In situ hybridization and promoter analysis reveal that cotton leaf curl Multan betasatellite localizes in the phloem

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Summary. – Begomoviruses (the family *Geminiviridae*) have either a monopartite or a bipartite (DNA A and DNA B) single-stranded DNA genome. DNA B contains the open reading frame for movement protein and enables some bipartite begomoviruses to invade non-phloem tissues, whereas monopartite begomoviruses are limited to the phloem. Betasatellite DNA replicates in association with some monopartite begomoviruses to produce severe symptoms and enhance replication of helper virus in some hosts. The cotton leaf curl Multan betasatellite (CLCuMuB) has been shown to substitute for the DNA B of a bipartite begomovirus by permitting systemic infection, but the tissue tropism and the role of betasatellites in releasing monopartite begomoviruses from the phloem to adjacent tissue has not been described. In this study, the tissue tropism of CLCuMuB and a monopartite helper virus, tomato leaf curl virus (ToLCV), has been investigated using *in situ* hybridization, promoter localization and analysis of transgenic 2β plants, which contain a dimer of the CLCuMuB genome. *In situ* hybridization showed that CLCuMuB and ToLCV were localized into the vascular tissue of infected plants. In addition, CLCuMuB was detected only in the vascular tissue of 2β transgenic plants after infection with ToLCV. β-glucuronidase (GUS) expression under the *βC1* promoter was also limited to the phloem tissues. In conclusion, we showed for the first time that CLCuMuB localizes only in the phloem tissues and unlike the DNA B component, CLCuMuB is unable to release the monopartite helper virus out of phloem tissues.

Keywords: betasatellite; geminivirus; phloem; tissue tropism

Introduction

Begomoviruses (the genus *Begomovirus,* the family *Geminiviridae*) have either monopartite or bipartite (DNA A and DNA B) genomes (Stanley *et al.*, 2005). The tissue tropism of begomoviruses is genetically determined (Morra and Petty, 2000) and different patterns of tissue tropism have been reported for bipartite begomoviruses. For example, Abutilon mosaic virus (AbMV) was detected and retained in the phloem tissues of infected plants, whereas two other bipartite begomoviruses, African cassava mosaic virus (ACMV) and tomato golden mosaic virus (TGMV), were detected in nearly all tissue types. The ability of ACMV and TGMV to move out of *Nicotiana benthamiana* phloem tissues was found to be correlated with the development of severe symptoms in comparison with mild symptoms produced by infection with the phloem-limited AbMV (Wege *et al.*, 2001). DNA B contains the open reading frame for movement protein and enables some bipartite begomoviruses to invade non-phloem tissues; whereas a number of monopartite begomoviruses are phloem-limited (Rojas *et al.*, 2001; Selth *et al.*, 2005) and similarly to most monopartite begomoviruses, DNA A of bipartite begomoviruses can invade only phloem tissue (Rojas *et al.*, 2005).

One of the factors that may support tissue tropism of a plant virus could be the promoter activity of viral genes (Yin

et al., 1997a). The activity of most viral promoters studied in transgenic plants was limited to the phloem cells; this may reflect the ability of most viruses, including geminiviruses, to multiply within these cells and, therefore, the systemic infection of the host plant and their dissemination by phloem-feeding insect vectors. For example, phloem-specific gene expression has been reported for geminiviruses such as wheat dwarf virus (Dinant *et al.*, 2004) and maize streak virus (Mazithulela *et al.*, 2000), both from the genus *Mastrevirus*, using the GUS (uidA) reporter gene in a stable system.

Tissue localization of plant viruses can be investigated using *in situ* hybridization (ISH), which is a robust technique for the detection of specific nucleic acid sequences and analysis of gene expression during development processes (McFadden, 1989). The principle of ISH is the same as for other nucleic acid hybridization methods. The major difference is that ISH involves the hybridization of nucleic acids located within fixed cells. This technique has been used successfully for detection of monopartite (Bian *et al.*, 2006) and bipartite begomoviruses (Weigel *et al.*, 2015).

Betasatellites are circular, single-stranded satellite DNA associated with certain monopartite begomoviruses. Betasatellites contain a single gene, *βC1*, which encodes a pathogenicity protein important for symptom expression (Cui *et al.*, 2004; Saunders *et al.*, 2004; Saeed *et al.*, 2005) and suppression of gene silencing (Gopal *et al.*, 2007; Eini *et al.*, 2012; Saeed *et al.*, 2015). Expression of this gene in 2β transgenic tobacco plants containing a dimer of cotton leaf curl Multan betasatellite (CLCuMuB) resulted in an abnormal phenotype (Saeed *et al.*, 2005). A more severe symptom was observed in plants infected with a complex of betasatellite and both cognate, cotton leaf curl Multan virus (CLCuMuV), and non-cognate, tomato leaf curl virus (ToLCV), helper viruses compared to the helper virus alone (Briddon *et al.*, 2001; Saeed *et al.*, 2005; Eini *et al.*, 2009a). In some host plants, betasatellite enhances the replication of helper virus (Briddon *et al.*, 2001), which can be explained by suppression of the host silencing system or supporting the release of the helper virus to non-phloem tissues.

Betasatellites functionally resemble DNA B from bipartite begomoviruses. For example, substitution of DNA B with CLCuMuB was shown to enable the systemic movement of DNA A from bipartite begomoviruses (Saeed *et al.*, 2007). The DNA B of the non-phloem-limited bean dwarf mosaic virus (BDMV) was shown to move the phloem-limited AbMV, a bipartite begomovirus, out of the phloem (Levy and Czosnek, 2003). However, a similar role for the betasatellites was not shown. In this study, we investigated tissue localization of CLCuMuB and a monopartite helper virus, tissue targeting of *βC1* promoter and the possible role of betasatellite in moving the helper viruses out of phloem tissues using *in situ* hybridization and promoter localization in infected and transgenic plants. We show for the first time

that CLCuMuB is localized in the vascular tissue of infected plants and that the *βC1* promoter is also phloem-specific. Unlike DNA B, CLCuMuB did not facilitate release of the monopartite helper virus from the phloem tissues.

Materials and Methods

Plant materials and virus clones. *N. benthamiana* plants were used in this study for tissue tropism of CLCuMuB and the helper virus. Transgenic tobacco lines, called 2β, carrying a head-to-tail dimeric construct of CLCuMuB have been described before (Saeed *et al.*, 2005). Agroinfectious constructs of CLCuMuB and ToLCV have been described previously (Dry *et al.*, 1993; Briddon *et al.*, 2000; Saeed *et al.*, 2005).

Virus inoculation and infectivity assays. To investigate tissue localization of CLCuMuB and helper viruses in infected plants, *N. benthamiana* plants at the 4–5 leaf stage were co-inoculated with an overnight culture of *Agrobacterium tumefaciens* (strain C58) containing the infectious viral clone of ToLCV together with infectious clone of CLCuMuB as described by (Kheyr-Pour *et al.*, 1991).

To test virus and betasatellite infection, developing leaves from individual plants (50–100 mg) were collected for dot-blot analysis as described (Stonor *et al.*, 2003). Then, young infected leaves were collected 3–4 weeks post-inoculation and processed for *in situ* hybridization as described below.

In another experiment, 2β transgenic tobacco plants were agroinoculated with infectious clone of ToLCV. Leaf tissues were collected from inoculated plants after four weeks of inoculation. These tissues were used for section preparation and *in situ* hybridization.

In situ hybridization. Leaf samples were collected from plants infected with ToLCV or CLCuMuB together with ToLCV and also from 2β transgenic tobacco plants infected with ToLCV. For *in situ* hybridization, the collected leaf tissues were fixed and sections, 6 to 8 μm thick, were placed on silane-coated slides (ProSciTech, Thuringowa Central, Australia). Prehybridization, hybridization, and posthybridization were performed as described (Guerin *et al.*, 2000). Probes were detected using Fast Red (Roche Diagnostics) or Western Blue (Promega Corp.) substrates. The slides were dehydrated, mounted in Entellan mounting medium, and observed under a light microscope (Carl Zeiss, Jena, Germany).

To prepare nonradioactive probes for *in situ* hybridization, ToLCV primers corresponding to the C4 (Table 1, P1 and P2) ORF were used to amplify viral DNA fragments by PCR. The *βC1* ORF from CLCuMuB was also amplified by PCR using P3/P4 primers (Table 1). The amplified DNAs were ligated into a pGEM-T Easy vector (Promega Crop, U.S.A.). The clones were used as templates for transcription using T7 or SP6 DNA-dependent RNA polymerase (Promega Corp.) in the presence of $\alpha^{32}P$-UTP according to the manufacturer's instruction. RNA probes used for *in situ* hybridization were labeled with fluorescein-12-dUTP (for the probe from the complementary-sense gene) and digoxigenin-11-dUTP (for the probe from the virion-sense gene) using fluorescein or DIG

Table 1. Oligonucleotide primers used in this study

Primers	Description	Size (nt)	Sequences (5′ to 3′)
P1	ToLCV C4-F	26	GGG<u>GAATTC</u>AGCAAGCGA CCAGCAGAT
P2	ToLCV C4-R	23	GG<u>CCATCC</u>TTAATTC TGAATCGA
P3	CLCuMB C1-F	27	CCG<u>CATATG</u>TCATACATC TGAATTCAT
P4	CLCuMB C1-R	27	CCG<u>ATGACA</u>ATCAAAT ACAACAACATG

Underlined sequences correspond to specific restriction enzyme sites.

RNA Labeling Mix, respectively (Roche Diagnostics, Castle Hill, Australia). The viral genome organization, position and orientation of the probes are shown in Fig. 1.

Plant transformation. A Binary construct harbouring the full-length *βC1* promoter fused to β-glucuronidase (GUS), called β989, was prepared (Eini *et al.*, 2009a). This construct was introduced into *A. tumefaciens* LBA4404 by electroporation with a Gene Pulser apparatus (BioRad). Agrobacterium-mediated transformation of tobacco leaf discs (*N. tabacum* cv. Samsun) was carried out essentially as described (Horsch *et al.*, 1985). Regenerated kanamycin resistant plants were grown in the glasshouse and the presence of the CLCuMuB promoter was confirmed by PCR. GUS activity and expression was tested in T1 plants.

RNA isolation and analysis. RNA was extracted from five lines of transgenic β989 and PBI121 tobacco leaves using RNA extraction reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (10 µg) was electrophoresed and blotted onto nylon membrane (BioRad,

Hercules, CA, USA). The blotted membrane was hybridized with ^{32}P-dCTP- labelled full length GUS gene as described (Sambrook and Russell, 2001).

Histochemical localization and quantification of GUS activity. Leaf tissues from five individual lines that express GUS driven by the full length *βC1* promoter were used for GUS staining as described (Jefferson *et al.*, 1987). Thin sections of the GUS-stained transgenic β989 tissues were prepared. Briefly, plant tissues were fixed, washed and then dehydrated through a graded acetone series to 100% and embedded in Spurr's resin (Spurr, 1969). Blocks were trimmed and photographed using bright field microscopy.

Quantitative GUS fluorometric assays with a Hitachi F-4010 fluorometer were conducted according to Jefferson *et al.* (1987) for leaf tissues of β989 and pBI121-GUS transgenic plants. This experiment was repeated two times, using five tobacco plants for each treatment.

Results

Plant infectivity

N. benthamiana plants were infected with CLCuMuB and ToLCV at a high rate. Accumulation of CLCuMuB and the helper virus in infected plants was confirmed by dot blot assay (Fig. 2). These plants were used for further analysis.

CLCuMuB localizes with the helper virus in phloem tissues

Tissue tropism for CLCuMuB and ToLCV was studied by *in situ* hybridization. Both CLCuMuB and the helper virus,
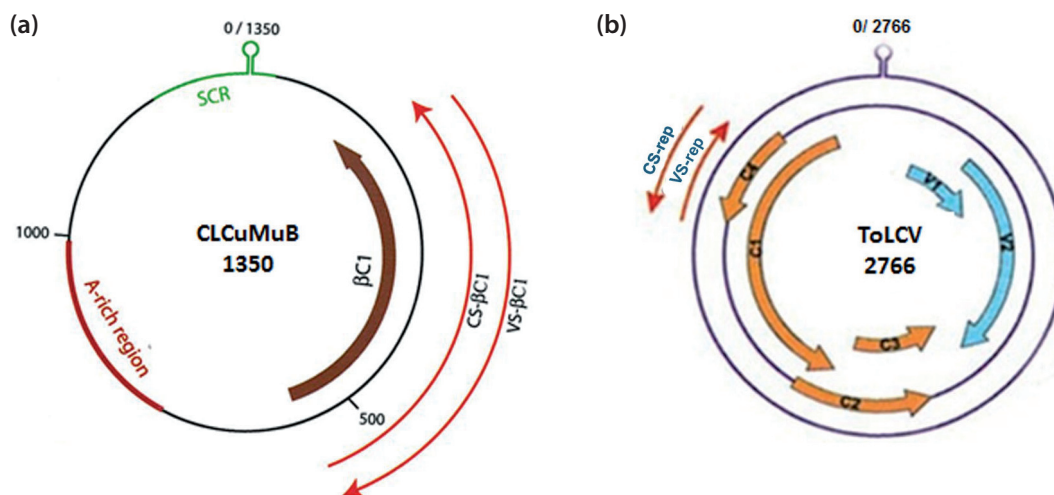
Fig. 1

Genome organization of CLCuMuB (a) and ToLCV (b) molecules that show the position and orientation of strand-specific RNA probes (thin arrows)

ORFs on the virion-sense (clockwise) strand and the complementary-sense (anticlockwise) strand are shown by arrows. The relative position of the satellite conserved region (SCR) and A-rich region are colored. VS = virion sense, CS = complementary sense.
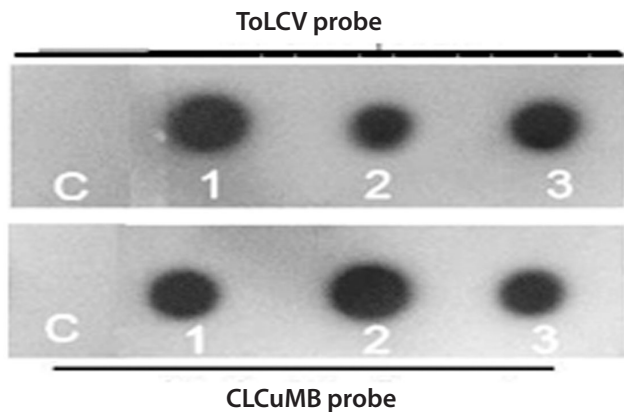
ToLCV probe

CLCuMB probe

Fig. 2

Dot blot hybridization for detection of CLCuMuB and helper virus, ToLCV, in the inoculated tobacco plants

The upper panel shows ToLCV accumulation in inoculated plants for three (1–3) individual plants. The lower panel shows CLCuMuB accumulation in three (1–3) individual plants. Healthy *N. benthamiana* plants were used as control (C).

ToLCV, were detected in the vascular tissues of systemically infected plants and signals were not detected in other cell types outside the vascular tissues (Fig. 3). Hybridization signals were not found in the sections of uninfected tissues of *N. benthamiana* plants (Fig. 3A).

To identify CLCuMuB and ToLCV signals in the same cell, fluorescein-labelled ToLCV-specific probe and DIG-labelled CLCuMuB-specific probes were used. Dual *in situ* hybridization exhibited a distinct purple chromogenic output due to the masking of red ToLCV signals by the blue CLCuMuB signal (Fig. 3D), and confirmed that CLCuMuB was detectable in almost every cell where ToLCV had accumulated.

Detection of CLCuMuB in the vascular tissue of 2β transgenic tobacco infected with ToLCV

It has been reported that CLCuMuB was rescued from a dimeric β transgenic tobacco plant (2β tobacco) upon ToLCV infection (Saeed *et al.*, 2005). CLCuMuB molecules should be present in all cell types of both vascular and non-vascular tissues of 2β transgenic tobacco. To investigate the localization pattern of ToLCV and rescued CLCuMuB molecules, *in situ* hybridization was performed on sections of 2β transgenic tobacco infected with ToLCV. Hybridization signals were found only in the vascular cells of infected 2β transgenic tobacco plants using a complementary-sense RNA probe either specific to CLCuMuB (Fig. 4B) or to ToLCV (Fig. 4C). Following hybridization with the CLCuMuB-specific probe designed to detect *βC1* specific RNA transcripts (virion-sense RNA probe from the *βC1* ORF), chromogenic signals were not detected in the section from the non-inoculated plant, suggesting that transcripts, if present, are probably below detection level (Fig. 4A).

Expression of βC1 from CLCuMuB in transgenic plants induces abnormal cell division.

Transgenic plants containing a dimer of CLCuMuB, called 2β, showed abnormal phenotypes (Saeed *et al.*, 2005). When these abnormalities in 2β transgenic plants were examined at the cellular level, large clusters of hyperplastic cells were frequently observed to be associated with the vascular tissue (Fig. 5). These abnormal cell structures were not found outside the vasculature. Such hyperplastic cells were also not observed in non-transgenic tobacco.

Tissue specificity of CLCuMuB βC1 promoter in transgenic plants

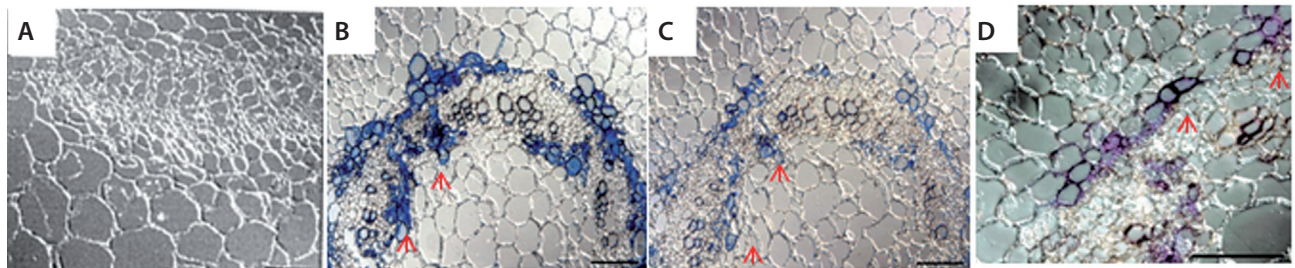The GUS expression pattern conferred by the β989 in mature leaf tissue was determined in five different transgenic

Fig. 3

***In situ* localization of CLCuMuB with the helper viruses, ToLCV, in infected plants**

Transverse sections of *N. benthamiana* uninfected plant (A), infected with ToLCV alone (B) or together with CLCuMuB (C and D). Sections were hybridized with digoxigenin (DIG)-labelled complementary-sense ssRNA from the C4 ORF of ToLCV (B) and βC1 ORF from CLCuMuB (C). Fluorescein-labelled ToLCV-specific probe and DIG-labelled CLCuMuB-specific probes were used in dual *in situ* hybridization that exhibits a distinct purple chromogenic output due to the masking of red ToLCV signals by the blue CLCuMuB signals (D). Red arrows represent the hybridization signals. Bars = 100 μm.
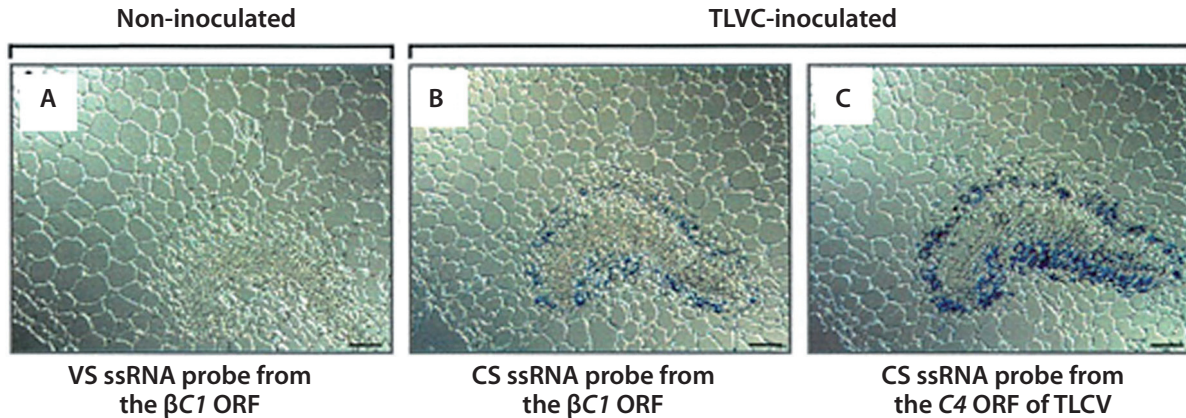
Non-inoculated

TLVC-inoculated

VS ssRNA probe from the β*C1* ORF

CS ssRNA probe from the β*C1* ORF

CS ssRNA probe from the *C4* ORF of TLCV

Fig. 4

***In situ* localization of CLCuMuB in 2β transgenic plants inoculated with ToLCV**

Transverse sections of 2β transgenic plants (A–C). Section shown in (A) was hybridized to detect *βC1* mRNA in non-inoculated plants. Sections (B) and (C) show the accumulation of ClCuMuB and ToLCV in the infected plants, respectively. Viral infections and type of probe used are indicated. VS = virion sense, CS = complementary sense. Bars = 100 μm.
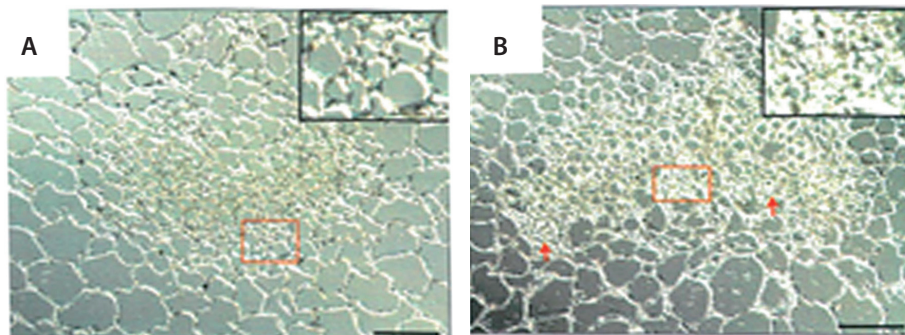

Fig. 5

Hyperplastic cells in 2β transgenic plants

Transverse sections of 2β transgenic plants showing groups of hyperplastic cells indicated by red arrows (B). A section from a non-transgenic plant is shown in the left panel (A). A higher magnitude of tissues is provided on the right corner of each panel. Bars = 100 μm.

tobacco lines. All lines showed GUS staining associated with the vascular tissue. Line β989-3 was used for detailed histochemical analysis. In leaf cross sections, GUS expression was strongest in vascular tissue, mainly in the phloem tissues (Fig. 6a). No GUS activity was detected in mesophyll and epidermal cells. These expression patterns were compared with 35S promoter from CaMV, which is active in all types of plant tissues (Fig. 6a).

βC1 promoter activity in transgenic tobacco

To analyse the promoter activity of CLCuMuB in a stable system, transgenic tobacco plants containing a full-length βC1 promoter-GUS (called β989) cassette were generated. The expression of GUS was tested by Northern blot. A significantly lower level of *GUS* expression was observed in β989

transgenic lines compared to the pBI121 plants that drive GUS expression under a strong and ubiquitous promoter, 2× 35S from CaMV (Fig. 6b).

We also used another approach to investigate the activity of βC1 promoter in β989 transgenic lines. Quantitative GUS fluorometric assays also showed a low level of GUS production in the tested plants compared to the pBI121 plants (Fig. 6c). This may indicate a weak promoter activity for βC1 promoter or activity of this promoter in a limited part of the plant tissue.

Discussion

Most characterized betasatellites are associated with monopartite begomoviruses (Zhou, 2013), except for a novel
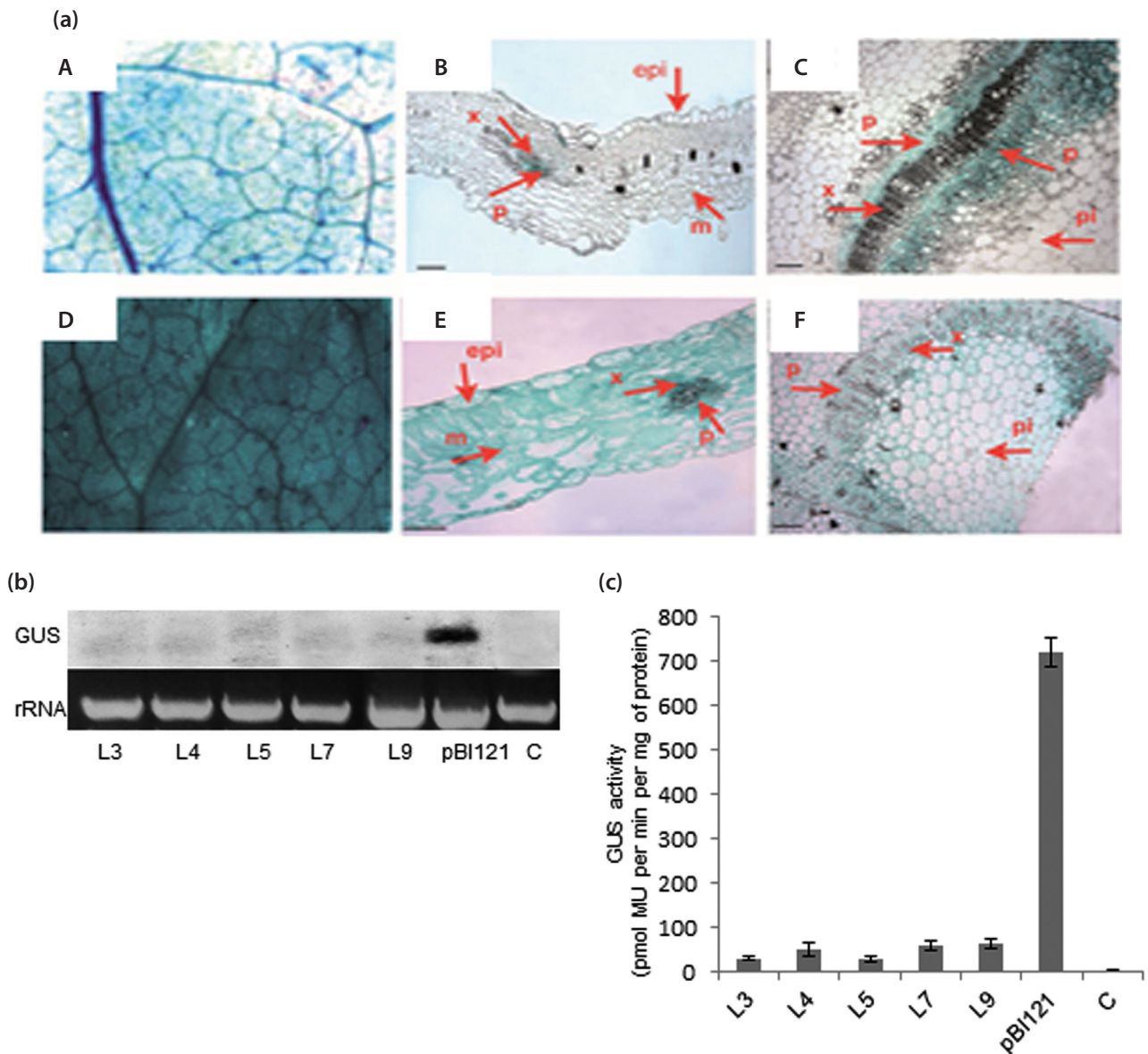
(a)

(b)

(c)

Fig. 6

Expression pattern and activity of βC1 promoter compared with CaMV 35S promoter in transgenic tobacco plants

(a) Histochemical localization of GUS in transgenic tobacco plants containing full length CLCuMuB promoter-GUS (β989) in the leaf slice (A) and transverse sections through the transgenic tobacco leaf (B) and stem (C) compared with the constitutive expression of GUS under CaMV 35S promoter in the leaf disc (D) and transverse section of leaf (E) and stem (F). p, phloem; x, xylem; epi, epiderm; pi, pith; m, mesophyll. Scale bars = 100 μm. **(b)** Northern blotting assay shows expression of GUS in five individual lines of transgenic β989 plants, pBI121 and wild-type plants. The lower panel shows the level of total RNA loaded for each sample. **(c)** Quantitative GUS fluorometric results shows the GUS activity in five individual lines of β989 and pBI121 plants. Wild type plants were used as control, C. Six replicates were used for each line; means ± SD are shown.

class of DNA satellites, which have been recently found to be associated with a bipartite begomovirus in malvaceous plants (Fiallo-Olivé *et al.*, 2012) and a betasatellite, which has been reported to be associated with wheat dwarf Indian virus, a mastrevirus (Kumar *et al.*, 2014). Betasatellites in monopartite begomoviruses are analogous to DNA B in

bipartite begomoviruses (Briddon and Stanley, 2006). The βC1 protein of CLCuMuB was shown to bind to long single stranded and double stranded DNA (Saeed *et al.*, 2015). It has been suggested that this protein mediates virus movement in plants (Cui *et al.*, 2005; Saeed *et al.*, 2007). Substitution of DNA B with a betasatellite also supported systemic infection

of DNA A in tomato leaf curl New Delhi virus, a bipartite begomovirus (Saeed *et al.*, 2007). It has been suggested that DNA B of bipartite begomoviruses can be involved in mesophyll invasion (Rojas *et al.*, 2005) and co-infection with a mesophyll-invasive bipartite begomovirus, TGMV, was shown to release bean golden mosaic virus from phloem to mesophyll tissues in infected plants (Morra and Petty, 2000). Therefore, we tested the possibility that betasatellite may affect tissue specificity of helper viruses, which was shown for DNA B components of bipartite begomoviruses. However, two lines of evidence in this study, including *in situ* hybridization and localization of ToLCV in the vascular tissue of 2β transgenic plants infected with this virus, did not support this assumption. Indeed, both cognate, CLCuMuV (data not shown), and non-cognate, ToLCV, helper viruses remained in the phloem tissue in the presence of CLCuMuB. Localization of CLCuMuB and the helper virus in phloem cells is reported for the first time in this study.

The evolutionary origin of betasatellites is not known. It has been assumed that betasatellites may have evolved from a pre-existing component by association with a monopartite begomovirus (Mansoor *et al.*, 2003). A recombination between DNA A and betasatellite of ageratum yellow vein betasatellite (AYVB) has been reported to produce a viable betasatellite recombinant (Saunders *et al.*, 2001). Analysis of the sequences of CLCuMuB also showed extensive sequence variation, most likely resulting from recombination (Akhtar *et al.*, 2014). A prerequisite for such a recombination is the replication of different viruses within the same cell. The result of this study suggests that tissue specificity may provide the opportunity for such an evolutionary process.

The βC1 gene of CLCuMuB is a suppressor of host gene silencing (Eini *et al.*, 2012). Ectopic expression of a silencing suppressor, *p23* from citrus tristeza virus, was found to modify tissue tropism of the virus from the phloem tissue to the mesophyll tissue (Fagoaga *et al.*, 2011), whereas expression of *βC1* in infected plants was unable to release the phloem-limited helper viruses to non-phloem tissues. This indicated the functional variation between p23 and βC1 from two distinct groups of plant viruses. Therefore, other genetic elements from CLCuMuB and helper viruses may determine tissue tropism in infected plants. Indeed, compatibility between two movement proteins, BL1 and BR1, was found to be required for mesophyll invasion in TGMV, a bipartite begomovirus, (Morra and Petty, 2000). This suggests that *βC1* alone may not support mesophyll invasion and needs another compatible viral protein.

The properties of a viral promoter can determine the tissue specificity of the virus (Yin *et al.*, 1997a). For example, the non-coding region upstream of the BR1 ORF of TGMV together with AL2/3, BL1 and BR1 has been shown to determine tissue tropism (i.e. mesophyll cells) of the virus (Morra and Petty, 2000). A phloem-specific promoter has been reported from geminiviruses, including wheat dwarf virus (Dinant *et al.*, 2004), maize streak virus (Mazithulela *et al.*, 2000), the C2/C3 promoter of a monopartite begomovirus ToLCV (Dry *et al.*, 2000), and also from coconut foliar decay virus (Rohde *et al.*, 1995) and rice tungro bacilliform virus (Yin *et al.*, 1997b). In histochemical GUS staining experiments, expression of GUS under the full length of CLCuMuB βC1 promoter showed phloem-specific activity in transgenic β989 tobacco lines. Similarly, phloem-specific promoter activity for tomato yellow leaf curl China betasatellite has been reported (Guan and Zhou, 2006). Therefore, phloem-limited helper viruses, ToLCV and CLCuMuV (data not shown), are not the sole determinant for limiting the CLCuMuB into the phloem cells.

A low level of *GUS* expression under the *βC1* promoter of CLCuMuB in transgenic plants is consistent with the earlier observation that accumulation of *βC1* mRNA is low in 2β transgenic tobacco (Saeed *et al.*, 2005). It needs to be noted that phloem-specific activity of *βC1* promoter limits the expression of the GUS gene in β989 plants and *βC1* in 2β plants to a small part of the leaf tissue. Therefore, comparing a *βC1* promoter with 2× 35S promoter, which is an ubiquitous promoter, may not reflect the intrinsic strength of the *βC1* promoter.

Further support for vascular localization of CLCuMuB and its tissue-specific promoter avidity come from specific cell proliferation in vascular bundles in 2β transgenic plants (Fig. 4), which also resembles cell proliferation in vascular bundles of transgenic plants expressing another *βC1* gene from Ageratum yellow vein betasatellite (AYVB) (Saunders *et al.*, 2004). Indeed, both CLCuMuB (Saeed *et al.*, 2005; Eini *et al.*, 2009b) and AYVB (Saunders *et al.*, 2004) produced a specific vein-greening and vein-swelling phenotype in infected tobacco plants that supports their tissue specificity.

Two lines of evidence, including localization of the helper virus and phloem-specific activity of CLCuMuB promoter, lead to the conclusion that CLCuMuB is localized in the phloem tissue. Unlike DNA B in bipartite begomoviruses and some viral suppressor proteins, CLCuMuB did not affect the tissue tropism of the monopartite helper begomovirus. A more comprehensive study is required to understand the association of this phloem-specific begomovirus-betasatellite with leaf curling disease complex to design control strategies for this increasing threat to global agriculture.

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