INTRODUCTION

Hibiscus chlorotic ringspot virus (HCRSV), a member of the genus Carmovirus, has a (+)-strand RNA genome of 3911 nt containing seven open reading frames (ORFs); the coat protein (CP) is encoded by the 1.47 kb subgenomic RNA (Huang et al., 2000; Li & Wong, 2006). The atomic structure of HCRSV (http://viperdb.scripps.edu/info_page.php?VDB=hcrv) shows that the CP subunit has three structural domains: a partly disordered RNA-binding (R) domain (aa 1–99), a shell-forming (S) domain (aa 100–258) and a protruding (P) domain (aa 259–345). The S and P domains are connected by a flexible hinge region (four residues in aa 242–251). The R domain is believed to interact with RNA inside the virus particle. Mutation of eight site-specific amino acids in the HCRSV CP rendered the virus avirulent in kenaf (Hibiscus cannabinus L.) plants, but the mutant virus was able to accumulate in kenaf protoplasts (Liang et al., 2002a), indicating that the complete HCRSV CP is involved in pathogenicity. Recently, the HCRSV CP has been shown to be a gene-silencing suppressor (Meng et al., 2006). These observations imply that HCRSV CP may interact with some unidentified host factor(s) during the virus–host interaction to complete these functions. Thus, a kenaf cDNA library was constructed and screened for interacting proteins by the yeast two-hybrid system (YTHS). Sulfite oxidase (SO) from kenaf (HcSO) was one of the proteins identified, and the interaction was confirmed in yeast and kenaf protoplasts. Transcripts and activity of HcSO were upregulated in HCRSV-infected kenaf plants.

METHODS

Plant materials and construction of plasmids. Kenaf seedlings were grown under 16 h light and 8 h dark at 25 °C. The HCRSV CP gene was amplified by PCR using appropriate primers (see Supplementary Table S1, available in JGV Online), digested with EcoRI/BamHI and ligated into the EcoRI- and BamHI-digested pGBK7 vector to give pGBK7-CP. Three gene fragments corresponding to the R, S and P domains of HCRSV CP were amplified by PCR and inserted into EcoRI/BamHI-digested pGBK7 to give pGBK7-CP-R, -CP-S and -CP-P, respectively. Similarly, the complete ORF of HcSO was amplified and cloned into the pGADT7 vector.

The plasmids used in the bimolecular fluorescence complementation (BiFC) assay, pSAT1-nEYFP-C1, pSAT1-cEYFP-C1(B) and pSAT6-EYFP-C1, were purchased from the Biological Resource Center, Ohio State University, USA. The full-length coding sequences of HCRSV CP and HcSO were PCR-amplified, digested with EcoRI/ BamHI and ligated into the pSAT1-NEYFP-C1, pSAT1-cEYFP-C1(B) and pSAT6-EYFP-C1 vectors to generate the recombinant plasmids.
pSAT1-nEYFP-C1-CP, pSAT1-cEYFP-C1-(B)-HcSO and pSAT6-EYFP-C1-HcSO, respectively. The sequences of all primers used are listed in Supplementary Table S1.

Construction and screening of the kenaf cDNA library. The kenaf cDNA library was constructed by using BD Matchmaker Library Construction and Screening kits (Clontech) according to the manufacturer’s protocols. Total RNAs were extracted from kenaf leaves by using TRizol reagent (Invitrogen). Kenaf mRNA was extracted by using an mRNA isolation kit (Invitrogen) and used for cDNA library construction. The kenaf cDNA library was screened with pGBK7-CP to identify putative interacting proteins. Plasmids containing the cDNAs were selected from the cells of blue colonies, which indicated positive protein–protein interactions. The cDNA sequences were obtained following PCR and DNA sequencing and compared with sequences in GenBank by using the BLASTX program.

Interaction of HCRSV CP and HcSO and identification of HCRSV CP domain(s) important for the interaction. The Matchmaker Yeast Two-Hybrid system was purchased from Clontech. pGADT7-HcSO and constructs derived from pGBK7 were co-transformed separately into yeast strain AH109 competent cells, and the cells were grown for 3 days at 30 °C on minimal synthetic dropout (SD) solid medium lacking leucine, tryptophan, adenine and histidine (SD/−Leu/−Trp/−Ade/−His). pGBK7T-53+pGADT7-RecT was set as a positive control. PGBK7-Lam+pGADT7-RecT, pGADT7+pGBK7T-CP and pGADT7-HcSO+pGBK7T were set as negative controls. Paired plasmids were co-transformed into AH109 competent cells and plated onto SD/−Leu/−Trp/−Ade/−His solid medium. Yeast colonies were streaked onto the same solid medium supplemented with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and incubated at 30 °C for 24 h to monitor β-galactosidase expression.

Colocalization of HCRSV CP and HcSO using BiFC. Paired plasmids pSAT1-nEYFP-C1-CP+pSAT1-cEYFP-C1-(B)-HcSO, pSAT1-nEYFP-C1-CP+pSAT1-cEYFP-C1-(B) and pSAT1-nEYFP-C1-CP+pSAT1-cEYFP-C1-(B)-HcSO were transfected into kenaf protoplasts separately to study the interaction of HcSO and HCRSV CP in vivo. The sequences of all primers used are listed in Supplementary Table S1. The kenaf cDNA library was also transfected into kenaf protoplasts to monitor the subcellular localization of HcSO. The kenaf protoplasts were transfected with the constructs listed above by the polyethylene glycol method (Liang et al., 1998). The protoplasts were observed under a Carl Zeiss LSM510 META confocal imaging system. Yellow fluorescence was excited at 514 nm and detected by using a BP 530–600 filter, 22 h after transfection. Autofluorescence of the chlorophylls was also excited at 514 nm, but detected by using an LP 615 filter. Differential interference contrast (DIC) images of protoplasts were taken together under the same excitation wavelength.

For immunofluorescent labelling of peroxisomes, kenaf protoplasts were fixed in 4.5 % (w/v) paraformaldehyde and 0.3 % glutaraldehyde in wash buffer containing 600 mM mannitol in 2.3 % PBS (pH 7.0). Cells were fixed for 2 h and then washed three times for 10 min each in wash buffer; the first wash contained 0.2 % (w/v) Triton X-100. Fixed protoplasts were incubated with anti-SKL antibody (Usuda et al., 1999), diluted 1:400 in wash buffer containing 3 % BSA, overnight at 4 °C. Cells were washed three times for 10 min each and then incubated for 2 h with Alexa Fluor 488 goat anti-rabbit IgG (H+L) or goat anti-rabbit IgG (H+L), highly cross-adsorbed, DyLight 405 (Thermo Scientific), diluted 1:200 in wash buffer containing 3 % BSA. Cells were washed three times in wash buffer for 10 min each and then observed by confocal microscopy (Swanson et al., 1998).

Protein expression and pull-down assay of CP with HcSO. The clone of pGEX-4T-CP was transformed into Escherichia coli strain BL21. Glutathione S-transferase (GST) and GST fusion proteins were purified following standard protocols (GE Healthcare). The eluted GST–CP protein was mixed with 40 μl glutathione–Sepharose 4B beads in 1× PBS to a final volume of 500 μl. The mixture was incubated at 4 °C with gentle agitation for 1 h. HcSO was in vitro-translated from pGADT7-Rec-HcSO, which contained the HcSO gene isolated from the kenaf cDNA library, using the Tnt Quick Coupled Transcription/Translation system (Promega). The in vitro-translated product of HcSO labelled with biotinylated lysine was mixed with GST–CP. The mixture of the HcSO and GST or GST–CP and the in vitro-translated product from the pGADT7 vector was included as negative control. All mixtures with glutathione–Sepharose 4B beads were incubated at 4 °C with gentle agitation for 1 h, followed by washing four times with 500 μl 1× PBS. The bound proteins were eluted by using 100 μl elution buffer twice, and separated on an SDS-PAGE 12 % (w/v) gel. Western blot analysis was carried out and HcSO was detected and visualized on Kodak X-ray film by the Transcell Chemiluminescent Non-Radioactive translation-detection system (Promega).

Real-time PCR and sequence analysis. Upper leaves were sampled 3 weeks post-inoculation with HCRSV, when systemic infection and symptoms became apparent. For each reaction, kenaf total RNA was reverse-transcribed with an iScript cDNA synthesis kit (Invitrogen), using modified Moloney murine leukemia virus-derived reverse transcriptase (Bio-Rad) and a blend of oligo(dt) and random hexamer primers according to the manufacturer’s instructions. Quantitative RT-PCRs contained 1:10 (v/v) first-strand cDNA as template, specific primers (Supplementary Table S1) and iQ SYBR Green super mix (ABI) in a final volume of 10 μl. Amplification was performed for 40 cycles consisting of initial preheating at 95 °C for 3 min, 20 s at 95 °C, 20 s at 65 °C and 30 s at 72 °C. Fluorescence increments of each reaction were monitored simultaneously by using a Spectrum 48 real-time cycler (ESCO). To normalize the RT-PCR, one endogenous reference gene (18S rRNA) was used in each experiment. Results were expressed as a threshold cycle (Ct) value. Each RNA sample was assayed in triplicate and their Ct and SD values were averaged. These averaged Ct values and SD for the triplicates were used in all subsequent calculations. Gene expression was normalized to that of 18S rRNA by subtracting the Ct value of the 18S rRNA from that of the HcSO mRNA to give ΔCt. The ΔCt values of the HCRSV-infected samples were then subtracted from the ΔCt of the mock-inoculated sample to give a ΔΔCt value. The error estimated for the mean ΔΔCt value is propagated into each of the ΔCt values for the test samples. The relative gene transcript level was calculated by the 2−ΔΔCt method as described by Livak & Schmittgen (2001). In the final calculation, the error is estimated by evaluating the 2−ΔΔCt term using ΔΔCt ± SD and ΔΔCt ± SD. Lack of variation in PCR products and the absence of primer dimers were ascertained from the melting-curve profile of the PCR products. In all experiments, three biological replicates of each sample type were tested. Relative quantification of HcSO against an internal standard (18S rRNA) was compared between mock-inoculated and HCRSV-infected kenaf plants. The experiment was carried out twice. Sequence analysis was performed by using an ABI Prism BigDye Terminator cycle sequencing ready reaction kit in an ABI Prism 310 cycle sequencer (PE Applied Biosystems).

Determination of sulfate level in mock-inoculated and HCRSV-infected kenaf leaves. For sulfate level determination, both mock-inoculated and HCRSV-infected leaves were sampled after systemic symptoms appeared (eight-leaf stage and 2 weeks post-inoculation). The leaves were washed twice in running tap water followed by reverse osmosis (RO) water twice. The leaves were dried at 60 °C for 6 h and at 80 °C for 4 h until the dry mass remained unchanged. The
dried leaves were cooled and ground into powder using a mortar and pestle at room temperature. The leaf powder was filtered through a 250 μm filter and stored in a vacuum dryer for further use. The powder was dissolved in double-distilled water (1:20, w/v) and heated for 5 min at 95 °C. Sulfate levels were determined by ion-exchange chromatography (Hänisch et al., 2006). For each experiment, three replicates were included for each test sample and the experiment was repeated three times.

Biochemical assays of SO and H_{2}O_{2}-generating activities. SO activity of leaf extracts from mock- and HCRSV-infected plants was determined by ferricyanide reduction at 420 nm in 1 ml reactions containing 10 μg soluble protein, 0.395 mM ferricyanide, 0.4 mM sodium sulfite in 20 mM Tris–HCl buffer (pH 8) (Eilers et al., 2001). One unit of SO activity was defined as the conversion of 1 μmol sulfite into sulfate min~^{-1}~. H_{2}O_{2}-generating activity of leaf extracts from mock- or HCRSV-infected plants was assayed in reactions containing 10 μg soluble protein, 0.85 mM 4-aminopyrine, 3.4 mM 3,5-dichloro-2-hydroxobenzene sulfonate and 4.5 U horse-radish peroxidase ml~^{-1}~ in 1 ml phosphate buffer (50 mM. pH 7.5) (Brychkova & Sagi, 2007). H_{2}O_{2}-generating activity was assayed spectrophotometrically at 515 nm after adding sodium sulfite to a final concentration of 0.4 mM. For both assays, reaction mixtures without sodium sulfite were used as controls. Each sample had three replicates and each experiment was repeated three times. All leaves assayed were from the same leaf stage and grown under the same conditions.

Transmission electron microscopy (TEM) and immuno-EM study. HCRSV-infected kenaf leaves were processed for TEM by cutting leaves into 1 x 1 mm pieces and fixing in formaldehyde:glutaraldehyde (4:1) fixative buffer for 2 h on a rotator at room temperature. Samples were post-fixed overnight at 4 °C in 2 % (w/v) osmium tetroxide (Fisher Scientific), dehydrated in a graduated series of 50–100 % ethanol (v/v) and embedded in Spurr resin. Ultrathin sections were cut with a glass knife mounted on a microtome, collected on copper grids and post-stained with 2 % (w/v) uranyl acetate and Reynold’s lead citrate for 10 and 3 min, respectively. Immunogold labelling of ultrathin sections was prepared as described above except that 4 % (v/v) paraformaldehyde, 2.5 % (w/v) sucrose in 0.1 M phosphate buffer (pH 7.2) was used for fixation, post-fixation in osmium tetroxide was omitted and ultrathin sections were mounted onto nickel grids. Immunogold experiments were performed on ultrathin sections of kenaf leaves that were incubated in a blocking buffer consisting of 3 x PBS, glycine (0.05 %, w/v), Tween 20 (0.05 %, v/v; Fisher Scientific) and BSA (0.5 %, w/v). Samples were incubated for 1 h in HCRSV CP antisera diluted in blocking buffer, washed a second time in blocking buffer, followed by incubation with a goat anti-rabbit antibody conjugated with 15 nm gold particles for 1 h. The grids were washed three times with 1 x PBS and then 10 times with autoclaved RO water. The staining method was the same as described above. Immunogold-labelled particles were quantified by counting the number of gold particles in peroxisomes. All images were acquired with a JEOL JEM 3010 electron microscope.

RESULTS

Identification of proteins interacting with HCRSV CP and mapping of the interacting domains

A cDNA library in the yeast GAL4 activation-domain expression vector was constructed by using RNAs extracted from fully expanded upper leaves of HCRSV-infected kenaf plants 14 days after inoculation. The library was screened by using a full-length HCRSV CP fused with the GAL4 DNA-binding domain as bait. Of 68 positive colonies, 27 clones were selected and sequenced. The screened putative interacting proteins included SO, a putative major latex-like protein, a putative chaperone P13.9, a C2 domain-containing protein, a ricin-rich domain-containing protein and α-d-xylosidase (see Supplementary Table S2, available in JGV Online).

There were five clones that shared identical sequences, containing a single ORF of 1182 nt with the capacity to encode a putative protein of 394 aa. A BLAST search of the amino acid sequence revealed that it has a conserved Moco domain and shared approximately 80 % amino acid sequence identity with SO or putative SO from other plants, ranging from 73 % identity with the putative SO of Brassica oleracea (NCBI Protein accession no. ABD65019) to 83 % identity with the SO of Codonopsis lanceolata (accession no. BAE48793). This protein was named HcSO (GenBank accession no. FJ603465) (see Supplementary Fig. S1, available in JGV Online).

To confirm the interaction between HCRSV CP and HcSO, pGBK77-CP and pGADT7-HcSO were co-transformed into the yeast strain AH109. Plasmids pGBK77-HR, -HS and -HP were co-transformed separately with pGADT7-HcSO into AH109 competent cells. The cells transformed with pGBK77-P+pGADT7-HcSO and pGBK77 S+pGADT7-HcSO, but not pGBK77-R+pGADT7-HcSO, grew rapidly and turned blue when streaked onto selective medium SD/-Leu/-Trp/-Ade/-His supplemented with X-a-Gal (Fig. 1b, c). The plasmid combinations pGBK77+pGADT7, pGBK77+pGADT7-HcSO, pGBK77-CP+pGADT7 and pGBK77-Lam+pGADT7-ReC were used as negative controls and pGBK77-53+pGADT7-RecT was used as a positive control. Only transformants of pGBK77-CP+pGADT7-HcSO and the positive control grew on SD/-Leu/-Trp/-Ade/-His agar plates supplemented with X-a-Gal (Fig. 1c). Both the P and S domains showed a strong interaction with HcSO in yeast cells. This is consistent with the atomic structure of HCRSV, which shows that the P and S domains are exposed on the virion surface.

HcSO interacts with HCRSV CP in kenaf protoplasts

The full-length HcSO was fused with the C terminus of the N-terminal half of enhanced yellow fluorescent protein (eYFP) and the HCRSV CP was fused with the C terminus of cEYFP. Yellow fluorescence was observed when the two fusion proteins were expressed and interacted in the same protoplast (Fig. 2a). By overlaying with the DIC image of the protoplast (Fig. 2b), it was observed that the yellow fluorescence was not located within the chloroplasts (Fig. 2c). This yellow fluorescence was not observed in the negative-control experiments (Fig. 2d–f). The chlorophyll autofluorescence and chloroplasts colocalized (Fig. 2g–i). The subcellular localization of free green fluorescent protein (GFP), GFP–CP in kenaf leaf cells and EYFP–
HcSO in kenaf protoplasts is shown in Fig. 3(a–c), respectively. The peroxisomes labelled by anti-SKL antibody and goat anti-rabbit IgG (H+L), DyLight 405 showed green fluorescence (Fig. 3d). The yellow fluorescence from the interaction of CP and HcSO (Fig. 3e) colocalized with the peroxisomes (Fig. 3f). Immunostaining of peroxisomes by anti-SKL antibody and Alexa Fluor 488 goat anti-rabbit IgG (H+L) in kenaf protoplast (Fig. 3g–i) shared similar expression patterns with the colocalization of CP and HcSO (Fig. 2c). The

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negative controls showed non-specific green fluorescence of protoplasts by goat anti-rabbit IgG (H + L), DyLight 405 in the cytoplasm (Fig. 3j–l).

**HCRSV CP bound to HcSO in vitro**

To confirm the interaction of HCRSV CP and HcSO, an *in vitro* binding assay was performed using purified GST–HCRSV CP bound to glutathione–Sepharose 4B beads and the *in vitro* translation product of HcSO labelled with biotinylated lysine. As shown in Fig. 4, the GST–HCRSV CP fusion protein bound to the *in vitro*-translated HcSO. In contrast, no binding activity was detected either between GST–HCRSV CP and the *in vitro* translation product of plasmid without the HcSO insert, or between GST and *in vitro*-translated HcSO. The *in vitro*-translated HcSO also did not bind to the glutathione–Sepharose 4B beads directly. In the washes, the *in vitro*-translated HcSO showed two bands including HcSO and the *in vitro* translation product from the pGADT7 vector (Fig. 4a). The unbound *in vitro*-translated pGADT7 resulted in a lower-molecular-mass product, which was included as a negative control (Fig. 4a). Thus, the *in vitro* binding of HCRSV CP and HcSO was shown to be specific (Fig. 4b).

**HCRSV infection induces peroxisome proliferation and aggregation in kenaf cells**

A normal peroxisome was observed adjacent to two chloroplasts in mock-inoculated leaves by TEM (Fig. 5a). Peroxisome proliferation and aggregation were observed in HCRSV-infected leaves (Fig. 5b, d, each representative of 20 micrographs). Immunolabelling of CP with HCRSV antiserum and goat anti-rabbit secondary antibody conjugated to 15 nm gold particles was quantified by counting the number of gold particles on peroxisomes. On average, six gold particles were found over each peroxisome of mock-inoculated leaf cells (Fig. 5c), compared with 30 gold particles detected in each peroxisome of HCRSV-infected kenaf leaf cells (Fig. 5e). As HCRSV CP labelled with gold particles was detected in the peroxisomes of infected kenaf leaves, this indicates that HCRSV CP is able to enter peroxisomes of systematically infected leaves.

**Fig. 3.** Subcellular localization of HCRSV CP and HcSO in kenaf epidermal cells (a–c) and colocalization of the two proteins to peroxisomes, which were counterstained with a peroxisome-specific anti-SKL antibody in protoplasts (d–i). (a) Fluorescence of free GFP, which could be observed in both nucleus and cytoplasm. (b, c) Subcellular localization of fused GFP–HCRSV CP (reproduced from Zhou *et al.*, 2006) in a kenaf epidermal cell and of EYFP–HcSO in a kenaf protoplast (this study). The fused GFP–HCRSV CP and EYFP–HcSO were localized in the cytoplasm and the peroxisomes, respectively. (d) Protoplast labelled with anti-SKL antibody, followed by goat anti-rabbit IgG (H + L) DyLight 405; (e) yellow fluorescence resulting from the interaction of HCRSV CP and HcSO in the BIFC experiment, as shown in Fig. 2; (f) merged image of (d) and (e). (g) Positive control showing green fluorescence from anti-SKL antibody and Alexa Fluor 488 goat anti-rabbit IgG (H + L) labelling in a kenaf protoplast; (h) DIC image of the protoplast; (i) merged image of (g) and (h). (j) Negative control showing labelling of protoplasts by Alexa Fluor 488 goat anti-rabbit IgG (H + L) in the cytoplasm; (k) DIC image of the same protoplast; (l) merged image of (j) and (k). Bar, 10 μm.
HCRSV infection leads to an upregulation of HcSO gene transcript and SO activity

Steady-state levels of HcSO gene transcripts were upregulated approximately five times in the HCRSV-infected leaves compared with mock-inoculated leaves (Fig. 6). The SO activity in HCRSV-infected kenaf plants also was higher than that in the mock-inoculated plants (Fig. 7a). The H$_2$O$_2$-generating activity was higher in the virus-infected kenaf plants than in the mock-inoculated plants (Fig. 7b). Sulfite is difficult to measure, as it is maintained at low levels in plant tissues and it oxidizes rapidly in extracts (Tsakraklides et al., 2002). We therefore chose to monitor the sulfate concentration, which increased by approximately two- to threefold after HCRSV infection compared with control leaves (Fig. 8). This was consistent with the upregulation of SO activity and H$_2$O$_2$-generating activity.

DISCUSSION

Key interactions between specific virus and host components can be linked to the role of specific virus–host interactions during replication, movement or suppression of resistance, which affect disease symptoms (Whitham et al., 2006). Complexity of interactions may disrupt host physiology and function to promote virus replication and spread within a host (Culver & Padmanabhan, 2007). The CP of plant RNA viruses plays many important roles throughout the virus life cycle. In addition to the primary function of encapsidating genomic nucleic acids, the CP
also assists in virus replication and local and/or systemic movement, elicits host defence responses and modulates symptom development in infected plants (Callaway et al., 2001). It has been shown that the accumulation of CP of tobacco mosaic virus in chloroplasts of systemically infected leaves may affect photosynthesis by inhibiting photosystem II activity (Reinero & Beachy, 1989). Transgenic plants expressing a high level of potato virus Y CP in chloroplasts are more susceptible to infection by the virus (Naderi & Berger, 1997). These observations suggest that CPs interact with putative host factors. Thus, identification of host proteins capable of interacting with the CP is an important first step in understanding the mechanisms of the CP-mediated functions. Few host factors have been identified as interacting directly with the CP of plant viruses. Turnip crinkle virus (TCV) CP has been shown to interact with a TCV-interacting protein (TIP) (Ren et al., 2000), believed to be a transcription factor that regulates defence responses in Arabidopsis thaliana (Ren et al., 2005), but it is not involved in suppression of RNA silencing (Choi et al., 2004). A putative oxidoreductase, HCP1, interacts with the brome mosaic virus (BMV) CP and affects BMV infection in barley (Okinaka et al., 2003). It has also been reported that high expression of a tomato mosaic virus CP-interacting protein is necessary to assist virus long-distance movement (Li et al., 2005). However, none of these studies has linked CP to its interactions with host proteins in peroxisomes, which play an essential role in specific defence mechanisms conferring resistance against pathogens and also have important functions in metabolism (Koh et al., 2005; Lipka et al., 2005). The peroxisomal protein SO is important in detoxification of excess sulfite, consequently protecting plant cells against sulfite damage or sulfitolysis (Hänisch & Mendel, 2005; Nowak et al., 2004). In this study, we show for the first time that HCRSV CP interacts with HcSO and that SO activity is upregulated in HCRSV-infected kenaf leaves. Viruses can induce a variety of responses, from non-specific changes in gene expression due to general accumulation of virus proteins to those responses that are initiated by specific interactions between virus and host proteins in the host cells. The functions of genes with altered expression profiles in plant–virus interactions may lead to new hypotheses on how host cells are manipulated to create a favourable environment for infection (Whitham et al., 2006).

In this study, it is shown that HCRSV CP interacts with HcSO in yeast and kenaf protoplasts (Figs 1–4). The subcellular colocalization of CP and HcSO, which showed small, punctate structures between chloroplasts, shares similar expression patterns with protoplasts labelled by anti-SKL antibody and Alexa Fluor 488 goat anti-rabbit IgG. Although fixation and staining of the protoplasts expressing CP and HcSO together by BiFC were completed, the yellow fluorescence was weak after processing. The fixed protoplasts could be visualized by indirect immunofluorescence analysis (Fig. 3g). Because HCRSV CP is localized in peroxisomes, as shown by immuno-EM (Fig. 5e), it is reasonable to conclude that the interaction of CP and HcSO is associated with peroxisomes (Fig. 3f).

TEM analysis showed proliferation and aggregation of peroxisomes in HCRSV-infected kenaf leaves (Fig. 5b, d), suggesting that the genes responsible for modulating peroxisome activity have been upregulated under the stress of HCRSV infection. H$_2$O$_2$, which is a universal stress-

![Fig. 6. Quantitative analysis of transcript expression of the SO gene of mock-inoculated compared with HCRSV-infected kenaf leaves after normalization against kenaf 18S rRNA expression. Results shown are means of three samples for each treatment; error bars indicate SD. One of two independent experiments, which yielded similar results, is shown.](image)

![Fig. 7. SO activity and H$_2$O$_2$-generating activity from mock-inoculated and HCRSV-infected kenaf leaf extracts (10 µg). SO activity was assayed by using the ferricyanide reduction technique (a) followed by an H$_2$O$_2$-generation assay (b). The results of both assays were measured by using a spectrophotometer, at 420 and 515 nm, respectively; error bars indicate SD.](image)
signal molecule that induces the upregulation of peroxisome biogenesis genes in plants (Lopez-Huertas et al., 2006), is produced from plant SO (Hänsch et al., 2006). Thus, HcSO may play an important role in upregulating H2O2-generating activity (Fig. 7b), which upregulates genes required for peroxisome biogenesis in HCRSV-infected leaves. The increased level of H2O2 may inhibit catalase activity (Hänsch et al., 2006), which may cause necrosis of the leaves (Willekens et al., 1997). The upregulated H2O2-generating activity (Fig. 7b) may cause cell death of the leaves and prevent systemic movement of virus to other cells.

Steady-state levels of HcSO gene transcripts and SO activity were upregulated in HCRSV-infected leaves compared with mock-inoculated leaves (Figs 7 and 8). Plant SO is the smallest molybdenum-containing enzyme known and SO is required to oxidize sulfite to sulfate in the peroxisomes in plant cells. Sulfite is able to attack diverse substrates as a nucleophilic agent, where it opens disulfide bridges and thereby causes inactivation of the relevant enzymes. This reaction is called sulfitolysis (Peiser & Yang, 1985). Brychkova & Sagi (2007) demonstrated that SO-silenced A. thaliana lines accumulated relatively low concentrations of sulfate following application of SO2; this was associated with enhanced induction of senescence and wounding-associated RNA transcripts, leaf necrosis and chlorophyll bleaching. In addition, SO-overexpressing lines accumulated relatively high sulfate concentrations and showed little or no necrosis after SO2 was applied. These observations suggest that an increase in SO may be involved in accommodating the overflow of sulfite that accumulates upon decomposition of sulfur-containing amino acids or sulfated metabolites in the sulfite-oxidizing pathway. Interestingly, we also found that older HCRSV-infected leaves with severe necrosis exhibited much higher SO activity and sulfate levels than the younger, non-necrotic leaves (unpublished data). The increased amount of sulfate (Fig. 8), which is oxidized from sulfite, in the HCRSV-infected kenaf leaves may indicate the overflow of sulfite in the virus-infected cells. In order to reduce sulfite damage to the cells, the plant upregulated SO transcripts (Fig. 6) and activity in the HCRSV-infected leaves (Fig. 7a) to detoxify the excess sulfite. It is speculated that, when HcSO is silenced in a transgenic kenaf plant, SO activity will decrease and it will lead to increased virus virulence and result in more severe necrotic symptoms.

The increased number of peroxisomes and upregulated HcSO activity in the HCRSV-infected kenaf cells are a plant response to virus infection. The interaction of HCRSV CP with HcSO and the association of the interaction with the peroxisomes may play a role in increasing the sulfite level and upregulating SO activity. Thus, the production of SO-silenced and SO-overexpressed transgenic kenaf plants will help us to better understand the function of SO in protecting the plant during virus infection.

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Fig. 8. Comparison of sulfate levels between mock-inoculated (empty columns) and HCRSV-infected (filled columns) kenaf leaves. Results are means of three samples for each treatment in three independent experiments (1–3); error bars indicate SD.


