Maize Elongin C interacts with the viral genome-linked protein, VPg, of Sugarcane mosaic virus and facilitates virus infection

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Summary

- The viral genome-linked protein, VPg, of potyviruses is involved in viral genome replication and translation. To determine host proteins that interact with Sugarcane mosaic virus (SCMV) VPg, a yeast two-hybrid screen was used and a maize (Zea mays) Elongin C (ZmElc) protein was identified.
- ZmELC transcript was observed in all maize organs, but most highly in leaves and pistil extracts, and ZmElc was present in the cytoplasm and nucleus of maize cells in the presence or absence of SCMV. ZmELC expression was increased in maize tissue at 4 and 6 d post SCMV inoculation. When ZmELC was transiently overexpressed in maize protoplasts the accumulation of SCMV RNA was approximately doubled compared with the amount of virus in control protoplasts.
- Silencing ZmELC expression using a Brome mosaic virus-based gene silencing vector (virus-induced gene silencing) did not influence maize plant growth and development, but it did decrease RNA accumulation of two isolates of SCMV and host transcript encoding ZmElf4E during SCMV infection. Interestingly, Maize chlorotic mottle virus, from outside the Potyviridae, was increased in accumulation after silencing ZmELC expression.
- Our results describe both the location of ZmElc expression in maize and a new activity associated with an Elc: support of potyvirus accumulation.

Introduction

The members of the genus Potyvirus (family Potyviridae) cause significant yield and quality losses in a broad range of crop plants (Riechmann et al., 1992; Revers et al., 1999; Gibbs & Oshima, 2010; Adams et al., 2012). Within grass species, the potyvirus Sugarcane mosaic virus (SCMV) is widespread and induces severe disease in maize (Zea mays L.), sugarcane (Saccharum officinarum) and sorghum (Sorghum vulgare) (Fuchs & Grünzig, 1995; Shi et al., 2005; Uzarowska et al., 2009). It is known as the major causal agent of maize dwarf mosaic disease in China, and the Beijing isolate (SCMV-BJ) belongs to a prevalent strain of SCMV in China (Fan et al., 2003). Yield losses can be as high as 30–50% and understanding the mechanism of infection by SCMV is critical for the identification of novel methods to control its accumulation and spread.

Potyviruses possess a single-stranded, positive-sense RNA genome c. 10 kb in length. A viral genome-linked protein, VPg, is attached to the 5’ terminus of the genomic RNA and a polyadenylate tract resides at the 3’ end of the genome. The viral genome can be translated to yield a polyprotein that is cleaved into ten mature proteins by three viral proteases (Urucuqui-Inchima et al., 2001). These proteins are responsible for virus accumulation and spread, suppression of RNA silencing and vector transmission (Urucuqui-Inchima et al., 2001; Adams et al., 2012). An additional protein, P3N-PIPO, resulting from a translation frame shift within the P3 cistron, was discovered and reported to influence virus cell-to-cell movement (Chung et al., 2008; Wei et al., 2010b; Vijayapalani et al., 2012). The continued analysis of the functions of these potyviral proteins is critical for exploring methods for virus control.

The VPg of potyviruses is covalently linked to the 5’ terminus of the genomic RNA via a tyrosine residue (Murphy et al., 1996; Anindya et al., 2005). VPg is an intrinsically disordered protein (Grzela et al., 2008; Rantalainen et al., 2008, 2011), and this property enables it to have multiple functions during virus infection (Rantalainen et al., 2008; Jiang & Laliberté, 2011). Potyviral VPg is a component of the virus replication complex and has been suggested to be the primer for negative-strand RNA synthesis because of its uridylylation, like the VPg of picornaviruses (Pusztin & Makinen, 2004). Other studies determined that VPg is involved in virus translation by either recruiting translation factors to promote viral RNA translation or sequestering translation factors to inhibit the formation of the translation initiation complex for host mRNAs (Léonard et al., 2000; Michon et al., 2006; Khan et al., 2008; Eskelin et al., 2011). VPg also

Key words: Brome mosaic virus, elf4E, Elongin C, maize (Zea mays), Sugarcane mosaic virus (SCMV), virus accumulation, virus-induced gene silencing (VIGS), viral genome-linked protein (VPg).
influences potyvirus movement (Rajamaki & Valkonen, 2002; Dunoyer et al., 2004).

Several host proteins that interact with the VPg have been reported in the past decades. The best characterized is the eukaryotic translation initiation factor 4E (eIF4E) or its isoform, eIF(iso)4E. Arabidopsis thaliana eIF(iso)4E was the first identified VPg-interacting host protein (Wittmann et al., 1997). Later, a large number of VPg-eIF4E/eIF(iso)4E interactions were discovered from multiple hosts (Wang & Krishnaswamy, 2012). It is known that potyviral VPgs from some virus species selectively bind to specific isoforms of eIF4E (Lellis et al., 2002; Sato et al., 2005; Ruffel et al., 2006; Jenner et al., 2010). Studies showed that eIF4E or eIF(iso)4E was required for viral RNA translation (Khan et al., 2008; Miyoshi et al., 2008; Eskelin et al., 2011). Thus, knockout or mutation of either the eIF4E or eIF(iso)4E gene in the host can result in resistance to potyvirus infection (Duprat et al., 2002; Yeam et al., 2007; Charron et al., 2008; Rubio et al., 2009; Gallois et al., 2010; Hébrard et al., 2010; Ashley et al., 2011; Nieto et al., 2011). In addition to host translation proteins, cysteine-rich protein (Dunoyer et al., 2004), poly (A)-binding protein (PABP) (Léonard et al., 2004; Beauchemin & Laliberté, 2007; Dufresne et al., 2008), DEAD-box RNA helicase (AtrFH8) and peach DDX-like protein (PpDDXL) (Huang et al., 2010), were identified as VPg interactors. Those interactions are reported to be crucial for virus infection and accumulation, although the underlying mechanisms for their actions remain unclear.

Elongin C was originally identified as a member of the mammalian transcription factor SIII that increases the rate of transcription by suppressing RNA polymerase II pausing (Bradsher et al., 1993a,b; Aso et al., 1995). As a central member of several multiprotein complexes, Elongin C is involved in a variety of activities including von Hippel-Lindau (VHL)-mediated tumor suppression (Duan et al., 1995; Yu et al., 2003) and cytokine signaling (Bullock et al., 2006; Babon et al., 2008) in mammalian cells. Other studies determined that it also acts as an E3 ligase within the ubiquitin-mediated proteolysis pathway in mammalian cells through binding with Elongin B (Gerber et al., 2004; Willems et al., 2004). In yeast, Elongin C is not involved in transcriptional stimulation (Koth et al., 2000). Yeast two-hybrid analysis demonstrated that yeast Elongin C interacts with a specific set of proteins involved in stress responses (Jackson et al., 2000). Yeast Elongin C, like its mammalian counterpart, is also known to be a component of E3 ligase complexes, in this situation influencing the DNA repair process (Ramsey et al., 2004; Gillette et al., 2006; Ribar et al., 2006, 2007; Lejeune et al., 2009). More recently, Elongin C was shown to participate in the spread of repressive histone modifications in Chlamydomonas reinhardtii (Yamasaki & Ohama, 2011). The only investigation of Elongin C in plants determined that A. thaliana Elongin C null mutants grew normally under experimental conditions, suggesting that it is dispensable for plant growth (Hua & Vierstra, 2011).

In this study, we identified a maize Elongin C (ZmElc) protein which interacts with SCMV VPg in both yeast and maize cells. We determined that the expression of ZmELC was induced in maize plant at 4 and 6 d post inoculation (dpi) with SCMV and ZmElc facilitated SCMV RNA accumulation in maize protoplasts when it was transiently overexpressed. By contrast, silencing its expression in maize plants through virus-induced gene silencing (VIGS) significantly reduced the accumulation of two different isolates of SCMV but increased the accumulation of Maize chlorotic mottle virus (MCMV), which is not within the Potyviridae. We also report that silencing ZmELC resulted in a decrease of ZmELF4E expression in the presence of SCMV, although ZmElc did not interact directly with ZmElF4E in our yeast or plant cell analyses.

Materials and Methods

Plasmid construction

Maize has two ELC members and the ELC we amplified is located on chromosome 6 (GenBank accession number: KJ811537) (determined through sequence analysis of the maize genome at Phytozome (http://www.phytozome.net/search.php)). Our primer-pairs used in this study were specific for the ELC identified in the yeast-two hybrid (Y2H) assay.

All of the constructs were sequenced before use. Information about the construction of all the plasmids is provided in Table 1. Sequences of all the primers used in this study are listed in Supporting Information Table S1.

Virus and virus inoculations

SCMV-BJ and MCMV were from previously published sources (Fan et al., 2003; Zhang et al., 2011). Crude extracts were prepared by homogenizing the SCMV-BJ-, the Ohio isolate of SCMV- (SCMV-OH, provided by Dr Margaret G. Redinbaugh, Wooster, OH, USA) or MCMV-infected maize leaf tissues in 0.01 M phosphate buffer (pH 7.0) at 1 : 5 (w/v) ratio. The crude extracts were rub-inoculated individually to Bromovirus (BMV)-inoculated maize leaves at 8, 5 or 6 dpi, respectively. The inoculated plants were again covered with plastic domes and grown inside a glasshouse set at 24°C.

Yeast two-hybrid screen

The maize cDNA library screening was performed using a BD Matchmaker Library Construction and Screening Kit (Clontech, Mountain View, CA, USA) as instructed by the manufacturer. Positive colonies were isolated to obtain plasmid for sequencing and the sequences were analyzed through BLASTX searches.

Particle bombardment

Particle bombardment was conducted as described (Finer et al., 1992), with the following specific modifications. To prepare a tungsten particle stock solution, 70 mg of tungsten particles (M17; Bio-Rad, Hercules, CA, USA) was mixed with 1.2 ml 75% ethanol in an Eppendorf tube by vortexing. After a 15-min incubation at room temperature, the tungsten particles were
Table 1 Construction of plasmids

| Construct                  | Primer         | PCR template                      |
|----------------------------|----------------|----------------------------------|
| For Y2H assay (constructs were based on pGBK7 and pGAD7 (Clontech)) |                |                                  |
| pGBK7-SCEM-V-VPg           | VPG-1F, 1R     | cDNA from virus infected maize tissue |
| pGBK7-SCEM-VPg             | VPG-1F, 1R     | cDNA from virus infected maize tissue |
| pGBK7-PEMV-VPg             | VPG-2F, 2R     | cDNA from virus infected maize tissue |
| pGBK7-TVBMV-VPg            | VPG-3F, 3R     | cDNA from virus infected Nicotiana benthamiana tissue |
| pGBK7-SCEM HC-Pro          | Cheng et al. (2008) |                                  |
| pGAD7-ZmEIC                | ELIC-1F, 1R    | maize cDNA                       |
| pGAD7-ZmEIC               | elF4E-1F, 1R   | maize cDNA                       |
| pGAD7-ZmEIC(5:4E)          | elf(iso)-E4F, R | maize cDNA                       |
| For BiFC assay (constructs were based on pUC-SPYNE, pUC-SPYCE (Walter et al., 2004) and pGD (Goodin et al., 2002)) |                |                                  |
| SCVM-BJ VPG-YFP           | VPG-4F, 4R     | cDNA from virus infected maize tissue |
| PenMV VPG-YFP             | VPG-5F, 5R     | cDNA from virus infected maize tissue |
| TVBMV VPG-YFP             | VPG-6F, 6R     | cDNA from virus infected maize tissue |
| SCVM HC-Pro-YFP           | Cheng et al. (2008) |                                  |
| pGD-SPYCE                 | YPFC-2F, R     | pUC-SPYCE                        |
| YFPC-ZmEIC                | ELC-2F, 2R     | maize cDNA                       |
| (pGD-YFPC-ZmEIC)          | elfE4E-2F, 2R  | maize cDNA                       |
| ZmEIC-YFPC               | elF4E-2F, 2R   | maize cDNA                       |
| ZmEIC-YFPC(5:4E)          | elf(iso)-E4F, R | maize cDNA                       |
| For transient expression (constructs were based on pCAMBIA1390-GFP-N1, pCAMBIA1390-GFP-C1 (provided by Dr Elson Blancasflor) and pGD) |                |                                  |
| pCAMBIA1390-GFP-ZmEIC     | ELC-3F, 3R     | maize cDNA                       |
| pCAMBIA1390-GFP-ZmEIC-GFP | ELC-4F, 4R     | maize cDNA                       |
| pCmV-GFP                  | GFP-1F, 1R     | pCAMBIA1390-GFP-N1               |
| pCmV-GFP-ZmEIC            | ELC-2F, 2R     | maize cDNA                       |
| For BMV-VGS (constructs were based on a pC13/F3-13 m (Sun et al., 2013)) |                |                                  |
| pC13/F3-13 m: ELC         | ELC-5F, 5R     | maize cDNA                       |
| pC13/F3-13 m: GFP         | GFP-2F, 2R     | pCAMBIA1390-GFP-N1               |

pelleted by centrifugation at 13 800 g for 5 min and the pellet was rinsed twice in RNase-free H2O. The pellet then was resuspended with 1.2 ml of 50% glycerol solution and stored at −80°C. For bombardment assays, 50 μl of the tungsten particle stock solution was mixed with 5 μl (c. 5 μg) plasmid DNA in an Eppendorf tube. After 5-min incubation on ice, 20 μl of 0.1 M spermidine solution was added to the tube followed by 10 μl of 2.5 M CaCl2 solution. The mixture was vortexed at low speed and then incubated at room temperature for 10 min. A 75% ethanol solution (700 μl) was added to the tube and the tungsten: plasmid DNA particles were pelleted at 17 000 g for 20 s. After two washes in 1 ml of 100% ethanol, the tungsten:plasmid DNA particles were pelleted again and then resuspended by pipetting in 400 μl of polyvinylpyrrolidone (PVP)/ethanol solution (1.6 μl PVP (20 mg PVP in 1 ml H2O) in 400 μl of ethanol). The tungsten:plasmid DNA solution was loaded into a Tefzel tube using a syringe. The tubing with the syringe attached was incubated for 10 min at room temperature. The PVP/ethanol solution was carefully withdrawn from the tubing and the tubing was allowed to dry inside a desiccator for 1 h. The dried tubing was cut into small pieces, inserted into individual slots inside a cartridge, and then the tungsten:plasmid DNA particles were bombarded (220–250 psi) into maize leaves using the Helios Gene Gun system (Bio-Rad) as instructed.

Preparation of MCMV RNA transcripts

The full-length cDNA clone of MCMV (pMCM41) was provided by Dr Kay Scheets (Stillwater, OK, USA). RNA transcripts of MCMV were synthesized as described (Scheets et al., 1993). The RNA transcripts were then treated with RNase-free DNase I (TaKaRa Bio Inc., Otsu, Japan) and purified with RNase-free DNase I (TaKaRa Bio Inc., Otsu, Japan) followed by two phenol/chloroform extractions and one chloroform extraction. The resulting supernatant was mixed with 3 M NaAc (pH 5.2) (10 : 1, v/v) and ethanol (1 : 2, v/v) followed by precipitation for 1 h at −80°C. The RNA transcripts were pelleted at 12 000 g for 5 min at 4°C and the pellet was washed twice with 1 ml 75% ethanol. The pellet was then resuspended in RNase-free double-distilled (dd)H2O.

Maize protoplasts isolation and transfection

Protoplasts were isolated from leaves of maize inbred line Zheng 58 and transfected using a polyethylene glycol (PEG)-mediated method (Sheen, 1991) with modifications. Approximately 100 μl of maize protoplasts (1 × 107) were gently mixed with 10 μg pGDP-GFP or pGDP-GFP-ZmELC, 5 μg of SCVM-BJ viral RNA from purified virus or MCMV RNA produced by in vitro transcription and 110 μl PEG/Ca solution (4 g PEG (MW 4000, Fluka), 2.5 ml 0.8 mannitol, 1 ml 1 M CaCl2 or Ca(NO3)2 in 3 ml H2O) and incubated at room temperature for 15 min. The protoplasts were washed in 440 μl cold W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 2 mM MES, pH 5.7) and then centrifuged at 150 g for 1 min. The pelleted protoplasts were resuspended in 1 ml W5 solution and incubated in the dark at 25°C. The transfected protoplasts were harvested at 12–18 h post transfection (hpt) and used for further analysis. Three independent experiments were conducted and 14 Eppendorf tubes with 1 × 105 protoplasts each were used for each treatment within each experiment. Protoplasts in 10 tubes of the same treatment were pooled and used for protein extraction and Western blot assay as described previously (Cao et al., 2012). Protoplasts from the other four tubes were pooled for RNA isolation followed by qRT-PCR analysis.

Confocal microscopy

For bimolecular fluorescence complementation (BiFC) assays and visualization of GFP in maize protoplasts and leaves, fluorescence signals were visualized under a Nikon Eclipse TE 2000-6 laser-scanning confocal microscope (Nikon, Tokyo, Japan). To visualize subcellular expression of GFP-ZmElc in maize cells,
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Agrobacterium tumefaciens cultures carrying pC13/F1+2, pC13/F3-13:m: ELC or pC13/F3-13:m: GFP were prepared as described (Sun et al., 2013). After induction, the Agrobacterium cultures were individually resuspended in an infiltration buffer (10 mM MES and 10 mM MgCl₂) solution till OD₆₀₀ = 2.0. An Agrobacterium culture harboring pC13/F1+2 was mixed with equal amount of an Agrobacterium culture harboring either pC13/F3-13:m: ELC or pC13/F3-13:m: GFP, and infiltrated into Nicotiana benthamiana leaves using needleless syringes. The 214-bp fragment of ZmELC (representing nucleotides 341–554 from the ATG start codon) amplified and inserted into the BMV vector was from the 3′-untranslated region of the gene which had 43.97% identity with the analogous region from the other maize ELC. In addition, there was no 21 nt or greater stretch of identity between the sequence inserted into the virus vector and the other maize ELC. Therefore, under accepted criteria for silencing (Burch-Smith et al., 2004), the BMV vector expressing our amplified ELC fragment would not directly induce silencing of the second maize ELC located on chromosome 9 (GenBank accession number: KJ811538).

At 3 dpi, BMV virions were isolated from the infiltrated N. benthamiana leaves as previously described (Lane, 1981) with the following modifications. The harvested leaf tissues were ground in liquid nitrogen and then homogenized in an extraction buffer (0.5 M NaAc and 0.8 M HAc) at a 1 : 2 ratio (w/v). The crude extract was loaded into 2 ml tubes, vortexed for 20 s, incubated on ice for 30 min and then centrifuged at 8 000 g for 10 min at 4°C. The resulting supernatant was transferred into clean tubes and mixed (3 : 1, v/v) with 40% PEG (MW 8000, Sigma) containing 0.8 M NaCl followed by a 1-h incubation on ice with slow rocking. BMV virions were pelleted at 15 000 g for 15 min at 4°C and the pellets were resuspended in a small volume of 0.1 M phosphate buffer, pH 7.0. After 30 min incubation on ice, concentrations of partially purified BMV virions were estimated at OD₂₆₀ using a BioPhotometer (Eppendorf, Hamburg, Germany).

BMV viral RNA was extracted from the virions as previously described (Dijkstra & de Jager, 1998) with the following specific modifications. Approximately 100 µl of partially purified BMV virions were mixed with 160 µl RNAse-free ddH₂O, 200 µl viral RNA extraction buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM EDTA) and 40 µl 10% SDS, and incubated at room temperature for 5 min. After two phenol/chloroform extractions and one chloroform extraction, the resulting supernatant (c. 400 µl) was mixed with 3 M NaAc (pH 5.2) (10 : 1, v/v) and ethanol (1 : 2, v/v) followed by precipitation for 1 h at −80°C. Viral RNA was pelleted at 12 000 g for 10 min at 4°C and the pellet was washed twice with 1 ml 75% ethanol. Then the pellet was resuspended in 30 µl RNase-free ddH₂O. Approximately 500 ng BMV viral RNA, the BMV-R primer (Table S1) and the M-MLV reverse transcriptase, was used to synthesize the first-strand cDNA as instructed (Promega). Two microliters of cDNA and primers BMV-1F and BMV-1R (Table S1) were used for PCR analyses to visualize the maintenance of foreign inserts in different BMV VIGS vectors.

Approximately 20 µg of partially purified BMV virions containing the full-length foreign inserts were rub-inoculated to each 1-wk-old Va35 maize seedling. The inoculated plants were covered with plastic domes and grown inside a growth chamber set at 18°C for 7 d before being transferred into a glasshouse set at 24°C with the domes removed.

Results

Identification of a VPg-interacting Elc from maize

In order to identify maize proteins that interacted with SCMV-BJ VPg, a Y2H screen of a maize leaf cDNA library (Cheng et al., 2008) was performed using SCMV-BJ VPg as a bait. A total of 40 positive colonies were isolated for sequencing. One of them contained an intact ORF with 100% amino acid sequence identity to a maize Elc. The coding sequence of the Elc was cloned into pGADT7 and co-transformed with pGBKT7-VPg to yeast strain AH109. Using this cloned sequence in yeast the interaction between VPg and ZmElc in yeast was confirmed (Fig. 1a). To ensure that subsequent analyses were specific for this ELC, strictly specific primers were designed to amplify this ELC for transient expression and silencing studies and analysis of the ELC transcript amounts (see the Materials and Methods section).

In order to determine whether VPg and ZmElc interacted in planta, a BiFC assay was performed in maize protoplasts. The coding sequences of SCMV VPg and ZmElc were cloned into BiFC vectors pUC-SPYNE (YFPY) and pGD-SPYCE (YFPc) to
generate SCMV VPg-YFP<sup>N</sup> and YFP<sup>C</sup>-ZmElc, respectively. SCMV VPg-YFP<sup>N</sup> and YFP<sup>C</sup>-ZmElc were co-transfected into protoplasts. Two other combinations of constructs, SCMV VPg-YFP<sup>N</sup> and YFP<sup>C</sup>, and YFP<sup>N</sup> and YFP<sup>C</sup>-ZmElc, were co-transfected into protoplasts as negative controls. YFP fluorescence was observed in both the nucleus and cytoplasm of the SCMV VPg-YFP<sup>N</sup> and YFP<sup>C</sup>-ZmElc co-transfected protoplasts by 12–18 hpt (Fig. 1b). No YFP signal was detected in the negative controls (Fig. 1b). To determine the frequency of the interaction between SCMV VPg and ZmElc, we examined 100 protoplasts per experiment and calculated the numbers of the protoplasts exhibiting YFP signal from three experiments. The average percentage of protoplasts exhibiting fluorescence signal was 64 ± 4.5% (grand mean for three experiments ± SD). This result indicated that VPg and ZmElc interacted in maize cells.

In order to investigate the interaction specificity between ZmElc and VPgs, interactions between ZmElc and the VPgs from other members of the genus *Potyvirus*, *Pennisetum mosaica virus* (PenMV; Fan *et al.*, 2004) and *Tobacco vein banding mosaic virus* (TVBMV; provided by Dr Xiangdong Li, Tai’an, Shandong), were analyzed through Y2H and BiFC assays. Positive interactions between ZmElc and both VPgs were observed in both assays (Fig. 1a,b). Y2H and BiFC assays also were used to determine whether ZmElc interacted with non-VPg proteins such as HC-Pro, P1 and CP of SCMV. No positive interaction was observed (Fig. 1a,b and data not shown).

Expression analysis of *ZmELC*

In order to analyze whether *ZmELC* showed organ- or tissue-specific expression patterns, the relative amounts of *ZmELC* transcripts were determined by quantitative reverse transcription (RT)-PCR (qRT-PCR) using total RNA extracted from leaf blades, sheaths and roots of 14-d-old maize (cv Va35). The expression levels of *ZmELC* were similar in leaf sheath and root (Fig. 2a). However, the expression level of *ZmELC* was significantly higher in leaf blade than in leaf sheath and root (Fig. 2a).

A second analysis of *ZmELC* mRNA expression pattern was performed with total RNA extracts from both reproductive and vegetative organs of 70-d-old maize (cv Va35) plants. The *ZmELC* transcript quantity was highest in the pistil, with the stamen having a similar quantity to that in the leaf. The root had a lower quantity of *ZmELC* transcript than the other organs (Fig. 2b).

In order to study the subcellular localization of ZmElc in maize, leaves of maize (cv Va35) were bombarded with tungsten: plasmid DNA particles to express a fusion of ZmElc with GFP. The influence of the position of the fusion between ZmElc and GFP on subcellular location was evaluated by fusing ZmElc to either the N or C terminus of GFP (e.g. p1390-ZmELC-GFP and p1390-GFP-ZmELC) and then introduced into leaf cells through bombardment. Green fluorescence was observed at 36 h post bombardment. ZmElc distributed in both the cytoplasm and nucleus of the maize epidermal cells for both fusion proteins (Fig. 2c), suggesting that the location of the fusion, N or C terminus, had no effect on the subcellular location and was similar to that exhibited by free GFP (Fig. 2c). To understand the capacity for ZmElc to localize to the nucleus, a search at the NLSdb (https://rostlab.org/services/nlsdb), a database of nuclear localization signals (Nair *et al.*, 2003), was performed and no nuclear localization signal was found within the ZmElc sequence.
In order to determine whether the nuclear localization of ZmElc was further enhanced during SCMV infection, presumably due to the known nuclear location of VPg (Rajamäki & Valkonen, 2009; Fig. 1b), maize protoplasts were transfected with pGD-GFP-ZmELC and SCMV-BJ RNA or pGD-GFP-ZmELC alone. Green fluorescence was observed at 12–18 hpt. ZmElc localized in both cytoplasm and the nucleus in maize protoplasts transfected with pGD-GFP-ZmELC alone (Fig. 2d), similar to the results obtained during its expression in uninfected maize epidermal cells (compare Fig. 2c with 2d). After co-transfection with SCMV-BJ RNA, the subcellular location of GFP-ZmELC was unchanged in the majority of protoplasts (>99%, n > 60) (Fig. 2d).

ZmELC expression was up-regulated at 4 and 6 d post SCMV inoculation

In order to determine whether ZmELC expression was induced by virus infection, 1-wk-old maize (cv Va35) seedlings were inoculated with SCMV-BJ or phosphate buffer (mock). Total RNA for qRT-PCR analysis was isolated from the inoculated leaves at 4 and 6 dpi, and from first systemically infected leaves at 6 and 10 dpi, respectively. ZmELC transcript amounts were c. 50% and 25% higher in virus-inoculated leaves than in mock-inoculated leaves at 4 and 6 dpi, respectively (Fig. 3a). The ZmELC transcript quantity was 87% higher in the first systemically infected leaves than in equivalent leaves from the mock-inoculated plants at 6 dpi (Fig. 3b). This difference disappeared at 10 dpi (Fig. 3b).

Transient overexpression of ZmELC increased SCMV-BJ RNA accumulation in maize protoplasts

It is known that the VPg plays a key role in potyvirus replication. To investigate the possible role of ZmElc in SCMV replication, maize protoplasts were co-transfected with pGD-GFP-ZmELC and SCMV-BJ RNA. Protoplasts co-transfected with pGD-GFP and SCMV-BJ RNA were used as a control. GFP-ZmElc and GFP were expressed at 18 hpt as determined by Western blot using an anti-GFP antibody (Fig. 4a). qRT-PCR analyses were then conducted on RNA extracts at 18 hpt to quantify the relative expression levels of ZmELC transcript.
and SCMV-BJ RNA. When ZmELC transcript was overexpressed 3.9-fold, the SCMV-BJ RNA amount doubled compared with the control values (Fig. 4b). These findings indicated that transient overexpression of ZmELC was correlated with an increase in SCMV-BJ RNA accumulation.

In order to investigate whether transient overexpression of ZmELC influences the accumulation of another virus species in protoplasts, in vitro transcripts of MCMV, a member of the genus *Machlomovirus* (family *Tombusviridae*) and without a VPg, were co-transfected with either pGD-GFP or pGD-GFP-ZmELC into maize protoplasts. By 18 hpt, transient overexpression of ZmELC was correlated with significantly less accumulation of MCMV RNA (Fig. S1).

**Knockdown of ZmELC expression impaired SCMV infection in maize plants**

In order to investigate further the role of ZmElc during SCMV infection, a newly modified DNA-based BMV VIGS vector (Benavente et al., 2012; Sun et al., 2013) was transformed into *A. tumefaciens* strain C58C1. *Agrobacterium* cultures harboring the virus vector containing a fragment of ZmELC were infiltrated into *N. benthamiana* leaves. VIGS vectors can lose foreign gene inserts over time post inoculation (Bruun-Rasmussen et al., 2007a) and this may be a major cause of the transient nature of the gene-silencing phenotype in grasses (Bruun-Rasmussen et al., 2007a; Ramanan et al., 2013). In addition, the virus without an insert accumulates to higher amounts than those still containing the insert (Bruun-Rasmussen et al., 2007a). To mimic the accumulation and stability characteristics of our BMV vector containing the ZmELC fragment, *Agrobacterium* harboring the BMV vector containing a 205-bp fragment of GFP gene was infiltrated into *N. benthamiana* leaves as a control. BMV virions containing full-length inserts were isolated from infiltrated *N. benthamiana* leaves at 3 d post agro-infiltration and rub-inoculated to leaves of 1-wk-old maize (cv Va35) seedlings (Fig. 5). Initial BMV infection symptoms were seen in the first systemically infected leaves by 5–7 dpi. In a preliminary experiment to determine the influence of BMV-GFP infection on expression of ZmELC in maize, it was determined that there was some inhibitory effect on ZmELC expression when compared with levels in mock-inoculated plants (Fig. S2). This further indicated the worth of the BMV-GFP vector as a control: to account for nonspecific effects of virus infection on ZmELC expression. In the study comparing ZmELC expression levels after inoculation with BMV-ZmELC and BMV-GFP, the second systemically infected leaves at 14–20 dpi were harvested from individually inoculated plants and analyzed for ZmELC silencing through qRT-PCR. ZmELC transcript amounts were decreased by c. 50% in BMV-ELC-inoculated plants compared with the amounts in BMV-GFP-inoculated plants (Fig. 6a). The plants silenced for ZmELC expression did not show any unusual visual phenotype compared with the BMV-GFP infected control plants (Fig. 6b).

In the subsequent experiments, BMV-inoculated leaves were challenged with SCMV-BJ at 8 dpi. The second systemically infected leaf of each assayed plant was harvested at 5 and...
7 dpi to determine ZmELC transcript knockdown efficiency and SCMV-BJ RNA accumulation. The results showed that at 5 dpi with SCMV, a 30% decrease in ZmELC mRNA levels (Fig. 7a) was associated with a 30% decrease in SCMV-BJ RNA accumulation (Fig. 7b). By 7 dpi, although the silencing of ZmELC in the BMV-ELC-inoculated plants was recovering (Fig. 7d), the inhibition of SCMV-BJ RNA accumulation was still significant (Fig. 7e). Simple linear regression analyses showed that there was a positive correlation between ZmELC expression and SCMV RNA accumulation at both 5 (Fig. 7c) and 7 dpi (Fig. 7f).

In order to determine if a similar relationship between ZmELC transcript amount and SCMV accumulation occurred across SCMV isolates, the relationship between ZmELC expression and the accumulation of the SCMV isolate, SCMV-OH, was analyzed. The interaction between SCMV-OH VPg and ZmElc was confirmed through the Y2H system (Fig. S3). As for the SCMV-BJ studies, silencing of ZmELC through VIGS resulted in decreased SCMV-OH accumulation, both effects decaying with time post inoculation (Fig. S3). Taken together, these results indicated that the knockdown of ZmELC was correlated with decreased accumulation of different isolates of SCMV.

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In order to determine whether knockdown of ZmELC expression only inhibited accumulation of viruses encoding a VPg, leaves inoculated 6 d previously with the BMV silencing vectors were challenged with MCMV. Surprisingly, knockdown of ZmELC expression led to increased, rather than decreased, accumulation of MCMV at 7 dpi (Fig. S4).

Knockdown of ZmELC expression in the presence of SCMV decreased Zmelf4E expression

It is well known that eIF4E or its isoform, eIF(iso)4E, is necessary for potyvirus accumulation and their absence provides recessive resistance to potyviruses in plants (Hebrard et al., 2010; Ashby et al., 2011; Mazier et al., 2011; Nieto et al., 2011). eIF4E belongs to a small multigenic family and two eIF4E isoforms, eIF4E and eIF(iso)4E, have been identified in maize (Manjunath et al., 1999). It was reported that potyviruses need only one specific eIF4E isoform to multiply in a specific host, although some potyviruses use both of them (Ruffel et al., 2006; Hwang et al., 2009). To identify which eIF4E isoform was needed by SCMV, a Y2H assay was performed and a positive interaction was found only between ZmeIF4E and VPg (Fig. 8a). This interaction was further confirmed by the BiFC assay in maize protoplasts (Fig. 8b). These results implied that ZmeIF4E may be employed by SCMV during its infection in maize.

In order to investigate if there is a relationship between the amounts of ZmeIF4E and ZmELC transcripts during SCMV infection, we inoculated maize plants using the same BMV-VIGS vector and SCMV-BJ inoculation procedure described above. The second systemically infected leaf of each assayed plant was
harvested at 5 dpi to determine the ZmELC transcript knockdown efficiency, SCMV-BJ RNA accumulation and expression of ZmElF4E transcript by qRT-PCR. The result indicated that, in the presence of SCMV and after knockdown of ZmELC, the expression of ZmElF4E was decreased (Fig. 8c). To determine whether the decrease of ZmElF4E expression also occurred in the absence of SCMV, developmentally similar leaves from plants infected only with the BMV-VIGS vector were sampled for qRT-PCR analysis at 13 dpi. Expression of ZmElF4E transcript was not affected by the knockdown of ZmELC in the absence of SCMV (Fig. 8d). To exclude the possibility that the decreased expression of ZmElF4E was due to SCMV infection, the ZmElF4E transcript quantity was detected in the SCMV- and mock-inoculated leaves at 4 and 6 dpi, and the first systemically infected leaves of these plants at 6 and 10 dpi. The transcript quantity of ZmElF4E in the SCMV-inoculated plants was similar to that in the mock-inoculated plants at most of these time points, although ZmElF4E transcript quantity in first systemically infected leaf was c. 30% higher than that in the same leaf from the mock-inoculated plants at 6 dpi (Fig. 8e). This result indicated that SCMV infection alone did not downregulate ZmElF4E expression. In addition, the Y2H and BiFC assay showed that there was no direct interaction between ZmElc and ZmElF4E (Fig. 8a,b). Taken together, these data suggest that in the presence of SCMV, knockdown of ZmELC caused less ZmElF4E expression.

Discussion

Early studies on Elongin C showed that it was important for suppressing the pause by RNA polymerase II during the elongation phase of transcription in mammalian cells (Bradsher et al., 1993a,b). It later was determined that the elongin complex with
Elongin C did not stimulate transcription elongation (Koth et al., 2000), but did play roles in stress responses by targeting specific factors that regulated protein kinase activities in yeast (Jackson et al., 2000). In mammalian cells it was demonstrated that this small protein worked as a core component of the Skp1-Cullin-F-box (SCF)-like ubiquitin ligase (E3 ligase) (Iwal et al., 1999). To date, the expression pattern and physiological function of Elongin C is not known in plants. In our study, we identified a ZmElc that interacted with SCMV VPg in both yeast and plant cells (Fig. 1a,b). The expression levels of ZmELC transcript were higher in the leaf blade than in the leaf sheath and root in 14-d-old maize seedlings (Fig. 2a). In adult maize plants the highest ZmELC transcript quantity was in the pistil (Fig. 2b). In maize cells, ZmElc located in both cytoplasm and nucleus (Fig. 2c,d). These findings provide the first evidence of Elongin C organ and subcellular localization in plants. Our observation that plants knocked down for ZmELC expression through VIGS did not show an abnormal visible phenotype (Fig. 6b) agreed with a previous report that Elongin C Arabidopsis null mutants were unaffected in growth (Hua & Vierstra, 2011).

The ZmELC we identified in this study is located in maize chromosome 6 and belongs to a maize two gene family. The other ZmELC is located in chromosome 9 and shares a low nucleotide sequence identity with chromosome 6 ZmELC in the 3’ untranslated region, although both ZmELCs have high nucleotide identity in the first 75% of the ORF from the start codon (Fig. S5a). All of our expression analyses were specific for the ELC encoded on chromosome 6 and thus, the lack of an altered visual phenotype in maize plants silenced for expression of this ZmELC may be due to the residual expression of this gene in our silenced plants and/or to functional complementation by the other member in this gene family. However, functional complementation by the ZmELC encoded in chromosome 9 might be minor, because its RNA expression level in leaf blades (at similar
Plant viruses have small genomes that encode a very limited number of proteins. Therefore, they depend on host factors to complete their infection cycles. As an important host factor involved in SCMV accumulation, ZmElc may be hijacked by the virus from its normal role in the plant to act as an enhancer during viral RNA replication. This speculation is supported by the fact that the ZmElc interacted with SCMV VPg, the suggested primer for viral RNA replication, and the accumulation of SCMV RNA was increased when ZmELC was transiently overexpressed in maize protoplasts (Fig. 4b). This may also explain why ZmElc was not further re-located into the nucleus by VPg through its interaction after SCMV infection (Fig. 2d): cytoplasmic localization is needed for potyvirus replication (Wei & Wang, 2008; Cotton et al., 2009; Laliberté & Sanfaçon, 2010; Wei et al., 2010a).

We also determined in this study that ZmElc interacted with the VPg from other potyviruses, PenMV and TVBMV (Fig. 1a, b). This suggests that these viruses may also require this protein for normal replication. However, the positive influence of Elongin C on virus accumulation may be limited to the potyviruses, because overexpressing or silencing ZmELC resulted in, respectively, reduced or enhanced accumulation of MCMV (Figs S1, S4). In this instance, Elongin C may compete with MCMV proteins for host factors necessary for virus multiplication. It will be meaningful to carry out additional investigations to determine the role of Elongin C during the life cycles of different virus species, which, in turn, may further define the function of plant Elongin C in the absence of virus infection.

VPg is reported to interact with several proteins of both viral and host origin. Within the interaction network, the intrinsically disordered VPg acts as a hub protein that regulates many processes during virus infection (Jiang & Laliberté, 2011). Potyvirus VPg functions in viral RNA translation through its interaction with host elf4E or its isof orm, elf(iso)4E. Host elf4E binds to the 5’ cap structure of mRNA and it is best known for its essential function in the initiation of mRNA translation (Jackson et al., 2010). We determined that SCMV VPg bound ZmeIF4E in addition to ZmElc, but the two host proteins did not interact directly with each other (Fig. 8a,b). It is possible that ZmElc facilitates SCMV accumulation by interacting with the VPg during viral RNA replication before an interaction between VPg and ZmeIF4E, which is necessary for viral RNA translation. In this scenario, the downregulation of ZmELC, which was shown to inhibit SCMV RNA accumulation, would provide less viral RNA for translation and less requirement for ZmeIF4E and its transcript. Indeed, ZmeIF4E transcript amounts were decreased after silencing ZmELC and challenging with SCMV (Fig. 8c,d). It was shown that the expression of Brassica oleracea elf4E protein can be induced by TuMV infection (Léonard et al., 2004). It is still unknown if elf4E transcript amounts could be induced by potyvirus infection, but our evidence showed that it can be induced, at least transiently (Fig. 8e). Because elf4E transcript can be induced during virus infection, it is possible that ZmeIF4E transcript amounts would decline when less virus accumulates due to ZmELC silencing.

Many recessive resistance genes against potyviruses have been identified in the last decade, such as sbm-1 and sbm-2 in pea (Johansen et al., 2001; Gao et al., 2004), ppr 1, 2 and 6 in pepper, (Ruffel et al., 2002; Kang et al., 2005), mol1 and mol2 in lettuce (Nicaise et al., 2003), rym 4 in barley (Kanyuka et al., 2005) and wlv in white lupin (Bruun-Rasmussen et al., 2007b). Most of these genes encode elf4E or its isof orm elf(iso)4E. In this study, we showed that ZmElc could facilitate virus accumulation; thus, maize mutants lacking ZmElc should be more resistant to virus infection. Future experiments should challenge the Arabidopsis Elongin C null mutants with a potyvirus to determine if they are more resistant. In addition, investigations should be made into whether knockdown of ELONGIN C can confer a broad resistance against other potyviruses in other plant species.

In the first report of VIGS in maize using the BMV-based vector, in vitro transcribed BMV RNAs were used to inoculate plants (Ding et al., 2006). In a later study the silencing vector was propagated in N. benthamiana, an intermediate host for BMV, before inoculation to the target grass plant (Ding et al., 2007). To obtain more uniform infection in various BMV-VIGS experiments, a modified inoculation procedure was established through normalizing virus titers between N. benthamiana extracts by qRT-PCR before inoculating maize leaves (van der Linde et al., 2011). In a more recent report a DNA-based BMV-VIGS vector was described and used to silence genes in rice and maize through Agrobacterium-mediated vacuum infiltration or vascular puncture inoculation (Benavente et al., 2012; Sun et al., 2013). However, the reported Agrobacterium-mediated vacuum infiltration was not successful in maize and therefore we further modified the inoculation method described by van der Linde et al. (2011) for maize. After propagating the virus in N. benthamiana leaves, virions were partially purified from the infiltrated leaves and virions maintaining full-length inserts were quantitated before inoculation to individual maize plants (Fig. 5). This modified inoculation method is easy to perform and ensures more uniform infection and gene silencing in maize. Barley stripe mosaic virus (BSMV) infection was reported to change the expression of common plant defense-related genes and resulted in a decreased susceptibility of wheat to Magnaporthe oryzae (Tu fen et al., 2011). Although the effect of the BMV-VIGS vector on host defense-related genes remains unexplored, using a similar amount of BMV inocula in different treatments should equalize, and thus, minimize any confounding general effects caused by BMV infection alone during VIGS studies. In addition this new method allows the production of large amounts of recombinant BMV with full-length foreign inserts at a low cost.

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