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SHORT COMMUNICATION

Macromolecular trafficking between *Nicotiana tabacum* and the holoparasite *Cuscuta reflexa*

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Abstract

Transgenic tobacco plants expressing green fluorescent protein (GFP) under the control of the companion cell-specific promoter, *AtSUC2*, were parasitized by the holoparasite *Cuscuta reflexa* (dodder). GFP, moving in the translocation stream of the host, was transferred to the *Cuscuta* phloem via the absorbing hyphae of the parasite. An identical pattern of transfer was observed for the phloem-mobile probe, carboxyfluorescein. Following uptake by the parasite, GFP was translocated and unloaded from the *Cuscuta* phloem in meristematic sink tissues. Contrary to published data, these observations suggest the presence of a functional symplastic pathway between *Cuscuta* and its hosts, and demonstrate a considerable capacity for macromolecular exchange between plant species.

Key words: *Cuscuta*, green fluorescent protein, macromolecular transport, *Nicotiana*, plasmodesmata, symplastic transport.

Introduction

The parasitic relationship of *Cuscuta* (dodder) with its hosts has been the subject of numerous investigations (De Bock and Fer, 1992; Dörr, 1968; Haupt and Neumann, 1996; Israel *et al*., 1980; Jacob and Neumann, 1968; Jeschke and Hilpert, 1997; Rothe *et al*., 1999; Schumacher, 1934). *Cuscuta* has little photosynthetic activity and draws most of its nutrients from the host. For example, in parasitized faba bean plants *Cuscuta* is an extremely powerful competing sink for assimilates, and is capable of completely preventing fruit set and pod development (Wolswinkel, 1974).

During the infection process *Cuscuta* produces an haustorium, a highly elaborate adventitious root (Dawson *et al*., 1994) that penetrates the host tissue. From the tip of the haustorial cone, 'searching hyphae' grow extracellularly through host tissues in a manner similar to pollen tubes. On reaching individual sieve elements of the phloem the terminal hyphae cells differentiate into 'absorbing hyphae' (Dörr, 1969) which produce finger-like protrusions that surround the sieve element (Dörr, 1972). The conversion of a searching hypha into an absorbing hypha starts at the point of host sieve-tube attachment and advances towards the haustorial organ, eventually connecting with the haustorial sieve elements differentiating in the opposite direction. On the inside of the absorbing hypha cell the wall is thrown into numerous infoldings reminiscent of transfer cells while the cytoplasm develops a conspicuous smooth endoplasmic reticulum network (Dörr, 1990) resembling that found in the SE.

Although plasmodesmata occur between searching hyphae and parenchyma elements of the host cortex (Dörr, 1969), both plasmodesmata and sieve pores are thought to be absent between absorbing hyphae and sieve elements (Dörr, 1990), necessitating apoplastic transfer of solutes between the phloem systems of the host and the parasite at this interface (Tsivion, 1978; Wolswinkel, 1978). This transfer has been suggested to involve an active mechanism of solute release (Jeschke *et al*., 1994; Wolswinkel, 1974).

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Given the apparent symplastic isolation of host sieve elements and absorbing hyphae, it appears paradoxical that *Cuscuta* is an effective vector for the transfer of several viruses (Bennett, 1944; Hosford, 1967; Roos and Aldrich, 1988; Schmelzer, 1958) and phytoplasmas (Heintz, 1989; Macrone *et al*., 1999). These studies suggest that a symplastic link occurs at some point in the host–*Cuscuta* interface to allow the passage of pathogenic RNAs, or RNA–protein complexes, from the phloem of one species to another.

Recently it was shown that GFP, synthesized in source tobacco companion cells under the control of the *Arabidopsis SUC2* promoter, entered sieve elements and was translocated to, and unloaded within, sink tissues (Imlau *et al*., 1999; Oparka *et al*., 1999). In the transport phloem of the stem, in which the SE-CC complexes are virtually symplastically isolated, the GFP was restricted to the phloem (Oparka *et al*., 1999). As *Nicotiana tabacum* can be parasitized effectively with *Cuscuta* (Bennett, 1944), the authors investigated whether phloem-mobile GFP could be transferred to the phloem of *Cuscuta* at the sites of haustorial attachment. Here, an extensive transfer of GFP from host to parasite was demonstrated and it was shown that the protein is unloaded in sink tissues of *Cuscuta*. The pattern of GFP transfer was identical to the movement of the low-molecular-weight probe carboxyfluorescein, indicating a common symplastic pathway of transfer for both solutes and macromolecules. The data demonstrate a considerable capacity for macromolecular exchange between holoparasitic angiosperms and their hosts.

Materials and methods

Plant material

Plants of *Nicotiana benthamiana* and transgenic plants of *Nicotiana tabacum* expressing GFP under the control of the *Arabidopsis thaliana* sucrose transporter promoter (*At*SUC2-GFP, see Imlau *et al*., 1999) were grown from seeds in a heated glasshouse and used for experiments when the plants were between 28 and 56-d-old. *Cuscuta reflexa* was cultivated on *Vicia faba* as a host under the same greenhouse conditions with 15 h light and 9 h darkness.

For experiments, *Cuscuta* shoots of 30–35 cm length were cut from the stock culture and carefully twisted around the stems or petioles of older source leaves. Parasitized plants were examined 16 d after infection with *Cuscuta*.

Phloem transport

Phloem transport between host and parasite was imaged on intact plants using the fluorescent probe carboxyfluorescein diacetate (CFDA), exactly as described earlier (Roberts *et al*., 1997). The plants were imaged after translocation in the light for between 1 h and 1.5 h.

Sectioning

Prior to confocal imaging parasitized petioles were cut free-hand into longitudinal or transverse sections through the middle of the haustorium. The sections were then mounted immediately in silicon oil and covered with a cover slip.

Confocal laser scanning microscopy (CLSM)

To image GFP, and to follow the movement of the fluorescent probe CFDA, a Bio-Rad MRC 1000 (Bio-Rad, Hemel Hempstead, UK) confocal laser scanning microscope (CLSM) was used. Both probes were excited by the 488 nm line produced by a 25 mW argon laser.

The individual petiole sections were 'mapped' using a Nikon X2 long working distance lens, and the images subsequently reconstructed using Photoshop® software (Adobe, Mountain View, CA).

Results and discussion

In the parasitic interaction between *Cuscuta* and its hosts, elongated 'searching hyphae' of the parasite penetrate the cortex and make contact with the host phloem. These 'absorbing hyphae' are intimately associated with sieve tubes, forming finger-like wall extensions that wrap around individual sieve elements (Dörr, 1972). When transgenic tobacco plants expressing *At*SUC2-GFP were parasitized with *Cuscuta*, extensive movement of GFP was observed between the phloem of tobacco and that of *Cuscuta* at 14–16 d after attachment of the parasite (Fig. 1a, b). In addition, GFP was detected in the absorbing hyphae cells of the haustorial complex (Fig. 1b). Longitudinal sections of *Cuscuta* stem, taken above the point of attachment of the haustorium, revealed GFP to be restricted mainly to the phloem and to a limited extent to the neighbouring parenchyma tissues of the *Cuscuta* stem (Fig. 1d). Close to the apical meristem of *Cuscuta* considerable unloading of GFP was apparent, the protein moving from cell to cell throughout developing leaf primordia (Fig. 1f).

When GFP is expressed in source companion cells of tobacco or *Arabidopsis*, it enters sieve elements and is translocated to sink regions of the plant where it is unloaded symplastically (Imlau *et al*., 1999; Oparka *et al*., 1999). As the plasmodesmata in sink regions appear to be modified to allow the passage of small macromolecules (Fisher and Cash Clark, 2000; Oparka and Santa Cruz, 2000), the symplastic unloading of GFP in sink tissues of *Cuscuta* was not unexpected.

When the fluorescent probe CF was applied to source host leaves in ester form, the impermeant moiety was translocated to sink tissues, as shown previously (Knoblauch and Van Bel, 1998; Roberts *et al*., 1997; Wright and Oparka, 1996). In host plants parasitized by *Cuscuta*, the dye was unloaded extensively at the site of haustorial attachment and subsequently entered the stem phloem of *Cuscuta* (Fig. 1c, e). When the haustorial

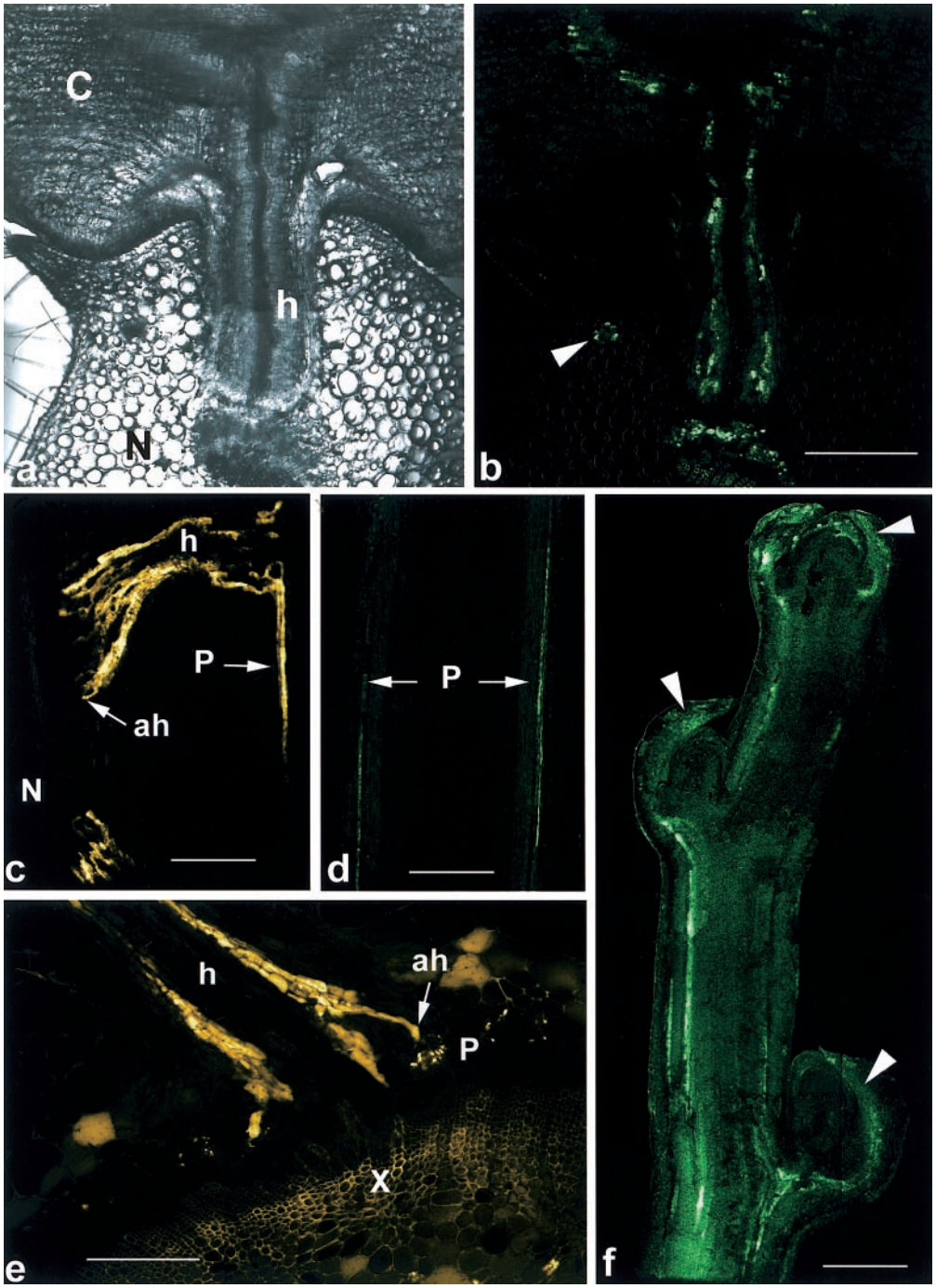

Fig. 1. (a) Transmitted light image of a cross section of a petiole from an *At*SUC2-GFP *Nicotiana tabacum* source-leaf petiole, parasitized by *Cuscuta reflexa*. The haustorium of the parasite is connected to the central vascular bundle of the host. (b) CLSM image of the same section, showing that GFP has been transferred from the phloem of the central bundle of the host into the *Cuscuta* phloem via the haustorium of the parasite. In the small (unparasitized) lateral bundle of the host (dart) GFP is restricted to the phloem. Scale = 1 mm. (c) CLSM image of a longitudinal section of stem parasitized by *Cuscuta reflexa*. CF is being transferred from the host phloem to the *Cuscuta* phloem via the absorbing hyphae at the tip of the haustorial complex. Scale = 0.5 mm. (d) CLSM image of a longitudinal section of a *Cuscuta* shoot that was parasitizing an *At*SUC2-GFP transgenic *Nicotiana tabacum*. GFP is transported exclusively within the phloem of the parasite. Scale = 0.5 mm. (e) CLSM image of a cross section of host stem parasitized by *Cuscuta reflexa*. CF, translocated within the host phloem, is transferred into the absorbing hyphae of the *Cuscuta* haustorium. The xylem shows autofluorescence. Scale = 0.5 mm. (f) CLSM image of a longitudinal section of the shoot tip of a *Cuscuta reflexa* plant that was parasitizing a transgenic *At*SUC2-GFP *Nicotiana tabacum* plant. The image shows the unloading of GFP within the developing leaf primordia of the parasite (darts). Scale = 1 mm. C, *Cuscuta reflexa*; N, *Nicotiana tabacum*; P, phloem; Pa, parenchyma cell; X, xylem; ah, absorbing hypha; h, haustorium.

complex was examined in longitudinal section, dye could be seen in the phloem of the host and also within the connecting absorbing hyphae of the parasite (Fig. 1c). A consistent feature was a lack of phloem labelling below the point of attachment of *Cuscuta*, suggesting that the translocating phloem of the host had been effectively 'drained' of solutes by the parasite (data not shown). In transverse sections, individual cell–cell contacts between the host SE-CC complexes and absorbing hyphae were apparent (Fig. 1e). In such cases dye continuity was apparent across the interface.

Because it is possible for some membrane-impermeant dyes to cross membranes (Oparka, 1991), the observations of dye transfer were considered to be equivocal evidence that a symplastic pathway was operating in the exchange of solutes between host and parasite. However, the unrestricted movement of GFP (27 kDa) from tobacco to *Cuscuta* provides strong evidence for a symplastic pathway between the absorbing hyphae of *Cuscuta* and the SE-CC complexes of the host. Ultrastructural studies of *Cuscuta* suggest that plasmodesmata are rare or absent at this interface (Dörr, 1972, 1990), making the extensive transfer of GFP observed here appear unusual. In other holoparasites, such as *Orobanche*, symplastic continuity is clearly established between the phloem of the host and parasite by direct linkage of sieve elements (Dörr and Kollmann, 1995). The observation that phloem-mobile GFP can be transferred from tobacco to *Cuscuta* suggests that a symplastic pathway is most probably utilized in the transport of macromolecules in this system. It seems likely that viral RNA is also transferred to other plants via this pathway (Bennett, 1944). While it remains possible that sucrose may be retrieved from the apoplast by absorbing hyphae (Jeschke *et al*., 1994; Wolswinkel, 1974), it seems unlikely that an apoplastic step is involved in the transport of GFP from the host SEs to the absorbing hyphae of *Cuscuta* for a number of reasons. First, the mature sieve elements of the host have a highly restricted endomembrane system and lack the vesicular machinery necessary to package and secrete GFP to the apoplast (Oparka and Turgeon, 1999). Second, GFP was not detected in the apoplast by confocal microscopy at any of the stages of the host–parasite interaction. At present, however, the possibility cannot be ruled out that the adjoining membranes of host SEs and absorbing hyphae are exceptionally permeable to a wide range of solutes and proteins at this contact point.

Although solute exchange between plant hosts and parasitic angiosperms has been studied extensively, little attention has been paid to the capacity for macromolecular exchange between plants via parasitic plant vectors. The capacity for GFP to exchange between *Nicotiana* and *Cuscuta* suggests that a wide range of proteins might exchange between plants of different species. The upper size exclusion limit for transport of macromolecules between *Nicotiana* and *Cuscuta* is currently being investigated.

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