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Molecular Analysis of Geminivirus ORFs on Symptom Development

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Mutants of the monopartite geminivirus beet curly top virus (BCTV) have been screened for infectivity, systemic movement, replication and symptom development in *Arabidopsis thaliana*. As known by coding for coat protein, R1 mutant was not infectious and did not move systemically. R2, R3 and L2/L3 mutants produced milder symptoms compared to wild type BCTV but the infectivity was reduced by 40% to 60%. R2 ORF is thought to be involved in the regulation of ssDNA and dsDNA accumulation because only dsDNA was accumulated on R2⁻ infected organs. Disruption of ORF L4 resulted in reduced infections, but the viral DNA was accumulated in infected organs from roots to shoot tips as much as wild type BCTV on Sei-O. In addition, 4 mutants did not produce callus-like tissues on infected organs, suggesting that L4 ORF may play a role in the induction of host cell divisions by virus infection. This result was supported by the patterns of mRNA expression and promoter analysis of the cell cycle marker gene, *cyc1*, on Arabidopsis. *cyc1* mRNA was accumulated on symptomatic organs by wild type BCTV infections but not by L4 mutant. We conclude that the BCTV L4 ORF is essential for symptom development, specially callus-like formation on infected organs.

***Keywords* :** Arabidopsis, callus-like tissue, *cyc1*, BCTV, L4.

Members of the geminivirus group are characterized by a circular single-strand (ss) DNA genomes of 2.5 to 3 kb and a unique geminate capsid morphology (Stanley, 1985; Lazarowits, 1992). The majority of geminiviruses may be conveniently divided into three subgroups on the basis of host range, insect vector, and genome organization (Mattews, 1991). Subgroup I geminiviruses include leafhopper-transmitted, monopartite viruses which, with the exception of tobacco yellow dwarf virus (Morris et al., 1992), are restricted to monocot plant hosts. Subgroup III contains whitefly-transmitted geminiviruses which infect dicot plant hosts. Most subgroup III viruses have two genomic components (designated A and B) of about 2.7 kb each, both of which are generally required for infectivity. Exceptions include isolates of tomato yellow leaf curl and tomato leaf curl virus which either lacks a DNA B component (Navot et al., 1991; Dry et al., 1993), or if present, do not require DNA B for infectivity (Rochester et al., 1990). Subgroup II is currently composed of only a single characterized member, beet curly top virus (BCTV, Fig. 1), which has a single genomic component of 2.7 to 3.0 kb. Although BCTV is monopartite and leafhopper-transmitted, BCTV may be differentiated from subgroup I viruses upon the basis of a unique genome organization (Stanley et al., 1986) and the ability to infect a wide range of dicot plant hosts.

Three BCTV isolates have been cloned and characterized in some detail by couple of groups (Stanley et al., 1992; Hormuzdi, 1993; Stenger, 1994). The genome organization and nucleotide sequence of the BCTV Logan strain (Stenger et al., 1990; Hormuzdi, 1993) were found to differ little from those of the California isolate (Stanley et al., 1986). Both the Logan and California BCTV strains contain seven open reading frames capable of encoding pro-

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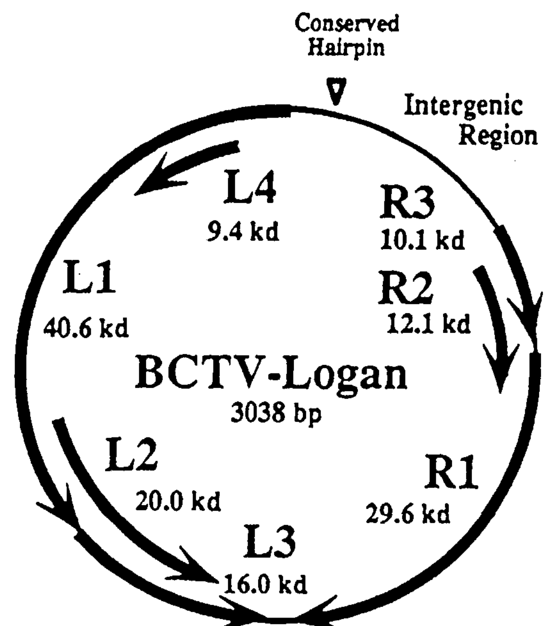

Fig. 1. Physical map of the BCTV-Logan genome. Solid arrows denote locations and polarity of conserved ORFs.

teins larger than 10 kD (Fig. 9) and are highly homologous at the nucleotide sequence level. The nucleotide sequence of the biologically distinct CFH strain of BCTV (2927 nucleotides) has also been determined and was found to have a genome organization very similar to that of the Logan and California strains (Stenger, 1994). In all three BCTV strains, there are four complementary sense ORFs (leftward ORFs L1, L2, L3 and L4), and three virion sense ORFs (rightward ORFs R1, R2 and R3).

This experimental system is based on the interactions of BCTV with *A. thaliana* that can be used for both molecular and genetic studies of plant cell divisions induced by virus infection. Since previous studies have shown that mutations in specific BCTV ORFs can cause different phenotypes depending on the plant host, it was necessary to test the phenotypes of host cell divisions by specific BCTV mutants in *A. thaliana* to determine which BCTV ORFs may be involved in host cell divisions. In this report, the infectivity, symptom development, viral DNA accumulation on *A. thaliana* ecotype Sei-O agroinoculated with wild type BCTV and 4 mutants as well as the induction of a cell division marker gene in *A. thaliana* inoculated with BCTV mutants were analyzed.

Materials and Methods

Plant growth and virus inoculation. A susceptible ecotype of *Arabidopsis thaliana,* Sei-O (The Ohio State University, USA) and a transgenic Arabidopsis, Cycl (Gent Univrsity, Belgium) which contains construct with *cycl* gene promoter fused to the *gusA* gene encoding β-glucuronidase (GUS), were grown in controlled environmental growth chambers as described (Lee et al., 1994).

The wild type BCTV-Logan strain (Stenger et al, 1990) was kindly provided by Dr. Stenger (Northern Illinois University). Mutants of the BCTV-Logan rightward ORFs R1, R2 and R3 (Hormuzdi and Bisaro, 1993) and a double mutant of BCTV-Logan leftward ORF (L2/L3) were supplied by Dr. Bisaro (The Ohio State University). BCTV L4 mutant was prepared by site-directed mutagenesis (Park and Lee, 1998). $R1^-$ mutation was constructed by cleaving pBCTV with *Csp45*I at nucleotide 1205, followed by end-filling with klenow fragment of DNA polymerase I and re-ligation. This result in a 2 bp insertion and a frameshift within the R1 ORF. Mutation $R2^-$ was created using the oligonucleotide, which generates a 2 bp deletion and introduces a frameshift mutation. Mutation $R3^-$ was constructed using the oligonuclotide, which results in the insertion of a single residue that introduces a frameshift and a *Bgl*II site into ORF R3. Mutation $L2/L3^-$ genome was constructed by cleaving pBCTV with *BspE*I at nucleotide 1767, followed by end-filling with klenow fragment of DNA polymerase I and re-ligation. This resulted in a 4 bp insertion. $L4^-$ was constructed by one base pare replacement (G to T at nucleotide 2727), followed by the creation of stop codon within a L4 ORF. Four week old *A. thaliana* plants were agroinoculated with *Agrobacterium* cultures containing wild type BCTV-Logan and five mutants. Inoculum was applied to the center of the each rosette and then the center of rosette was pricked 15~20 times with an insect pin. Infectivity in these whole plant assays was determined by counting the number of plants exhibiting characteristic BCTV disease symptoms once a week during the four week period after inoculation.

DNA isolation and blot hybridization. Plant tissues (infection origins, roots and shoot tips) were collected, frozen in liquid N_2 and stored at -80°C. Total DNA was prepared as described (Junghans and Melzlaff, 1990) and DNA concentrations were determined by measuring the A_{260}. Samples containing 20 μg of total unrestricted DNA were subjected to agarose gel electrophoresis and analyzed by DNA blot hybridization as described (Lee et al., 1994) using a ^{32}P-labeled probe prepared by random primer labeling of pCLC, a pUC8 derivative containing single tandemly repeated copies of the Logan and CFH genomes (Stenger et al., 1994).

RNA extraction and RNA blot analysis. RNA isolation and analysis was performed essentially as described by Davis and Ausubel (1989). Organs (shoot tips, infection origins and roots) were harvested from 9 individual plants of hypersusceptible ecotype, Sei-O on the third week after inoculation with BCTV-Logan and five BCTV mutants with *A. tumefaciens* GV3111 containing pTiB6S3SE. The fine powder was added to a 1.5 ml microfuge tube containing 0.55 ml of RNA extraction buffer (0.2 M Tris-HCl, pH 9, 0.4 M LiCl, 25 mM EDTA, and 1% SDS) and 0.55 ml water-saturated phenol. RNA was precipitated by adding one-third volume of diethylpyrocarbonate (DEPC)-treated 8 M LiCl and leaving on ice for 3 hr. Precipitated RNA was pelleted by centrifugation, dissolved in 0.3 ml of DEPC treated H_2O, and ethanol-precipitated in the presence of 0.3 M sodium acetate, pH 5.2. RNA was collected by centrifugation, dried under vacuum, dissolved in 50 μl of DEPC-treated H_2O, and quantitated spectrophotometrically by measuring the A_{260}.

RNA blots were prepared by a slot blotter of 10 μg RNA onto nylon membranes (Duralon UVTM, Stratagene). Hybridizations were conducted at 42°C for 16~20 hr. Membranes were then washed at 50°C for approximately 30 min with two changes of 2X SSC containing 1% SDS. Washed filters were blotted dry, wrapped in plastic wrap, and exposed to X-ray film and used for autoradiography. The *cycl* clone (pCyclAt) was obtained from Dr. Dirk Inze (Gent Univ. Belgium) for making probe.

Protein extraction and GUS kinetics. Three organs from 9 individual Cycl transgenic plants infected with BCTV wild type and five mutants were harvested and ground in liquid N_2 with a pestle and mortar and thawed into GUS extraction buffer (50 mM $NaHPO_4$, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% SDS, 0.1% Triton X-100). Insoluble material was removed by centrifugation at 13000 rpm for 10 min, at 4°C, and the supernatant was mixed with an equal volume of 100% glycerol prior to storage at -20°C. GUS activities were determined using the slightly modified protocol developed by Jefferson (1987). GUS assay buffer (2 mM 4-methyl umbelliferyl-β-D-glucuronide [MUG] in extraction buffer) was dispensed as 200 μl aliquots, to which 20 μg protein of organ extracts were added. At 10 min intervals up to 30 min the reactions were stopped by adding 800

μl 0.2 M Na_2CO_3 and the reactions were determined by Fluorometer (Hoefer DyNA Quant 200). Means were determined from three independent reactions and represented by relative kinetics of GUS activities. Protein content was determined by the method of Bradford (1976) with bovine serum albumin (BSA) as the standard.

Results

Infectivity and symptom severity of BCTV mutants. Sei-O inoculated with BCTV-Logan developed symptoms within two weeks and exhibited significantly severe bolt curling and stunting. In addition, Sei-0 developed severely deformed inflorescence structures, and consistently developed callus-like outgrowths on symptomatic tissues (Lee et al, 1994). Hyperplasia and deformation of plant structures have previously been reported in BCTV-infected sugar beet (Lackey, 1953; Hoefert and Esau, 1967; Esau and Hoefert, 1978) and *N. benthamiana* (Stanley and Latham, 1992). However, the development of callus-like structures as seen on Sei-0 have not been reported for geminivirus infection of any plant host.

Infectivity assays of the BCTV R1 mutant on Sei-0 revealed that R1 mutant was an asymptomatic (Table 1). Therefore, a functional R1 ORF is required for symptom development. The R2, R3 and L4 mutants did cause typical BCTV symptoms when inoculated on the hypersusceptible ecotype Sei-0, albeit, symptom severity and infectivity rates were 40% to 60% reduced compared to wild type BCTV-Logan (Table 1). Inoculation of the L2/L3 double mutant produced typical BCTV disease symptoms on the inflorescence stems of Sei-O, however, symptom severity was slightly reduced compared to that observed with BCTV wild type (Table 1).

Organ-specific BCTV DNA accumulation. To further characterize the ability of BCTV mutants to replicate and move systemically in hypersusceptible *A. thaliana* ecotype, Sei-O, viral DNA levels were examined in three major organ systems of inoculated plants. Total DNA was also isolated from infection origins, shoot tips and roots which were collected from plants inoculated with wild type BCTV-Logan and each of the R1, R2, R3, L4 and L2/L3 mutants, and subjected to DNA blot hybridization analysis (Fig. 2).

Replicating forms of BCTV-Logan were detected in susceptible ecotype infected with the wild-type strain. Shoot tips, infection origins and roots of plants infected with wild type BCTV-Logan contained both the ssDNA and dsDNA viral genomes as well as variable amounts of subgenomic DNAs.

Plants of Sei-O infected with the L2/L3 double mutant accumulated replicating forms of BCTV DNA in all organ systems at slightly reduced levels to plants infected with the wild type BCTV Logan. This accumulation of viral DNA in the inflorescence structures was associated with the development of disease symptoms, however, no obvious symptoms were observed in roots. Arabidopsis ecotype Sei-O infected by L4 mutant contained two types of viral DNA, dsDNA and ssDNA on three organs tested. The accumulation patterns of viral DNA in three organs were very similar to wild type BCTV in Fig. 2 even though the symptoms were much reduced.

However, mutations in the rightward virus sense ORFs, R1, R2, and R3, did exhibit differences in viral DNA accumulation patterns in Sei-O ecotype when compared to plants infected with wild type BCTV-Logan. As previously described, the R1 mutant was not infectious when inoculated onto the hypersusceptible ecotype as measured by symptom development and DNA blot analysis (data not shown). However, analysis of total DNA from organ systems collected from these asymptomatic R1 mutant infected plants showed that replicating forms of BCTV were present in roots, although the levels of virus DNA detected was much reduced compared to that observed in plants infected with the wild type strain. Viral DNA was never detected in shoot tips, which was consistent with the lack of any visible symptoms in this organ system.

Experiments in which DNA was extracted from infection origins, shoot tips and roots of R2 mutant infected Sei-O were analyzed by DNA blot hybridization revealed that the only dsDNA replicating form was present in all three organs tested. Sei-0 plants infected with the R3 mutant accumulated lower levels of dsDNA in the shoot tips and roots, while viral DNA was not detected in the infection

Table 1. Infectivity and symptom development in *A. thaliana* ecotypes inoculated with BCTV-logan mutants

Exp	WT		L4		L2/L3		R1		R2		R3	
	A[a] (%)	B[b]	A (%)	B	A (%)	B	A (%)	B	A (%)	B	A (%)	B
1	100(9/9)	5	46(7/15)	1	100(8/8)	4.0	0(9/9)	0	72(13/18)	2	44(8/18)	2
2	100(8/8)	5	58(7/12)	1	89(8/9)	4.0	0(9/9)	0	47(7/17)	2	4197/17)	2
3	100(9/9)	5	47(8/17)	1	100(9/9)	4.0	0(9/9)	0	66(6/9)	2	56(5/9)	2

[a] A: Infectivity, Percentage of plants showing symptoms. Numbers in parenthesis represent the number of plants showing symptoms/total number of plants inoculated

[b] B: Symptom severity represents the average of all independent experiments (0, no symptom; 5 severe symptoms).

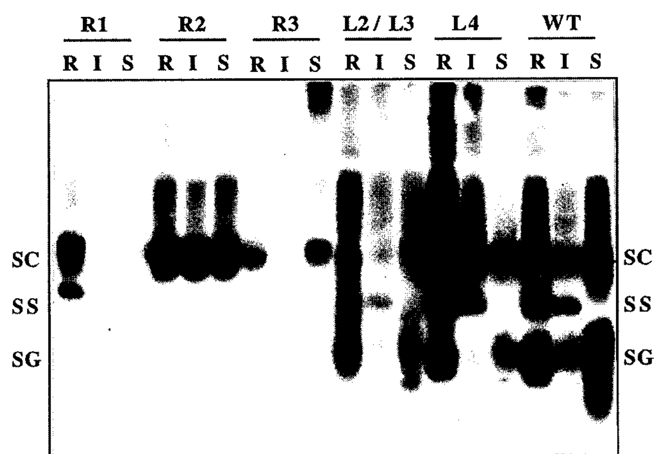

Fig. 2. Southern blot analysis of BCTV-Logan DNA forms in Arabidopsis Sei-O agroinoculated with BCTV-Logan and five BCTV mutants (R1, R2, R3, L2/L3 and L4). Equal amounts of total DNA isolated from three organs (R: roots, I: infection origins and S: shoot tips) of Sei-O infected with BCTV-Logan and five mutants on 3 weeks after inoculation were loaded in each lane (20 μg). DNA was separated on 1% agarose gel containing ethidium bromide, blotted onto nylon membrane and probed with ^{32}P-labeled, BCTV genome (pCLC). The position of supercoiled (SC), single stranded (SS) and subgenomic (SG) DNAs are indicated.

origins in Fig. 2. However, in other blots, viral DNA was often found on infection origins. The presence of viral DNA in the shoot tips was consistent with the observation that approximately 40% to 60% of the R2, R3, L4 and L2/L3 mutant inoculated plants exhibited mild curly top symptoms (Table 1).

Accumulation of cyclin transcripts during symptom development. The analysis of the morphological changes associated with symptom development in BCTV-infected *A. thaliana* plants showed a strong correlation with the induction of cell divisions (Park and Lee, 1998). To evaluate possible mechanism for this cell division activation by virus infection, studies of the expression patterns of a cell cycle gene were conducted.

Initial studies were done to examine the transcript abundance of the cell cycle gene, *cyc1,* in mock inoculated *A. thaliana* plants and plants inoculated with BCTV-Logan and five mutants. RNA blot analysis showed that the accumulation of *cyc1* transcript was positively correlated with the virus accumulation throughout symptom development. *cyc1* mRNAs was accumulated to 10 to 15 fold higher levels in infection origins and shoot tips of BCTV-infected Sei-O plants compared to the mock-inoculated control over the 4 weeks period after inoculation (Fig. 3). The largest differences between BCTV-infected and control plants were observed in the shoot tips. In the case of BCTV R1 mutant infected Sei-O, the *cyc1* gene accumulation was not changed compared to wild type virus. However, the other

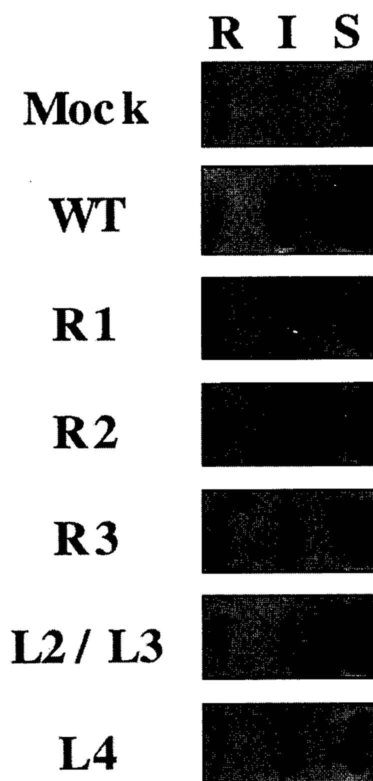

Fig. 3. Induction kinetics of *cyc1* mRNA accumulation during BCTV infection. Total RNA was isolated from three organs (R: roots, I: infection origins and S: shoot tips) harvested from Sei-O after BCTV-Logan and five BCTV-Logan mutants (R1, R2, R3, L2/L3 and L4). 10 μg of RNA were used to prepare for slot blot hybridization as described in text.

BCTV mutants which were able to develop the various symptoms on shoot tips depending on mutants showed positive correlation of *cyc1* mRNA accumulation to symptom severity. BCTV R2 and R3 mutants induced 2 to 4 fold higher levels of *cyc1* mRNA accumulation in shoot tips. There was 10 fold induction of *cyc1* gene expression on L2/L3 mutant infected shoot tips as expected by symptom severity and Southern blot data. In the case of L4 mutant infected Sei-O, viral DNAs were accumulated as much as wild type virus but this mutant virus contained *cyc1* mRNA of similar expression patterns shown on mock and R1 mutant inoculated organs (Fig. 3).

GUS activity in transgenic plant, Cyc1, inoculated with BCTV-Logan was detectable but not varied considerably in symptom development. Similar results were obtained when BCTV mutants were inoculated on transgenic plant, Cyc1. Cyc1 infected with BCTV R2, R3 and L2/L3 mutants showed similar patterns but reduced GUS activities on shoot tips compared to BCTV wild type (Fig. 4). But the GUS activities of R1 and L4 mutants were different from the activities of other mutants described above. As was the case with the *cyc1* mRNA accumlation, no increase in *cyc1*

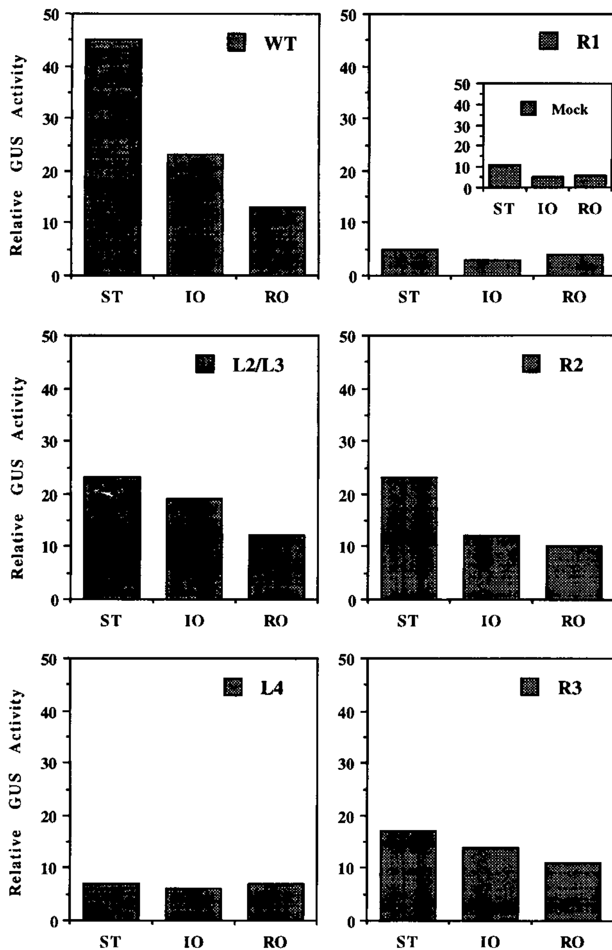

Fig. 4. GUS enzyme kinetics of Arabidopsis transgenic plant, Cyc1 which contains constructs with *cycl* gene promoter fused to *gusA* gene, during BCTV infection. Total protein was extracted from three organs (RO: roots, IO: infection origins and ST: shoot tips) harvested from Sei-O after BCTV-Logan and five BCTV-Logan mutants (R1, R2, R3, L2/L3 and L4). 20 μg of total proteins were used to prepare for the reaction mixtures for GUS enzyme assay. The inset represents the data for mock-inoculated controls plotted on a different scale.

promoter activity was observed in the shoot tips of BCTV L4 and R1 mutants.

Discussion

This paper describes the effects of disrupting the expression of several ORFs of the Logan strain of BCTV using *A. thaliana* as a new laboratory host. The results, in general, confirm the previous observations of Briddon et al. (1989), Stanley et al. (1992), Hormuzdi and Bisaro (1993) and Hormuzdi, (1993). In these previous reports, two different approaches were used for assessing the ability of specific mutants to replicate and move systemically throughout the plant and which gene could induce symptom development such as callus-like tissues.

For these experiments, a new experimental system was developed that allowed the examination of both viral DNA replication, movement and symptom development in using whole inoculated plants. This was accomplished by harvesting three major organ systems (shoot tips, infection origins and roots) from infected plants and examining viral DNA accumulation using DNA blot hybridization analysis. The presence of viral DNA at the infection origin is indicative of the ability of the virus to replicate and perhaps move short distances via cell to cell movement. Detection of viral DNA in the shoot tips and roots demonstrates that the virus is able to move systemically throughout the plant.

The previous papers (Lee and Park, 1997; Park and Lee, 1998) described the callus-like tissue formations shown on Sei-O infected with wild type BCTV are responsible for the abnormal cell divisions associated with BCTV infection. But those data did not pinpoint out how BCTV genes may induce host cell divisions directly. As shown in this paper, the molecular characterization of BCTV mutants indicated that BCTV L4 ORF might be one of the candidates for stimulating host cell divisions after virus infection. BCTV L4 mutants could replicate well on all three organs (Fig. 2) and move systemically from infection origins to both shoot tips and roots even though symptoms was much milder than wild type BCTV did. In addition, GUS kinetics and *cycl* mRNA accumulation data from L4 mutant infected Arabidopsis indicated that BCTV L4 ORF may play a role on callus-like tissue formation after virus infection. To demonstrate more accurate *in vivo* function of L4 ORF on Arabidopsis-BCTV system, we need to express L4 gene on Arabidopsis to analyze the role of this gene.

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