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Auxin Effects on Symptom Development of Beet Curly Top Virus Infected *Arabidopsis thaliana*

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Beet curly top virus is the DNA virus that is providing useful for basic studies of the infection of *Arabidopsis thaliana* with viral host and provides a system for studying both resistance and the molecular basis of symptom development. An important aspect of symptom development observed in BCTV-infected *A. thaliana* (ecotype Sei-O) was the induction of cell division on phloem and surrounding cortex cells. Analysis of the expression of GUS reporter gene activity in transgenic plants containing constructs with promoter of the auxin-inducible *saur* gene showed that *saur* promoter activity was induced concomitantly in symptomatic tissues at the inflorescence shoot tips of the transgenic lines. The auxin sensitivity tests showed that hypersusceptible ecotype, Sei-O produced more amounts of callus than susceptible ecotype, Col-O. These studies indicated that changes in auxin concentration were involved in the induction of cell division in BCTV-infected plants and clearly demonstrated that there was a strong correlation between auxin-induced gene expression and the activation of cell division.

Keywords : BCTV, *Arabidopsis,* callus, GUS, *saur,* cell division, hypersusceptible, auxin

Most plant viruses have names which include terms describing a major symptom that develop in infected plants or the name of a host from which the virus was first described (Matthews, 1991). Virus disease symptoms can be divided into two categories; local symptoms and systemic symptoms. Localized leision develop near the site of infection, and infected cells may lose chlorophyll and other pigments, giving rise to chlorotic local lesions (Fry and Tayler, 1954). In some cases, local lesions are formed when infected cells undergo hypersensitive cell death (Bol *et al.*, 1990). Systemic symptoms are often more complicated and vary greatly, depending on the combination of the particular virus and host being studied. Many virus symptoms fall into the systemic symptom class and include phenotypes such as wilting, spreading necrotic disease, ring spot diseases, yellow disease and a variety of developmental abnormalities such as stunting and leaf curling (Matthews, 1991).

Recently, studies have shown that viral genes must be involved in quite specific ways in the induction of disease. For example, two closely related strains of beet curly top virus (BCTV) produce distinct and mutually exclusive pathways of symptom development in several plant hosts (Stenger *et al.*, 1990). BCTV-Logan induces milder symptoms and has a longer latent period than BCTV-CFH even though they share 58% to 87% homology of leftward ORFs and 95% homology of rightward ORFs between them (Fig. 1, Stenger, 1994). To determine the functions of BCTV proteins, the production of recombinations between the genomes of these closely related strains and mutagenesis of specific ORFs have been used (Stenger *et al.*, 1994; Choi and Stenger, 1995). While these experiments have provided much useful information, they do not always clearly pinpoint a specific gene function since the cause of a particular phenotype may be somewhat ambiguous.

Interestingly, the symptoms caused by BCTV-Logan infection of *A. thaliana* are for the most part similar to those observed when *Nicotiana benthamiana* is the experimental host. One major exception was that in the hypersusceptible *A. thaliana* ecotype, Sei-O, more severe symptoms developed, including the novel symptom of callus-like structure

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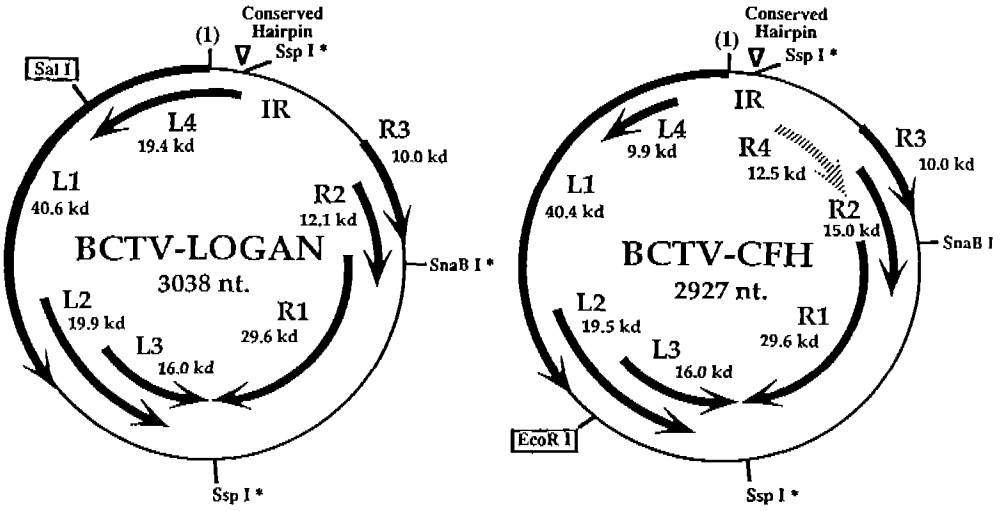

Fig. 1. Physical maps of the BCTV-Logan and BCTV-CFH genomes. Solid arrows denote locations and polarity of conserved ORFs; stripped arrow indicates a putative ORF unique to CFH. The locations of conserved endonuclease restriction sites (asterisk), unique endonuclease restriction sites used to clone unit-length genomes (boxes), the large intergenic region (IR), and the conserved hairpin (inverted triangle) are indicated. Nucleotide #1 of each strain (parenthesis) is defined as the first virion sense nucleotide upstream of the L1 ORF initiation codon (from Stenger *et al.*, 1994).

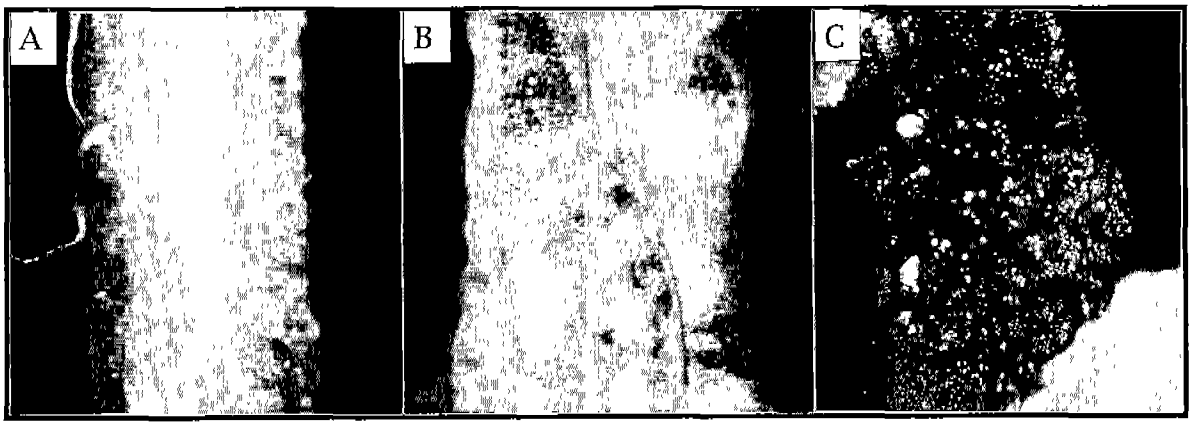

Fig. 2. Symptoms observed in mock inoculated and BCTV-Logan inoculated hypersusceptible *A. thaliana,* Sei-O. Sei-O callus was induced on inflorescence stems of Sei-O 4 to 5 weeks after inoculation. (A) mock inoculated control inflorescence stem (X 10), (B) swollen inflorescence stem (X 10), and (C) callus formation on inflorescence stem.

induction (Fig 2). The induction of these callus-like structures has never been reported for any other plant infected with a compatible geminivirus. Callus can be induced by in vitro tissue culture (Yeoman, 1970) or by infection with callus inducing pathogens (Braun and Laskaris, 1942; Klein and Link, 1952), and in both these cases, callus induction is caused by altering the plant tissues endogenous hormone levels. Thus, the induction of callus-like structures in BCTV-infected Sei-O could be due to an alteration in hormone levels which leads to new cell division. There are many possible ways in which virus infection could influence plant growth by increasing and decreasing the synthesis, translocation, or effectiveness of various hormones in different organs and at different stages of development (Smith *et al.*, 1968; Milo and Srivastava, 1969; Bailiss, 1974). Alternatively, the development of these callus-like structures could be due to the direct activation of plant cell division by the interaction of a viral protein with the regulatory machinery controlling the plant cell cycle.

In this study, we summarized the expressions of auxin-regulated genes during symptom development and amphasized the sensitivities of two different ecotypes of Arabidopsis to auxin treatments during

callus production.

MATERIALS AND METHODS

Plant materials and virus strains

A. thaliana ecotypes were obtained from Dr. F. Ausubel (Col-O) and Dr. R. Innes (Sei-O). Transgenic line (SAUR) was kindly provided by Dr. P. Green. This transgenic plant contain constructs with *saur* gene promoter fused to the *gusA* gene encoding β-glucuronidase (GUS). BCTV strains Logan and CFH were provided by Dr. D. Stenger. The conditions for plant growth and virus inoculations were the same as those described in Lee, *et al* (1994).

DNA isolation and DNA blot analysis

After virus inoculation of plants, inflorescence shoot tips, inflorescence stems, rosette leaves, infection origins and roots were harvested 4 weeks after inoculation, and stored at -81°C. Total DNA was prepared as described (Junghans *et al.*, 1990) and DNA concentrations were determined by measuring the A_{260}. Slot DNA hybridization was performed using ^{32}P-labeled probes prepared from pCLC as described in Lee, *et al.* (1994).

RNA extraction and RNA blot analysis

RNA isolation and analysis was performed essentially as described by Davis and Ausubel (1989). Organs (inflorescence shoot tips, inflorescence stems, infection origins and roots) were harvested from 9 individual plants every week after inoculation with BCTV-Logan or mock inoculation with *Agrobacterium tumefaciens* GV3110 containing pTiB6S 3SE. Total RNA was purified using a phenol sodium dodecyl sulfate extraction/LiCl precipitation procedure. RNA blot hybridizations were performed according to published procedures (Sharma and Davis, 1994). Heat denatured, gel purified inserts labeled by a random priming reactions (Rediprime kit, Life Technologies, Inc.) were used as probes. Hybridizations were conducted at 42°C for 16-20 hr. Membranes were then washed at 50°C for approximately 1 hr with two changes of 2X SSC containing 1% SDS. Washed filters were blotted dry, wrapped in plastic wrap and exposured to PhosphoImager screen (Molecular Dynamics Co.) and/or used for autoradiography. *A. thaliana* SAUR cDNA (p1013) was provided by Dr. P. Green and contains a 300 bp fragment comprising the complete SAUR-Aci coding region (Newman *et al.*, 1993).

Hormone treatments of inflorescence stem pieces

After surface sterilization with 20% commercial bleach containing 0.01% Triton X-100 for 20 min, inflorescence stem pieces were placed on agar plates containing 1X Murashige-Skoog (MS) salts (Gibco), 2% sucrose, 1X B5 vitamins supplemented with various concentrations of NAA (0, 1.0 and 2.0 μg/ml) or 2,4-D (0, 1.0 and 2.0 μg/ml) and kinetin (0, 0.05 and 0.1 μg/ml). The plates were placed in a tissue culture room operating at 21°C with 16 hr days being supplied by fluorescent light (50-100 $\mu E.m^{-2}.s^{-1}$). For the hormone sensitivity tests, the fresh weight of calli induced from inflorescence stem pieces were measured 3 weeks after hormone treatment.

To determine if BCTV-infected cells were induced producing higher levels of auxin, the ability of inflorescence stem pieces from BCTV-infected plants to form callus on media without exogenously supplied hormone (MSO) was examined. In 4 weeks after virus inoculation on Sei-O, inflorescence stems, inflorescence shoot tips, inflorescence stems with callus and swollen inflorescence stems were harvested and surface-sterilized These stem pieces were placed on callus inducing medium (CIM, MS salts containing 1X B_5 vitamin and 2% sucrose, 1 μg/ml 2,4-D and 0.05 μg/ml kinetin) and MSO medium.

Histochemical analysis of GUS activity

Histochemical assay for GUS activity were done essentially as described by Jefferson *et al.* (1987) and Ferreira *et al.*, (1994). Organs and whole plants of virus-infected transgenic plants were harvested from plants 3 to 4 weeks after inoculation. These plant materials were frozen at -80°C for 30 min and prefixed with cold 90% acetone for 1 hr at -20°C, washed twice with 100 mM sodium phosphate buffer, pH 7.4, and immersed in the enzymatic reaction mixture containing 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc, Life Technologies, Inc.) with 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide as catalysts in 100 mM sodium phosphate buffer, pH 7.4. The reaction was conducted at 37°C in the dark for a period 1 hr to overnight, depending on how quickly the color reaction developed in a given tissue.

RESULTS AND DISCUSSION

Organ specific viral DNA accumulation in susceptible and hypersusceptible ecotypes

To develop a more detailed description of the accumulation of BCTV DNA in infected susceptible plants, DNA blot hybridization studies were done using total DNA isolated from specific organ systems. The result obtained showed that BCTV DNA levels increased more quickly, and reached higher levels in every organ system tested in Sei-O compared to Col-O (Fig. 3). This was consistent with previous experiments using total DNA isolated from whole plants (Lee *et al.*, 1994) and was correlated with the more severe symptom development typically observed in Sei-O.

A careful comparison of viral DNA accumulation in the two ecotypes indicated that virus replication could be detected within one week after inoculation in the infection origins and the two poles of plants, the inflorescence shoot tip and roots. Viral DNA levels increased through 2 weeks after inoculation, particularly in the inflorescence shoot tips and roots. Sei-O and Col-O showed the same general pattern of viral DNA accumulation in each organ, although the timing and DNA levels were different. During the first week after inoculation, virus DNA levels were 2- to 4-fold higher in Sei-O tissues compared to Col-O. This difference became greater during the second week when inflorescence shoot tips and roots of Sei-O contained 8-fold more viral DNA than Col-O. Inflorescence shoot tips, inflorescence stems and infection origins of Sei-O showed similar kinetics of virus accumulation in which increased during the first two or three weeks after inoculation were followed by a decrease at the 4th week. In contrast, all organs of Col-O, except rosette leaves, showed increases in viral DNA accumulation up to 4 weeks after inoculation. Roots of the two ecotypes showed a rather distinct pattern of viral DNA accumulation compared to the other organs systems examined (Fig. 3). Viral DNA levels increased rapidly and were maintained at high levels for the entire 4 week experimental period. Analysis of viral DNA accumulation in rosette leaves of the two ecotypes revealed a major difference in the ability of BCTV-Logan to move into leaf tissues. During the first two weeks after inoculation, there was no evidence for viral DNA accumulation in mature rosette leaves in either Sei-O and Col-O. However, viral DNA accumulation in Sei-O leaves could be detected at 3 weeks after inoculation and continued to increase by week 4. In contrast, viral DNA was never detected in rosette leaves of Col-O (Fig. 3).

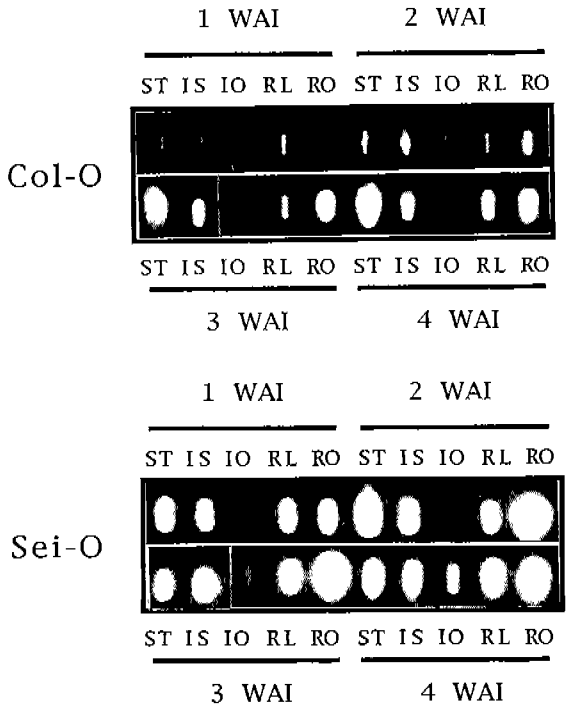

Fig. 3. Viral DNA accumulation in different organs of Col-O and Sei-O infected with BCTV-Logan. Total DNA was isolated from each organ and subjected to DNA slot blot analysis using BCTV pCLC probe. (ST; shoot tips, IS; inflorescence stem, IO; infection origin, RL; rosette leaves and RO; roots).

Spatial and temporal expression of auxin-regulated mRNAs 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 division. This abnormal activation of cell division could be due to a direct activation of plant cell division, or could be due to a more indirect effect of the virus causing phloem necrosis and the subsequent disruption of hormone transport. To evaluate possible mechanisms for this cell division activation, studies of the expression patterns of a auxin-regulated gene were conducted. Initial studies were done to examine the transcript abundance of a small auxin upregulated RNA (SAUR) in mock inoculated *A. thaliana* plants and plants inoculated with BCTV-Logan (Fig. 4). RNA blot analysis showed that the accumulation of *saur* tran-

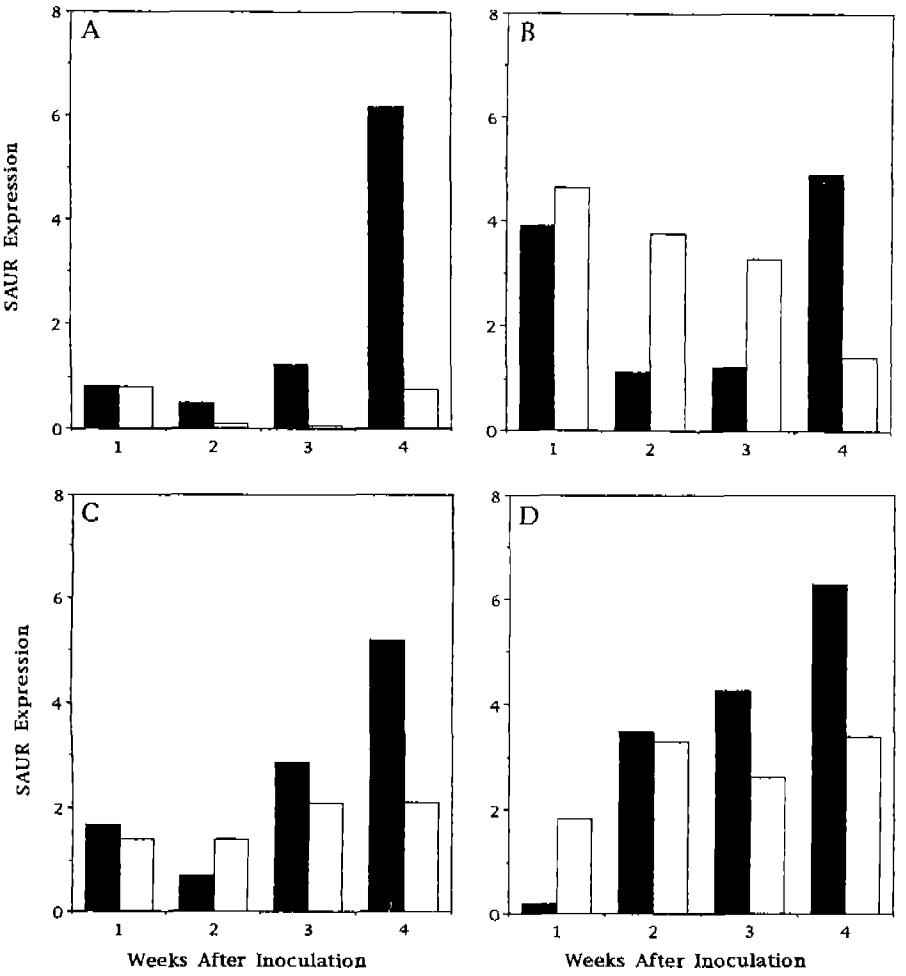

Fig. 4. Induction kinetics of *saur* mRNA accumulation during BCTV infection. Total RNA was isolated from various organs harvested from hypersusceptible ecotype Sei-O at 1, 2, 3, and 4 weeks after BCTV-Logan or mock inoculation. 10 μg of RNA were used to prepare RNA blots as described in text. The RNA blots were probed with a labeled 300 bp *saur* cDNA fragment. Black bars represent the relative amounts of *saur* mRNA in Sei-O infected with BCTV-Logan and open bars represent the relative amounts of *saur* mRNA in mock treated Sei-O.

script was positively correlated with virus accumulation throughout symptom development (Fig. 3). *saur* mRNA accumulated to 2- to 5-fold higher levels in most tissues of BCTV-infected Sei-O plants compared to the mock inoculated control over the four week period after inoculation. Most of these differences were not evident until 3 weeks after inoculation. The largest differences between BCTV-infected and control plants were observed in the inflorescence shoot tips, roots and infection origins. These were the same organ systems which were previously reported to accumulate significant levels of viral DNA (Fig. 3).

The RNA blot analysis indicated that auxin-regulated transcripts was induced in tissues known to contain viral DNA. This observation showed that there was a clear correlation among virus multiplication, virus inducible symptom development and *saur* gene expression.

Hormone sensitivity and callus induction in BCTV-infected Arabidopsis tissues

The previous study demonstrated that cell cycle and auxin-regulated gene expression were both induced in symptomatic tissues of BCTV-infected *A. thaliana* plants (Lee, *et al.*, 1996). These results indicated that the activation of cell division observed in symptomatic tissues was caused by an alteration of auxin levels. One unresolved question relating to these results was whether BCTV directly causes infected cells to produce auxin locally, or whether the

changes in auxin concentrations were due to the disruption of the phloem, resulting in changes in auxin transport throughout the plant.

To determine if BCTV-infected cells were indeed producing higher levels of auxin, the ability of inflorescence stem pieces from BCTV-infected plants to form callus on media without exogenously supplied hormones (MSO) was examined. It was anticipated that if the callus-like structures normally found on symptomatic Sei-O inflorescence stems were caused by local production of auxin by BCTV-infected cells, these callus structures would still form on media without hormones. The results obtained are summarized in Table 1. Inflorescence stem pieces from mock inoculated controls and BCTV-infected Sei-O did not develop any detectable callus when placed on MSO media alone. Explants from the control and BCTV-infected plants places on callus inducing medium (CIM) readily developed callus. One interesting difference between explants from control and BCTV-infected plants was observed. Explants from control plants placed on either MS or CIM elongated during the first few days of culture prior to callus formation. Explants from BCTV-infected plants did not elongate on either medium. These results were consistent with the hypothesis that BCTV infection altered auxin levels by disrupting long distance auxin transport rather than by causing infected cells to produce auxin. Additional experiments showing that excised calli from BCTV-infected plants were not hormone autonomous support this idea.

These observation raised the question of why Sei-O exhibited more severe symptoms compared to the other susceptible ecotypes, particularly with respect to the development of callus-like structures. This increased symptom severity in Sei-O could be due to a more severe disruption of phloem and a concomitantly larger change in hormone transport and/or an increased sensitivity to auxin. To test the latter possibility, section of inflorescence stems of uninoculated Col-O and Sei-O were cultured on MSO media supplemented with different combinations of auxin (NAA and 2,4-D) and cytokinin (kinetin). Results from a preliminary experiment indicated that inflorescence stem pieces from both Col-O and Sei-O were able to form callus when cultured on media containing any of the auxin and cytokinin concentrations tested (Table 2). On media without auxin, the stem pieces of both ecotypes failed to produce callus. Measurements of fresh weight of the explants revealed that Sei-O may be more sensitive than Col-O to NAA treatments. NAA induced more calli and root hairs on explants from Sei-O compared to Col-O, again indicating that Sei-O was more sensitive to auxin than Col-O. Sei-O treated with NAA and kinetin exhibited fresh weights 7- to 11-fold higher than explants on MSO media without hormones. Explants from Col-O exhibited 3- to 9-fold higher dry weights compared to controls on media without hormones (Table 2). The differences between Sei-O and Col-O in NAA treatment were more dramatic at the highest concentration of NAA tested (2.0 μg/ml). There was no significant difference between Sei-O and Col-O explants with respect to fresh weight or root hair induction on media containing 2,4-D. Differences between Sei-O and Col-O were also noted with respect to the timing of callus formation. Sei-O

Table 1. Hormone Effects on different parts of BCTV-Logan infected and Mock inoculated *A. thaliana*

Explants	Culture Medium	Stem Elongation	Callus Induction	Stem Swelling
Shoot tip/Mock	MSO[a]	Yes	No	No
	CIM[b]	Yes	Yes	Yes
Stem/Mock	MSO	No	No	No
	CIM	No	Yes	Yes
Shoot tip/Logan	MSO	No	No	No
	CIM	No	Yes	Yes
Swollen stem/ Logan	MSO	No	No	No
	CIM	No	Yes	Yes

[a]MSO, MS salts containing 1X B_5 vitamin and 2% sucrose.
[b]CIM, callus inducing medium, MS salts containing 1X B5 vitamin and 2% sucrose supplied with hormone (2,4-D and kinetin).

Table 2. Callus induction in Sei-O and Col-O in responses to various hormone treatments

		Kinetin (μg/ml)					
		0.00		0.05		0.01	
		Sei-O	Col-O	Sei-O	Col-O	Sei-O	Col-O
NNA (μg/ml)	0.1	100	100	76	111	88	52
	1.0	892	573	868	848	798	963
	2.0	776	303	1134	692	790	447
		0.00		0.05		0.01	
		Sei-O	Col-O	Sei-O	Col-O	Sei-O	Col-O
2,4-D (μg/ml)	0.0	100	100	107	98	75	66
	1.0	293	395	338	310	302	293
	2.0	306	240	295	283	292	310

Results are based on the ratios (average weight of stem pieces cultured on different concentrations and combinations out of average weight of control stem pieces without hormone treatment, %).

callus induction occurred 3-5 days sooner than observed with Col-O explants cultures on either NAA and 2,4-D containing media.

Another aspect of symptom production in *A. thaliana* that may related to auxin effects was the severe symptoms and callus formation observed in BCTV-infected Sei-O. These severe symptoms could be caused by the accumulation of higher levels auxin in the inflorescence shoot tips, due to the massive disruption of phloem observed in this ecotype. In addition, it was possible that Si-O was simply more sensitive to changes in auxin concentrations. An examination of auxin sensitivity of Col-O and Sei-O revealed that hypersensitivities may indeed involve increased sensitivity to auxin. Sei-O produced larger amounts of callus growth compared to Col-O when cultured on media containing NAA, and callus initiation occurred more quickly in Sei-O cultured on NAA and 2,4-D. Although further studies must be performed to confirm this result, it appeared that Sei-O was more sensitive to auxin than Col-O.

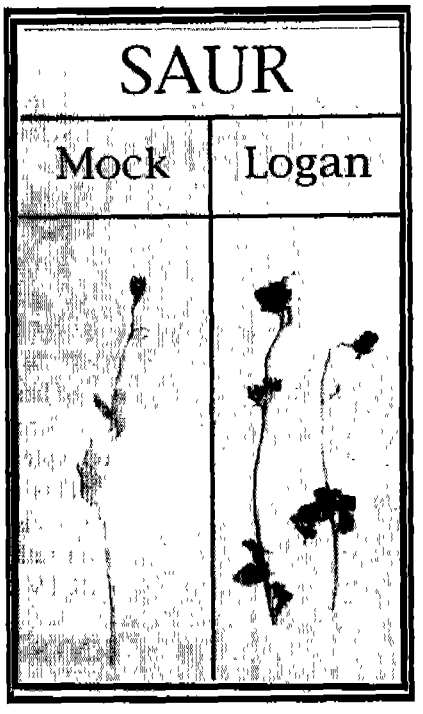

Fig. 5. Histochemical localization of SAUR promoter activity in inflorescence stems of transgenic *A. thaliana*.

Different patterns of GUS activity induced by BCTV

The RNA blot analysis indicated that auxin-regulated transcripts was induced in tissues known to contain viral DNA (Fig. 3 and Fig. 4). However, the results did not allow a precise localization of cell specific expression, and thus did not provide any information concerning whether or not the SAUR transcripts were expressed in these tissues. To better define the expression patterns of this gene, experiments was done using transgenic *A. thaliana* (SAUR) plants expressing reporter gene composed of promoter of the auxin-induced *saur* promoter fused to the *gusA* gene encoding GUS (Fig. 5). In mock inoculated control plants, *saur* promoter activity was limited to the elongating region of the inflorescence stem 4 weeks after inoculation and in developing flower buds (Fig. 5). No activity was observed in roots up to 4 weeks after inoculation (date not shown). This pattern of expression changed significantly in BCTV-infected plants. Strong GUS enzyme activity was observed in symptomatic tissues, particularly at the termini of the inflorescence branches 3 weeks after inoculation. Interestingly, by the GUS histochemical staining experiment, the region of elongation and some of the terminal flower buds were devoid of GUS activity.

An important aspect of phloem disruption observed in BCTV-infected *A. thaliana* was the induction of cell division within the phloem, and in the case of ecotype Sei-O, the induction of cell division in the phloem and surrounding cortex cells. Analysis of the expression of GUS reporter gene activity in transgenic plants containing constructs with promoters of the auxin-induced *saur* promoter showed that *saur* promoter activity was induced concomitantly with cell cycle gene promoter activities during BCTV infection described in another paper (Lee *et al.* 1996). Histochemical staining for GUS activity showed that cells in the symptomatic tissues at the inflorescence shoot tip of the CYC1 and CDC 2 transgenic lines were heavily stained blue (Lee, *et al.*, 1996). This strongly suggested that changes in auxin concentration were involved in the induction of cell division in BCTV-infected plants. The kinetics of induction of *saur* promoter activities after virus infection did not show any clear differences. Thus, the activation of these promoter activities were tightly linked in symptomatic tissues. Interestingly, levels of the SAUR transgenic line showed an increase of GUS activity after BCTV infection, whereas the levels of the CDC2 transgenic line showed a suppression of GUS activity after virus infection. Auxin was believed to be produced in shoot tips and leaf margins and then translocated through phloem cells or basipetal cell-to-cell movement. In

the virus infected plants, auxin may not move from source tissues because of the disrupted phloem (Lee, *et al.*, 1996). Therefore, high concentrations of auxin accumulated in the shoot tips and leaves. This was consistent with the GUS activities found in inflorescence shoot tips and leaves of the BCTV-infected SAUR plants. RNA blot analysis of *saur* transcript accumulation was for the most part consistent with the expression patterns observed in transgenic plants expressing the *saur* reporter genes. Accumulation of both transcripts induced by BCTV infection were also similar with respect to both the timing and magnitude of induction. These studies taken together clearly demonstrated a strong correlation between auxin-induced gene expression and the activation of cell cycle genes. This suggested that the activation of cell division was caused by increase in the local auxin concentration rather than a direct activation of the cell cycle by BCTV.

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