HC-Pro protein of sugar cane mosaic virus interacts specifically with maize ferredoxin-5 in vitro and in planta

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Symptom development of a plant viral disease is a result of molecular interactions between the virus and its host plant; thus, the elucidation of specific interactions is a prerequisite to reveal the mechanism of viral pathogenesis. Here, we show that the chloroplast precursor of ferredoxin-5 (Fd V) from maize (Zea mays) interacts with the multifunctional HC-Pro protein of sugar cane mosaic virus (SCMV) in yeast, Nicotiana benthamiana cells and maize protoplasts. Our results demonstrate that the transit peptide rather than the mature protein of Fd V precursor could interact with both N-terminal (residues 1–100) and C-terminal (residues 301–460) fragments, but not the middle part (residues 101–300), of HC-Pro. In addition, SCMV HC-Pro interacted only with Fd V, and not with the other two photosynthetic ferredoxin isoproteins (Fd I and Fd II) from maize plants. SCMV infection significantly downregulated the level of Fd V mRNA in maize plants; however, no obvious changes were observed in levels of Fd I and Fd II mRNA. These results suggest that SCMV HC-Pro interacts specifically with maize Fd V and that this interaction may disturb the post-translational import of Fd V into maize bundle-sheath cell chloroplasts, which could lead to the perturbation of chloroplast structure and function.

INTRODUCTION

Identification of interactions between viral and host proteins is essential to elucidate the molecular mechanisms that underlie the viral infection process and symptom development in plants. It has been suggested that common plant symptoms such as mosaic or chlorosis are caused largely by viral disturbance of normal chloroplast structure and function (Hull, 2001). Chlorosis could be related to reduced expression of chloroplast-targeted proteins caused by tobacco mosaic virus (TMV) infection (Lehto et al., 2003), and the interference with chloroplast protein functions may contribute significantly to the induction of defence responses and to the development of disease symptoms (Jiménez et al., 2006). To date, several chloroplast proteins of dicotyledonous plants, such as a 37 kDa protein (McClintock et al., 1998), PSI-K protein (Jiménez et al., 2006), Rieske Fe/S protein (Shi et al., 2007) and the chloroplast division-related factor NtMinD (Jin et al., 2007b), have been reported to interact with turnip mosaic virus (TuMV) CP, plum pox virus (PPV) CI protein, soybean mosaic virus Pinellia isolate (SMV-P) P1 protein and potato virus Y (PVY) helper-component proteinase (HC-Pro), respectively, but the mechanism by which host factors are involved in symptom development remains largely unknown, particularly for monocotyledonous plants.

Potyviral HC-Pro has several functions, being involved in self-cleavage from the polyprotein precursor (Carrington et al., 1990), aphid transmission (Atreya & Pirone, 1993), genome amplification, virus movement (Kasschau & Carrington, 2001), suppressing post-transcriptional gene silencing (Kasschau & Carrington, 1998), interfering with miRNA functions essential for the development of host plants (Kasschau et al., 2003) and symptom expression in host plants (Kasschau et al., 2003; Atreya & Pirone, 1993; Tribodet et al., 2005; Shiboleth et al., 2007). Thus, the multiple functions of HC-Pro would be expected to involve

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The GenBank/EMBL/DBJ accession numbers for the cDNA sequences of the precursors of maize ferredoxins 5, 1 and 2 reported in this paper are respectively EU328184–EU328186.

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multiple interactions with different host factors. Previous studies have revealed some dicotyledons plant factors that interact with HC-Pro (Anandalakshmi et al., 2000; Guo et al., 2003; Ballut et al., 2005; Jin et al., 2007a, b); however, to date no report has been published on any monocotyledonous host protein that interacts with a potyviral HC-Pro. Our previous studies showed that sugar cane mosaic virus (SCMV) (genus Potyvirus) was the major causal agent of maize dwarf mosaic disease in China, with the Beijing isolate (SCMV-BJ) from maize being the prevalent strain (Fan et al., 2003). Thus, the HC-Pro encoded by SCMV-BJ was used in this study as bait to screen a maize cDNA library for interacting proteins by the yeast two-hybrid system (YTHS). Our results show that SCMV HC-Pro could interact with the chloroplast precursor of maize ferredoxin-5 (Fd V) in yeast and living plant cells (maize protoplasts and Nicotiana benthamiana leaf epidermis); HC-Pro interacts specifically with Fd V, and not with the other two maize photosynthetic-type Fds (Fd I and Fd II). These results suggest that the post-translational import of Fd V into maize bundle-sheath cell (BSC) chloroplasts might be disturbed by the specific interaction between HC-Pro and Fd V, which, in turn, could lead to the perturbation of chloroplast structure and function.

METHODS

Plant materials and growth conditions. Maize inbred line Ye 107 was used for cDNA library construction and another inbred line Zong 31 was used for other experiments in this work. Maize and tobacco plants were grown in 10 cm pots filled with a mixture of 60 % vermiculite and 40 % meadow soil and cultured in growth chambers (16 h light/8 h dark at 25–26 °C).

Construction of plasmids. SCMV isolate BJ and its full-length cDNA (GenBank accession no. AY042184) were kept in our laboratory. The plasmids used in the bimolecular fluorescence complementation (BiFC) assay, pUC-SPYNE, pUC-SPYCE, pSPYNE-35S and pSPYCE-35S (for split YFP N-terminal/C-terminal fragment expression), were kind gifts from Dr Jörg Kudla (Walter et al., 2004). The primers used for construction of plasmids are listed in Supplementary Table S1 (available in JGV Online).

To construct plasmids for YTHS analysis, the coding sequences of the whole protein and N-terminal (amino acid residues 1–100), central (residues 101–300) and C-terminal (residues 301–460) fragments of SCMV HC-Pro were amplified separately using primer pairs F1/R1, F1/R2, F2/R3 and F3/R1, respectively. The PCR fragments were digested with NsiI/SalI and cloned into the vector pGBK7 (Clontech) to generate the recombinant plasmids pGBK-HC, pGBK-HC(1-100), pGBK-HC(101-300) and pGBK-HC(301-460), respectively.

The coding sequences of intact Fd V, its N-terminal fragment (residues 1–45), fer2 domain (residues 46–129) and mature chain (residues 42–138) were amplified from the insert obtained from the maize cDNA library with primer pairs F4/R4, F4/R5, F5/R6 and F6/R4, respectively. The PCR products were digested with EcoRI/BamHI and cloned into the GAL4 activation domain vector pGADT7 (Clontech) to generate recombinant plasmids pGAD-FdV, pGAD-FdV(1-45), pGAD-FdV(46-129) and pGAD-FdV(42-138), respectively.

cDNAs encoding the two other photosynthetic types of Fd in maize, Fd I and Fd II, were amplified from total RNA of maize (Zong 31) leaves by RT-PCR with primer pairs F7/R7 and F8/R8, respectively, which were designed according to maize mRNA sequences encoding Fd I (GenBank accession no. M73829) and Fd II (AB016810), respectively. The specific fragments were doubly digested with EcoRI/BamHI and then cloned into the pGADT7 vector to generate pGAD-Fd1 and pGAD-FdII, respectively.

For BiFC, the full-length coding sequence of SCMV HC-Pro was PCR-amplified with the primer pair F9/R9. The products were digested with SpeI/Xhol and ligated to pSPYNE-35S and pUC-SPYCE to generate recombinant plasmids pHc-YFPN and pHc-YFPc, respectively. The full-length cDNA of Fd V amplified with primer pair F10/R10 was digested with BamHI/Xhol and cloned into pSPYCE-35S and pUC-SPYNE to generate recombinant plasmids pHc-FdV-YFPc and pHc-FdV-YFPn, respectively. The combination pHc-FdV-YFPc/pFdV-YFPn was used for transient transfection of maize protoplasts, while pHc-FdV/pFdV-YFPc were used for co-infiltration into the leaf epidermis of N. benthamiana.

Maize cDNA library construction, screening and the identification of positive interactions in yeast. The construction and screening of the maize cDNA library and the analyses of positive interactions were performed by using BD Matchmaker Library Construction and Screening kits (Clontech) according to the manufacturer’s protocols. Total RNAs were extracted from maize (Ye 107) seedlings using an RNA isolation kit (Promega) and mRNA (1.0 μg) isolated with an mRNA isolation kit (Promega) was used for cDNA library construction. The maize cDNA library was screened with bait vector pGBK-HC, and positive clones were isolated. Positive interactions were confirmed in yeast by co-transformation into Saccharomyces cerevisiae strain AH109. All experiments were repeated at least three times, and identical results were obtained.

BiFC assay and confocal laser scanning microscopy. Transient transfection of maize protoplast cultures with the combination pHc-YFPc/pFdV-YFPn was performed according to the protocol provided by the Sheen Laboratory (http://genetics.mgh.harvard.edu/sheenweb/protocols_reg.html), while the combination pHc-YFPn/pFdV-YFpc was used as the negative control. YFP fluorescence was detected in maize protoplasts at 12–16 h after transfection. Co-infiltration of N. benthamiana leaves and confocal microscopy were performed essentially as described previously (Gissot et al., 2006). Agrobacterium tumefaciens strain GV3101 carrying either pHc-YFPn or pFdV-YFpc was separately cultured in a shaker overnight at 28 °C in LB medium containing 100 μg streptomycin and 50 μg kanamycin ml⁻¹, and the cells were resuspended to an OD₆₀₀ of 0.4 with MMA buffer (10 mM MES/NaOH, pH 5.6, 10 mM MgCl₂, 200 μM acetoxyrindole). For co-infiltration, equal volumes of the two cultures (containing pHc-YFPn and pFdV-YFpc) were mixed before agaroinfiltration, and the combination pHc-YFPn/pFdV-YFpc was used as the negative control. Observation of leaf epidermal cells for fluorescence was performed at 48–72 h after infiltration.

Confocal microscopy was performed on an inverted spectral confocal laser scanning microscope (Leica TCS-SP2-AOBS). Samples were excited using a 514 nm argon laser with an emission band of 530–560 nm for YFP detection and 650 nm for detecting chlorophyll autofluorescence of N. benthamiana epidermal cells.

Semi quantitative RT-PCR. Semi quantitative RT-PCR was used to analyse the transcript (mRNA) levels of the genes for Fd I, Fd II and Fd V in SCMV-infected and mock-inoculated (healthy) maize (Zong 31) plants with specific primer pairs F7/R7, F8/R8 and F4/R4, respectively. Total RNAs were extracted from the upper fourth or fifth uninoculated leaves. Reverse transcription was conducted at 37 °C for 1 h using oligo(dT) as the primer. Each PCR (25 μl) contained 2 μl
cDNA template, and the PCR amplification was conducted according to the following procedure: cDNA denaturation at 94 °C for 4 min; 30 cycles at 94 °C for 30s, 56 °C for 30s and 72 °C for 1 min and a final extension step at 72 °C for 10 min. The maize alpha-tubulin gene \((\text{tua4}; \text{GenBank accession no. X73980})\) was analysed as an internal control using primers F11 and R11 (Supplementary Table S1). PCR products were visualized in 1.2% agarose gels after staining with ethidium bromide and analysed with the built-in software of Alphalmager 2200.

**RESULTS**

**Identification of the interaction between maize Fd V and SCMV HC-Pro**

A cDNA library in the yeast GAL4 activation domain expression vector was obtained by using RNAs extracted from maize seedling leaves and stems. The library (consisting of \(2.0 \times 10^6\) independent clones) was then screened using the full-length SCMV HC-Pro fused with the GAL4 DNA-binding domain as a bait. Of 42 positive colonies, 36 clones were sequenced, most of which did not encode proteins of known function (not shown). Among four closely related clones (clones 12, 26, 27 and 37), two (26 and 27) shared an identical sequence of 665 nt (including a polyadenylated tail of 32 nt) containing a single open reading frame (ORF) with the capacity to encode a putative protein of 138 aa. The sequences of the other two clones (clones 12 and 37) also had the potential to encode a protein comprising 138 aa, with only one difference each from that of clones 26 and 27 (not shown). A BLAST query of the deduced amino acid sequence revealed that it has a conserved domain fer2 and that its sequence is closely related to maize ferredoxin isoproteins (Fd V, GenBank accession no. P27789, 94% amino acid sequence identity; Fd I, P27787, 81%; Fd II, BAA32348, 72.9%) (Fig. 1). Since our previous work on Fd I of tobacco and TMV showed that the decrease in Fd I was associated with the development of the chlorosis/mosaic symptoms (unpublished), we decided to further our investigation on maize ferredoxin isoproteins.

Compared with the previously published maize Fd V amino acid sequence (GenBank accession no. P27789), which includes a transit peptide (residues 1–38) and a mature chain (residues 39–135), the deduced amino acid sequence of the predicted protein contains a transit peptide of 41 aa, and the mature chain is of the same size but varies in five amino acid residues (Fig. 1). We also amplified and cloned the cDNA by RT-PCR using RNA extracted from the leaves of another maize inbred line, Zong 31 (Fig. 1; see also Fig. 6), with primer pair F4/R4, and this cDNA encodes the same predicted amino acid sequence as that of the two positive clones (clones 26 and 27) from Ye 107. Thus, we believe that this predicted protein should be maize Fd V, and the slight difference between the sequences could be due to variation in the genetic backgrounds of different maize lines. The cDNA sequence of this Fd V precursor (from Ye 107) was deposited in GenBank under accession number EU328184.

To confirm the HC-Pro–Fd V interaction, the coding sequence for Fd V was inserted into the GAL4 activation domain vector pGADT7 and retested for interaction with HC-Pro by co-transformation into yeast strain AH109; the plasmid combinations pGBK77/pGADT7, pGBK77/pGAD-FdV, pGBK77/pGAD-HC/pGADT7 and pGBK77-RecT were used as negative controls and pGBK77-53/pGADT7-RecT as a positive control. Only transformants of pGBK77/pGAD-FdV and the positive control could grow on agar plates of synthetic dropout (SD) medium lacking leucine, tryptophan, adenine and histidine (SD/-Leu/-Trp/-Ade/-His) (Fig. 2). Results obtained from five independent experiments confirmed the interaction between HC-Pro and Fd V in yeast cells.

**Confirmation of the interaction between Fd V and HC-Pro in living plant cells by BiFC**

BiFC is a valuable approach for studying protein–protein interactions (Walter et al., 2004). To confirm the
HC-Pro–Fd V interaction in plant cells, cDNAs encoding HC-Pro and Fd V were cloned into BiFC transformation vectors pSPYNE-35S (YFP<sup>N</sup>), pUC-SPYCE (YFP<sup>C</sup>), pSPYCE-35S (YFP<sup>C</sup>) and pUC-SPYNE (YFP<sup>N</sup>) to generate plasmids pHC-YFP<sup>N</sup>, pHC-YFP<sup>C</sup>, pFdV-YFP<sup>C</sup> and pFdV-YFP<sup>N</sup>, respectively.

Cultured maize protoplasts were transfected with pHC-YFP<sup>C</sup>/pFdV-YFP<sup>N</sup>, while the pair pFdV-YFP<sup>N</sup>/pYFP<sup>C</sup> was used as the negative control. *N. benthamiana* leaves were transformed through coinfiltration with *A. tumefaciens* GV3101 cells harbouring the combination pHC-YFP<sup>N</sup>/pFdV-YFP<sup>C</sup>, and pHC-YFP<sup>C</sup>/pYFP<sup>N</sup> served as the negative control. Samples were examined for YFP fluorescence using spectral confocal laser scanning microscopy. Reconstitution of YFP fluorescence in both maize protoplasts transfected with pHC-YFP<sup>C</sup>/pFdV-YFP<sup>N</sup> (Fig. 3a) and *N. benthamiana* leaf epidermis coinfiltrated with pHC-YFP<sup>N</sup>/pFdV-YFP<sup>C</sup> (Fig. 3b) confirmed the HC-Pro–Fd V interaction, whereas no or negligible fluorescence was observed in negative controls. In transfected maize protoplasts, subcellular localization of reconstituted YFP complexes was observed in the cytoplasm (Fig. 3a), suggesting that the HC-Pro–Fd V interaction occurred there.

Both the N and C termini of HC-Pro interact with the transit peptide of maize Fd V

The predicted Fd V amino acid sequence consisted of a transit peptide (residues 1–41) and a mature chain (residues 42–138), which contains a 2Fe–2S iron–sulfur cluster-binding domain, fer2 (residues 46–129).

Mutagenesis studies and sequence alignments suggest that HC-Pro can be divided schematically into three regions: the N terminus, central region and C terminus (Plisson et al., 2003). Thus, three deletion mutants each for Fd V (Fig. 4a) and HC-Pro (Fig. 4b) were created to analyse the interaction in more detail by YTHS. The coding sequences of HC-Pro and Fd V mutants were cloned into pGBKKT7 and pGADT7, respectively. To analyse the specific region of Fd V necessary for the HC-Pro–FdV interaction, combinations pGBK-HC/pGAD-FdV(1–45), pGBK-HC/pGAD-FdV(46–129) and pGBK-HC/pGAD-FdV(42–138) were co-transformed into the yeast strain AH109. Positive and reproducible interactions between HC-Pro and Fd V (aa 1–45) were detected in the yeast, whereas no interaction was observed with Fd V (aa 46–129) or with Fd V (aa 42–138) (Fig. 4c). Therefore, the region of Fd V involved in the interaction with HC-Pro is located within the N-terminal fragment (aa 1–41), i.e. the transit peptide. As to the region(s) of HC-Pro necessary for the interaction, it was observed that transformants of pGBK-HC(1–100)/pGAD-FdV, pGBK-HC(1–100)/pGAD-FdV(1–45), pGBK-HC(301–460)/pGAD-FdV and pGBK-HC(301–460)/pGAD-FdV(1–45) were able to grow on the SD/−Trp/−Ade/−His medium; however, like the negative controls, transformants of pGBK-HC(101–300)/pGAD-FdV and pGBK-HC(101–300)/pGAD-FdV(1–45) combinations were not able to grow (Fig. 4d). These results suggested that both the N terminus (residues 1–100) and C terminus (residues 301–460) of SCMV HC-Pro can interact with the precursor or transit peptide of Fd V, but the central region of HC-Pro (residues 101–300) is dispensable for the interaction.

SCMV HC-Pro interacts with Fd V but not other photosynthetic Fd types of maize

The three known photosynthetic-type Fd isoproteins (Fd I, Fd II and Fd V) are all present in maize leaves. To test whether HC-Pro could interact with the two other known Fds of the same type, cDNA fragments containing full-length ORFs encoding either Fd I or Fd II from maize (Zong 31) were amplified by RT-PCR using specific primers and sequenced (GenBank accession nos EU328185 for Fd I and EU328186 for Fd II; Fig. 1) before cloning in-frame into the vector pGADT7 to generate pGAD-FdI and pGAD-FdII, respectively. Cells of yeast strain AH109 were co-transformed with construct combinations pGBK-HC/pGAD-FdI and pGBK-HC/pGAD-FdII, and combinations pGBKKT7/pGADT7, pGBKKT7/pGAD-FdI and pGBKKT7/pGAD-FdII were used as negative controls and pGBKKT7-53/pGADT7-RecT as a positive control. It was concluded that SCMV HC-Pro could not interact with maize Fd I or Fd II, because transformants of combinations pGBK-HC/pGAD-FdI and pGBK-HC/pGAD-FdII were not able to grow on the selective medium (Fig. 5). These results suggest that SCMV HC-Pro interacts specifically only with maize Fd V.

SCMV infection resulted in downregulation of Fd V gene expression

Since SCMV HC-Pro was shown to interact specifically with maize Fd V, possible changes in maize Fd V, Fd I, and Fd II gene expression in SCMV-infected and mock-inoculated (healthy) maize plants were analysed by semiquantitative RT-PCR with specific primer pairs. PCR products were examined in an agarose gel (Fig. 6a) and the
corresponding relative mRNA levels in leaves of both healthy and SCMV-infected maize plants were analysed (Fig. 6b). The level of Fd V mRNA in SCMV-infected leaves decreased significantly (Fig. 6a) to about 30% of that in healthy plant leaves (Fig. 6b); however, no obvious changes were observed in Fd I or Fd II mRNA levels (Fig. 6a, b). Similar results were obtained in five independent experiments. These results suggest that SCMV infection significantly downregulates Fd V gene expression, but has no obvious effect on the expression of genes for Fd I and Fd II.

DISCUSSION

Several dicotyledonous plant factors have been reported to interact with potyviral protein HC-Pro, including a calmodulin-like protein involved in gene silencing (Anandalakshmi et al., 2000). Now, we report that a monocotyledonous host protein, maize Fd V precursor, interacts specifically with SCMV HC-Pro.

As a typical C₄ plant, maize has two types of photosynthetic cells, the BSC and the mesophyll cell (MC). The Calvin cycle is limited to the BSC, and BSC chloroplasts have high
active cyclic electron flow (CEF) around photosystem I (PS I) (Darie et al., 2006). In chloroplasts, Fd is a key component of PS I that mediates electron transfer. To date, six Fd isoproteins have been identified in maize (Hase et al., 1991; Matsumura et al., 1999; Sakakibara, 2003), including three photosynthetic isoproteins (Fd I, II and V) and three non-photosynthetic ones (Fd III, IV and VI). Fds of photosynthetic type are expressed predominantly in leaves; among them, Fd I and Fd II are expressed almost
Two chloroplast precursor proteins, the PS I-K protein (Jiménez et al., 2006) and the Rieske protein (Shi et al., 2002), our results show that HC-Pro
interacts specifically with maize Fd V in vivo which, together, results in the decrease in Fd V mRNA. This possibility has been raised by research using another potyvirus that has suggested that any viral product could affect the expression of host genes (Wang & Maule, 1995). The finding that dark periods destabilized Fd I mRNA (Petracek et al., 1998) indicates that blockage of photosynthesis affects RNA stability of a certain group of Fd isoforms; thus, the decrease in Fd V mRNA might also be caused by physiological changes during virus infection. The specific HC-Pro–Fd V interaction and the differential changes in levels of Fd V, Fd I and Fd II mRNA in leaves of SCMV-infected maize plants suggest that Fd V might play an important role in maize during SCMV infection. Therefore, the HC-Pro–Fd V interaction might play a role in symptom development in SCMV-infected plants. Chlorosis induced by different virus–host interactions is associated with reduced chlorophyll content of the leaves. The chloroplast is a dynamic organelle that needs to replace proteins continuously, some of which are encoded in the nucleus, to maintain its function (Dawson, 1992). When one or more of the protein components is not available for assembly of photosynthetic pigment–protein complexes, degradation of photosynthetic pigments would be enhanced (Lehto et al., 2003). From this point of view, the loss of chlorophyll is due mainly to perturbation of chloroplast structure and function (Hull, 2001). The HC-Pro–Fd V interaction might interfere with import of the precursor of Fd V into the chloroplast; in addition, SCMV infection apparently downregulated Fd V mRNA (Fig. 6), which might be caused by either the specific HC-Pro–Fd V interaction or multiple interactions between Fd V and SCMV proteins including HC-Pro. Thus, the level of Fd V available for PS I in BSC could eventually be reduced. Since Fd V is a key component of PS I in BSC chloroplasts, which catalyses CEF to produce extra ATP for the Calvin cycle, the decreased level of Fd V may lead to the perturbation of chloroplast structure and function and ultimately contribute to symptom expression. Previous studies have demonstrated that disruption of transport of nuclear-encoded proteins into the chloroplast in virus-infected cells (Dawson, 1992) or reduced expression of specific proteins of the PS II core complex (Lehto et al., 2003) might be related to the induction of chlorosis. In addition, our suggestion is also consistent with a finding that a decreased level of Fd led to a reduced total chlorophyll content in symptom development in SCMV-infected plants.
residues located at positions 400 and 419 in the C terminus of PVY HC-Pro (Tribolet et al., 2005), mutations in the N terminus (Atreya & Pirone, 1993), either the N or C terminus (Klein et al., 1994) of tobacco vein mottling virus HC-Pro, a 92 aa deletion in the N-terminal region of HC-Pro of a natural mutant of onion yellow dwarf virus (Takaki et al., 2006) and a single amino acid in the middle (FR180NK) of zucchini yellow mosaic virus HC-Pro (Shiboleth et al., 2007) have been reported to be related to symptom expression. Thus, the N terminus (residues 1–100) and C terminus (residues 301–460) of SCMV HC-Pro (Fig. 4d) necessary for the interaction with Fd V might be involved in symptom expression in maize.

In conclusion, our results show that SCMV HC-Pro interacts specifically with the maize chloroplast precursor protein of Fd V and that the interaction might disturb the post-translational import of Fd V into maize BSC chloroplasts, which could in turn lead to the perturbation of chloroplast structure and function.

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