestome) of systemic leaf was associated with partial resistance against RYMV in *Oryza sativa* cultivar Azucena (Ioannidou et al., 2000). The importance of ability to infect bundle sheath cells was also demonstrated for SCPMV in a non-permissive host *Phaseolus vulgaris*. It was detected in epidermal and mesophyll cells surrounding the vascular bundles when supported by co-infection of *Sunhemp mosaic virus* but not in bundle sheath cells and vascular tissues of inoculated leaf (Fuentes and Hamilton, 1993).

In the current study, the areas showing CfMV CP expression were expanding (Fig. 1d and e) during the progression of infection – at the end of the second week, the virus was spread all over the bundle sheath and leaf mesophyll forming large infected areas surrounding the vascular bundles. CfMV was more extensively detected in systemically infected (Fig. 1e) than in inoculated leaves (Fig. 1d). In vascular tissues of systemic leaves, it was seen only in phloem (mainly in the companion cells; Fig. 1e). In vascular tissues of inoculated leaves, viral CP was detected in phloem parenchyma and in small quantities also in xylem parenchyma (Fig. 1c and d). We suppose that the virus detected in inoculated leaf xylem parenchyma was most likely not originating from the roots but reached there through the adjacent bundle sheath cells as the infection of CfMV was always seen to start phloem-sided and it was found in xylem parenchyma not before the whole bundle sheath ring was involved. The distribution pattern of CfMV CP did not change significantly from the second week to the third week in inoculated leaves (Fig. 1f and g). Yet, the number of infected phloem companion cells increased (Fig. 1h) in systemically infected leaves. In the youngest systemically infected leaves that formed during the third week of infection, the virus was detected only in phloem (Fig. 1i) that might reflect the situation where the virus had reached to a new developing sink leaf but not yet unloaded. CP was not detected in xylem of systemically infected leaves supporting our opinion that the virus was not transported to the systemic leaves via xylem. The single earlier study analyzed CfMV localization at very late stages of infection (8 weeks after infection). The only tissue types reported to contain viral particles were leaf mesophyll and phloem companion cells (Chamberlain and Catherall, 1976). Thus, the preference for phloem pathway is presumably characteristic of the latter infection too. Also, it would be interesting to mention that this study was performed using wheat as the test plant and an English isolate as the example of CfMV. Therefore, we can say that the phloem preference of CfMV is not only characteristic of the Norwegian isolate of CfMV and oat plants.

We also analyzed the stem and root samples from the second week of infection. In stems, CP was localized in a few phloem-sided bundle sheath or companion cells (Fig. 2b). It was absent in stem xylem and was not detected outside the vascular bundles. After the straw was formed (3 weeks p.i.), the virus was detected in companion cells (Fig. 2c and d), in phloem-side mestome sheath cells (Fig. 2d) and rarely observed in xylem vessels and cortex of the straw (data not shown).

The infected root samples showed CP localization limited to vascular tissues. CP was detected both in phloem as well as in xylem (Fig. 2e and f) and was not observed in cortex. The restriction to vascular tissues in roots is also characteristic of other sobemoviruses like BSSV (Hartmann et al., 1973) and RYMV (Opalka et al., 1998).

Hence, it seems that CfMV preferably uses the phloem transport pathway as the virus was detected in xylem only after systemic infection had already been established. We do not rule out the use of xylem transport since the virus was seldom detected in xylem vessels of the straw (at the third week p.i.), but it seems marginal compared to phloem transport. French and Elder (1999) observed a variety viruses in guttate of cucumber plants systemically infected with them, and suggested that the occurrence of virus particles in guttate (originated from xylem exudate) was unrelated to virus particle size, morphology or genome organization. The use of xylem transport was suggested to be the result of successful in-time infection of xylem parenchyma or immature xylem vessel cells of root or leaf. Even some strictly phloem-limited polero- and luteoviruses (namely, RPV isolate of *Cereal yellow dwarf virus* and MAV isolate of *Barley yellow dwarf virus*) have been detected from xylem, probably due to co-infection (Gill and Chong, 1981). Most likely, it illustrates the ability of plant viruses to use xylem tissue as an alternative or a parallel transport pathway. That might be the case for SBMV and SCPMV for which xylem transport has been verified by stem steam-killing experiments (Schneider and Worley, 1959b; Gergerich and Scott, 1988) although the virus particles have been mainly found from phloem (Schneider and Worley, 1959a; Weintraub and Ragetli, 1970; Morales et al., 1995). However, the occasional xylem transport is most probably not the case for those sobemoviruses that are mainly detected from xylem during the course of infection.

One can ask why two sobemoviruses, e.g. CfMV and RYMV, which both infect monocots, target different vascular tissues? Is there any viral molecular determinant involved? For sobemoviruses, the most likely candidate for the viral movement determinant is considered to be their P1 protein. The viral RNA silencing suppressor P1 has been demonstrated to be essential for systemic movement of RYMV, SCPMV and CfMV (Bonneau et al., 1998; Sivakumaran and Hacker, 1998; Meier et al., 2006). When expressed transgenically in *Nicotiana benthamiana*, both RYMV and CfMV P1 proteins independently enhanced the spread but not the accumulation of crucifer-infecting strain of