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Lack of GFP Trafficking from Transgenic *Nicotiana benthamiana* to Parasitizing *Cuscuta pentagona*

[1,2,3]*A.E. Alakonya*, [2]*S.M. Runo*, [4]*K. Dan*, [3]*E. Monda*, [2]*J. Machuka and* [4]*N. Sinha*

[1]Jomo Kenyatta University of Agriculture and Technology,
Institute of Biotechnology Research, P.O. Box: 62000-00100 Nairobi, Kenya
[2]Department of Biochemistry, Kenyatta University and Biotechnology,
P.O. Box: 43844-00100, Nairobi, Kenya
[3]Department of Plant, Kenyatta University and Microbial Sciences,
P.O. Box: 43844-00100, Nairobi, Kenya
[4]Department of Plant Biology, University of California Davis,
One Shields Avenue, 95616, Davis, CA USA

Abstract: This work reports lack of GFP transport from transgenic *N. bethamiana* under the control of constitutive promoter CAMV 35S to *Cuscuta pentagona*. The data on *N. bethamiana* and *C. pentagona* host-parasite system suggests that macromolecular trafficking from host to parasite is host–parasite specific and such results should not be generalized outside the system studied. This data further suggests that molecular trafficking from host to parasite through either symplastic or apoplastic mechanisms is not fully understood. This is so for as especially the gating mechanisms involved. Macromolecular trafficking between host and parasite holds potential application in parasitic plant management and in basic research. It is therefore important to investigate why such contradictions exist in even very closely related host parasite systems like the genera *Nicotiana* and *Cuscuta*.

Key words: Nicotiana bethamiana · Cuscuta pentagona · Green fluorescent protein · Macromolecular trafficking · Parasitic plants

INTRODUCTION

Cuscuta spp have both direct vascular connections and cytoplasmic continuity with its hosts [1]. Haustorial searching hyphae have plasmodesmata that occur between them and the host cortical cells. Plasmodesmata and sieve elements are absent between absorbing hyphae and sieve elements hence apoplastic transfer of solutes between phloem systems of host and parasite has been proposed to involve an active mechanism of solute release [2, 3]. Water, minerals and carbohydrates, are vital for the survival of parasitic plants and earlier reports indicate varying degrees of their transfer/translocation across haustoria unidirectionally towards the parasitic plants [4]. In contrast to xylem transport, the transport of substances between host and parasite remains controversial and equally exciting. The earliest evidence of protein and nucleic acid movement across a haustorial boundary was the transmission of viral diseases between two host plants simultaneously parasitized by *Cuscuta* [5]. Movement of the nonviral green fluorescence protein (GFP) into *Cuscuta reflexa* Roxb. was observed following parasitism of *N. tabacum* plants expressing GFP [6]. These studies concluded that a symplastic connection exists between the *Cuscuta* spp and its hosts. But Christensen *et al.* [7] despite admitting that the symplastic connection between host and parasite are required also insisted that the transport of GFP was specific only for the investigated host parasite system (*N. tabaccum* and *C. reflexa.* Recently mRNA trafficking between Tomato and *C. pentagona* Engelmann through phloem and parenchyma cells was reported [8, 9] opening up an arena for further investigation geared towards understanding the mechanisms involved. In the results reported here, the transport of GFP was investigated between transgenic *N. bethamiana* and *C. pentagona,* a different host-

Corresponding Author: A.E. Alakonya, Jomo Kenyatta University of Agriculture and Technology, Institute of Biotechnology Research, P.O. Box: 62000-00100 Nairobi, Kenya. E-mail: aealakonya@gmail.com.

parasite system. This work contradicts earlier reports by showing that GFP does not traffick between transgenic *N. bethamiana and C pentagona.* The results buttress the importance of specificity of host- parasite sysem in macromolecular trafficking.

MATERIALS AND METHODS

Nicotiana Bethamiana - C. Pentagona Host Parasite System: *C. pentagona* seeds were rubbed with sand paper before dipping them in 100 % concentrated sulfuric acid for 1 hour. They were then cleaned with water and treated for 20 minutes with 100% bleach before rinsing four times for 2 minutes in distilled water. The seeds were then plated on moist filter paper placed in 11cm diameter petri dishes. After seven days, germinated dodder seedlings were placed next to 28 days old alfalfa (*M. sativa* L) seedlings in 30cm pots and left to establish for 42 days. *M. sativa* displays higher tolerance to *C. pentagonia* and therefore provides more tendrils for synchronized infection. None transgenic and transgenic tobacco (*N. benthamiana*) lines carrying the GFP open reading frame (ORF) designated GFP Y, GFP 8, GFP 16C, GFP 17B previously described in Brignetti *et al.,* [10] were obtained by Prof David Balcombe of The Sainsbury Laboratory, John Innes Centre, Norwich NR4 7UH, United Kingdom (10). Tobacco lines were raised from seeds grown directly in the soil. Bamboo sticks were erected in the *M. sativa* pots and about 10 cm of the auxiliary *C. pentagonia* strands coiled around them. The parasite developed pre-haustoria within 72 hours and was unwound from the stick and carefully coiled around stems of 4 week old transgenic and non transgenic tobacco plants then left to establish for 28 days. The plants were grown in a glasshouse set at 23°C and 50 % humidity at the University of California, Davis, USA.

Tissue Collection and Sectioning: Photographs of whole *N. bethamiana* plants parasitized by *C. pentagonia* were taken with Olympus SP-500UZ digital camera. Whole plant tissues of *N. bethamiana* plants parasitized by *C. pentagonia* were taken via the Zeis Discovery V12 dissecting microscope and Zeiss AxioCAm MRc digital camera. Longitudinal and transverse free hand excised *C. pentagonia* - *N. bethamiana* tissues sections were photographed using compound, stereo and confocal microscopy (Diagnostic Instrument, Sterling Heights, MI, USA). Prior to examination cover slips were mounted on distilled water covered sections.

Dissecting, Compound and Confocal Microscopy: To image GFP fluorescence and observe its presence in host-parasite tissues GFP filters on the dissecting and compound microscope were used. Dissecting microscope images were taken with Zeiss Discovery V12 microscope fitted with the Zeiss AxioCAm MRc digital camera. Compound microscope images were observed on a NIKON, Digital Sight DS SME Nikon Eclipse E600 (Nikon, Melville, New York, USA) and taken using the spot RT camera (Diagonistic Instruments Inc., Sterling Heights MI, USA). For confocal microscopy, a confocal laser scanning microscope (Leica, Munich Germany) was used with excitation at 488 nm. All images were processed in Adobe Photoshop CS3 (Adobe Systems Inc, New York, USA).

RESULTS

The germination protocol for *C. pentagona* used resulted in 95% germination of dodder seed (Figure 1A) hence successfully breaking the otherwise long dormancy period of *Cuscuta* seeds [1, 11]. Both transgenic tobacco plants and none transgenic were successfully infected by the parasite with the dodder tendrils forming yellow twines and haustoria at the infection points (Figure 1B and C). Upon sectioning and staining with alanine blue and examination under the microscope, haustoria (Hs) and searching hyphae were seen to have made connection with the *N. bethamiana* (Nb) vascular system (Figure, 1D). When *Cuscuta* spp attaches to the host the searching hyphae at the tip of the hastorium penetrate the host cell wall by producing cell wall degrading enzymes then move intercellularilly through the host cortical cells until they make vascular connections with the host vascular system [1]. Vaughn, [1] has also reported that the hyphae occasionally appear to grow through the host cells due to encasing by host cell walls over their entire surface. From this finding we concluded that successful infection process of transgenic and non transgenic (control) *N. bethamiana* by *Cuscuta* had occurred and went further to examine GFP expression. When transgenic tobacco is transformed with CAMV 35S-GFP they emit a green color under UV light wherever the GFP signal is expressed.

Upon mounting the hand excised transgenic plant sections, peels and examining under microscopes, GFP expression was observed in the guard cells, nucleus and cell wall (Figure 2A).

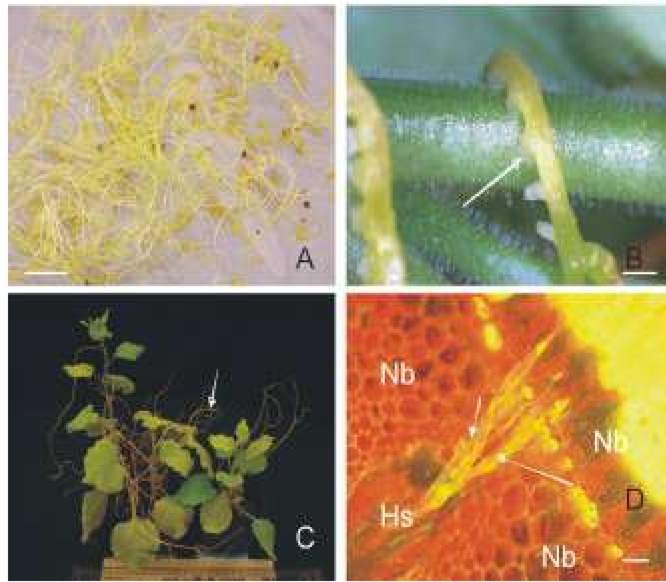

Fig. 1: Infection of *N. bethamiana* with *C. pentagona*. (A). three day old dodder germinated seedlings on a petri-dish. (B). Surface view of *C. pentagonia* stem coiled and hyphae coiled around *N.bethamiana*. (C). Dodder parasitizing 6 week old *N. bethamiana.* (D).Transmitted light image of Section of dodder haustoria and searching hyphae going through the *N.bethamiana* cortex and making vascular connections. Bars A (1cm), B (1mm), D (0.1mm).

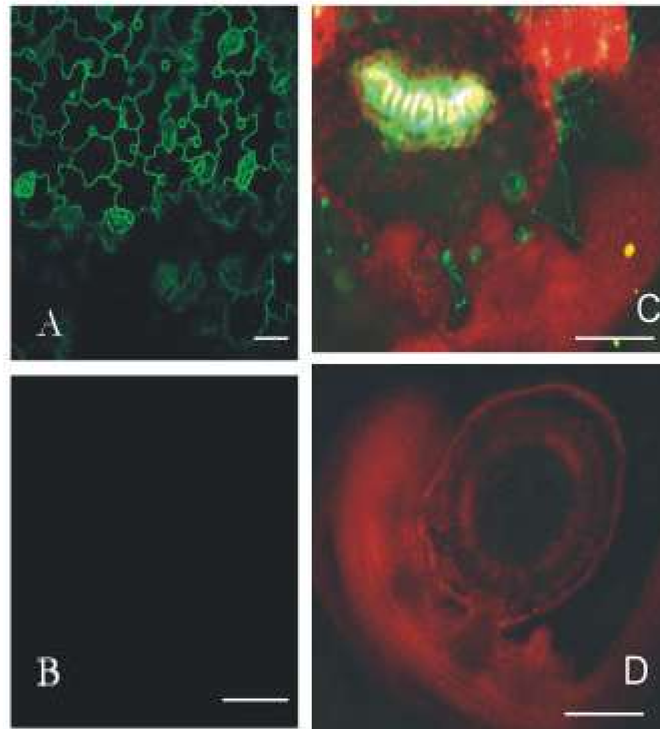

Fig. 2: Green Fluorescent protein expression in transgenic 35S-GFP and non transgenic *N. bethamiana* lines. (A) CLSM image of transgenic 35S-GFP *N. bethamiana* hand peels showing GFP expression in the nucleus and guard cells. (B) CLSM image of non transgenic *N. bethamiana* hand peels with no GFP expression (C) Transmitted light image of transgenic *N. bethamiana* section showing GFP expression in vasculature and surrounding cells (D) Transmitted light image of non transgenic section of *N. bethamiana* without GFP expression in vasculature and surrounding cells. Bars=500μm in A and B and 1mm C and D

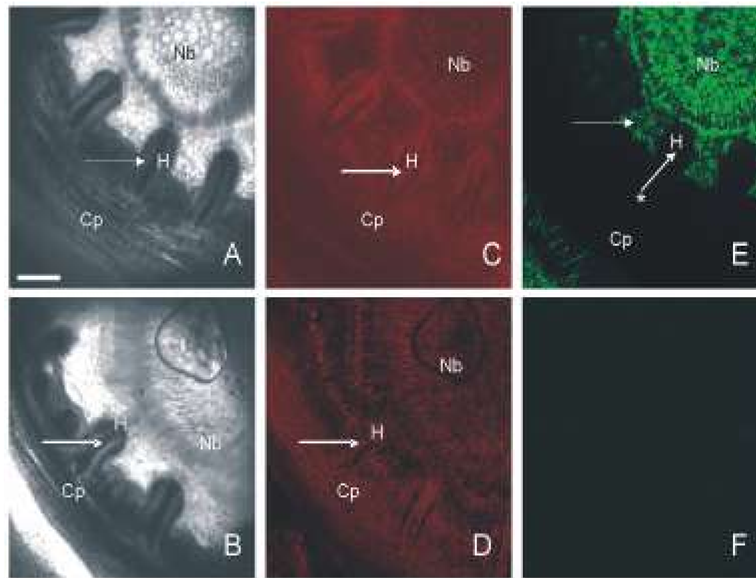

Fig. 3: GFP fluorescence in the *N. bethamiana – C. pentagona* composite tissue. Panel A) Bright field image of transgenic B) Bright field image of wildtype, C). D*ark field image of* transgenic D). D*ark field image of* wildtype E) GFP image of transgenic and F) GFP image of wild type N. *bethamiana – C. pentagona fused tissue.* H stands for *Cuscuta* haustoria, *Cp* means *C. pentagona and Nb* means *N. bethamiana* tissue. Bar=0.5cm

The vasculature had a lot of the GFP signal than any other plant cells. As expected, non transgenic plant cells or their compartments did not fluoresce green (Figure 2B). When transgenic tobacco plants expressing CAMV 35S-GFP were parasitized with *C. pentagona* and examined under the stereomicroscope and confocal laser capture microscopy, no GFP movement was observed between the phloem of tobacco and *C. pentagona* up to 28 days after attachment of parasite (Figure 2C to D). Furthermore no GFP was detected in the haustorial complex despite the signal being present in adjacent transgenic host cells. Interestingly, GFP signal was observed in the trichomes of the transgenic host (Figure 2A and D) this was probably due to the constitutive nature of the promoter used.

Except for the GFP signal visible only in transgenic lines the sections appeared similar under dark field (Figure 3A and B) and bright field (Figure 3C and D). The *Cuscuta* tissue (Cp), haustoria (H) and *N. bethamiana* tissues fluoresced in a similar manner (Figure 3A to D). Under GFP filters and during serial visualization under the CLCM the *C. pentagonia* tissue (Cp) including haustoria (H) had no signal but the *N. bethamina* (Nb) tissues gave a green GFP signal (Figure 3E). No GFP signal was visualized on both none transgenic *N. bethamiana* and *C. pentagona* parasitizing it (Figure 3F).

DISCUSSION

In this study, *C. pentagona* haustoria developed at points of contact between the host and the parasite and haustoria that penetrated the host stem or leaf petiole just as early reported [11]. Despite visualization of parasitic plant structures like the haustorium and searching hyphae (Lee and Lee, 1989) no GFP crossed from the host *N. bethamiana* and *C. pentagona*. This finding first confirms Christensen *et al.,* [7] reservations on generalizations of macromolecular studies in *Cuscuta* and its host outside the specific host–parasite system studied. It's interesting that even when *N. bethamiana* and *C. pentagona* are so related to *N. tabacum* and *C. reflexa*, the prior system has produced contradictory results from the later [6, 13, 14]. Direct phloem connections between *Cuscuta* and its hosts have actually been more difficult to demonstrate than plasmodesmatal connections. Of much interest is that direct anatomical phloem contact have never been shown, but rather the searching hyphae of *Cuscuta* that contact host sieve elements with finger-like projections that appear to grasp the host cell around the element [15]. Together, these studies and the current findings indicate mechanisms for macromolecular trafficking are not identical in different host-parasite systems.

Among the molecules reported to cross the interface between host and parasites are mRNA, genomic DNA, plasmid genes, proteins, sugars, GFP, mycoplasmas and phytoplasmas [7-9, 13, 14, 16-19]. The nature of haustorial connections are key to understanding macromolecular trafficking between hosts and parasitic plants. Dodders have clearly demonstrated to have xylem connections, but the species either differ or lack of a clearly demonstrated phloem/symplastic connection with the host [1]. This may range from transfer cells that absorb host resources from the apoplast to plasmodesmatal connections, to direct phloem–phloem continuity [1]. Dodders constitute the most studied and characterized haustorial connections, however even from this study this debate will not come to an end. It is postulated that variation in the exclusion size of the macromolecular substances could also be vital in determining what crosses the host parasite junction to enter the parasite. Such mechanisms could be structural, chemical or even molecularly controlled.

CONCLUSION AND RECOMMENDATIONS

It is clear that GFP protein can not traffick from transgenic *N. bethamiana* into *C. pentagona* and this emphasises the vast gating mechanisms regulating symplasic and apoplastic transfer betweeen plant host and parasite. The study results also call for further studies in different host parasite systems at the molecular, genetic and structural level in order to elucidate the factors that could be selectively influencing macro and micromolecular movement between different host and parasite plant systems.

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