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Short communication

The 2a protein of *Cucumber mosaic virus* induces a hypersensitive response in cowpea independently of its replicase activity

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ABSTRACT

Resistance in cowpea to infection with strains of *Cucumber mosaic virus* (CMV) involves a local hypersensitive response (HR), and previous studies indicated that the 2a replicase of CMV is involved in HR induction. In this study, we confirmed and extended this observation by demonstrating that the nonviral expression of the 2a protein encoded by CMV is able to induce a cell death response in cowpea plants, whereas no other CMV-encoded proteins elicits such response. The 2a single-amino acid mutant, F631Y, no longer induces the necrosis response, yet the A641S mutant still induces cell death. The 2a double mutant, F631Y and A641S, does not induce HR. However, the three 2a mutants have comparable replicase activities in a fluorescence reporter assay. The $2a^{D610A}$ mutant that alters the highly conserved GDD motif abolishes the replicase activity, however it does not affect HR induction in cowpea. The $2a^{301–778aa}$ fragment introduced with the same D610A mutation in the GDD motif is also capable of inducing HR in cowpea. Collectively, these findings suggest that the 2a protein of CMV is sufficient to induce HR in cowpea independently of its replicase activity.

The hypersensitive response (HR) is one of the most extensively studied resistance reactions that occur during an incompatible interaction between a plant and pathogen (Greenberg, 1997; van Ooijen et al., 2007; Soosaar et al., 2005; Thomma et al., 2011). Genetic studies have established that the interaction between a plant-borne resistance (*R*) gene and a corresponding pathogen-borne avirulence (*Avr*) gene leads to the activation of a signal transduction cascade necessary to induce HR. An elicitor molecule, which could be a translation product of an *Avr* gene or a by-product of a reaction catalyzed by an *Avr* gene product, is thought to be recognized as a ligand by a receptor encoded by an *R* gene (receptor-ligand model) (van Ooijen et al., 2007; Baker et al., 1997; Thomma et al., 2011). Many *Avr* genes have been identified in viral, bacterial and fungal pathogens, and several *R* genes have been cloned from different plant species (van Ooijen et al., 2007; Soosaar et al., 2005; Hammond-Kosack and Jones, 1997; Thomma et al., 2011).

Cucumber mosaic virus (CMV) has an unusually large host range, encompassing virus replication in ~1000 species of plants in 365 genera of 85 families (Palukaitis et al., 1992). The genome of CMV contains five genes located on three genomic RNAs, designated RNA1, -2 and -3, which are expressed from either the genomic RNAs or two subgenomic RNAs. RNA2 encodes the 5′-proximal 98 kDa 2a protein, a component of the viral 'replicase' (Hayes and Buck, 1990), and the 3′-proximal 11 kDa 2b protein that is involved in host-specific long-distance trafficking and the suppression of gene silencing (Brigneti et al., 1998; Ding et al., 1995). Many strains of CMV induce HR on inoculated leaves of cowpea plants, and several studies have implicated the CMV 2a replicase protein in HR induction (Karasawa et al., 1999; Kim and Palukaitis, 1997; Tao et al., 2002). The amino acids of the 2a protein that are required for HR induction in cowpea have been identified using chimeric CMV viruses (Karasawa et al., 1999; Kim and Palukaitis, 1997; Tao et al., 2003). However, the use of replicating CMV in these studies revealed that the participation of additional viral components in HR induction could not be excluded. To gain a better insight into HR induction by CMV in cowpea, we employed a non-viral expression method to determine and characterize the essential CMV components required for inducing HR in cowpea.

The CMV constructs were generated by cloning the CMV-Fny cDNA sequences into a suitable plant binary vector (Fig. 1A). The full-length open reading frames (ORFs) of the *1a* and *2a* genes were amplified and cloned into the plant binary vector pCXSN (Chen et al., 2009). The genes for *2b*, movement protein (MP), coat protein (CP) and enhanced green fluorescence protein (EGFP) were amplified and inserted into pRTL2 plasmids (Restrepo et al., 1990).

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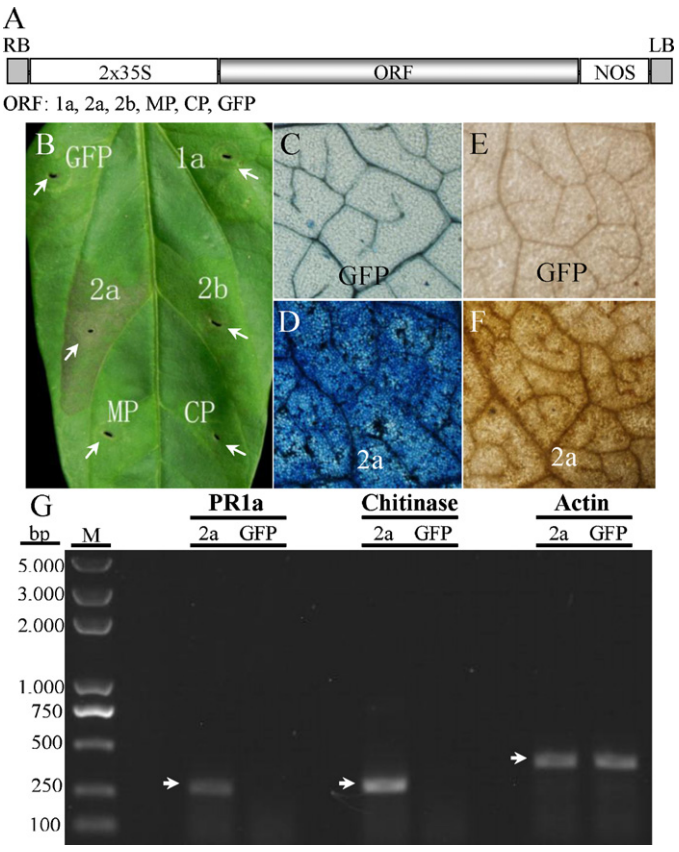

Fig. 1. CMV 2a protein is an elicitor of the hypersensitive response in cowpea. (A) Schematic diagrams of the CMV constructs used for agro-infiltration. The expression of GFP, *1a*, *2a*, *2b*, MP and CP is driven by the 35S promoter on the plant binary vector. RB and LB indicate the right and left borders of the T-DNA, respectively. (B) Infiltration of *Agrobacterium* GV3101 strains containing the GFP, 1a, 2a, 2b, MP, CP constructs into the leaves of cowpea plants. The photographs were taken 8 days after infiltration. (C, D) The cells of GFP (C)- or 2a (D)-infiltrated leaves were stained with Trypan blue. The images were taken using a light microscope at 100× magnification. (E, F) Detection of H_2O_2 accumulation in GFP (E)- or 2a (F)-infiltrated leaves was visualized using a light microscope at 100× magnification. (G) Expression of the *PR1a* and *Chitinase* genes was detected by semi-quantitative RT-PCR using PR gene-specific primers. Total RNA was isolated from 2a- and GFP-infiltrated cowpea leaves at 36 h post-infiltration (hpi). The *actin* gene was used as a control for the equivalent expression of constitutive genes.

The *2b*, MP and EGFP cassettes in pRTL2 were then transferred into the binary vector pBinPLUS (van Engelen et al., 1995), and pRTL2-CP was mobilized into the binary vector pLH7000 (Brauer et al., 1999). Site-directed mutagenesis was performed to alter amino acids Phe-631 to Tyr-631, Ala-641 to Ser-641, both sites, or Asp-610 to Ala-610 of 2a replicase using the two-step PCR procedure described by Higuchi et al. (1988). The replication reporter construct CP::erGFP contains the infectious cDNA of CMV RNA3 (Yao et al., 2011) for which the CP gene was replaced by an endoplasmic reticulum-targeted green fluorescent protein (erGFP) (Haseloff et al., 1997). The CP::erGFP reporter cassette (Fig. 2 A) was constructed with the mini binary vector pCB301-2x35S-HDVRZ-NOS and used for the replication assay by co-infiltration with 1a and 2a wide-type or 2a mutants. The *2a* deletion derivatives were generated by direct PCR amplification using a combination of specific primer pairs; the products were inserted into pCXSN using similar strategies as in the construction of the full-length 2a protein. All of the primers used in this study can be found in Supplementary Table S1. All of the constructs were transformed into competent *Agrobacterium* cells (GV3101) by electroporation. For the transient gene expression assay, *Agrobacterium* strains bearing the expression plasmids were grown to saturation in LB medium. Following centrifugation, the cells were resuspended in infiltration buffer (20 mM MES, pH 5.6, 10 mM $MgCl_2$, and 100 μM acetosyringone) at a concentration of OD_{600} = 1.0. After 3 h of incubation at room temperature, this suspension was pressure-infiltrated into the abaxial surface of cowpea leaves using a syringe. Prior to the infiltration, a small incision was made at the site of infiltration using a sterile needle to enhance the efficiency of infiltration. The infiltrated plants were grown at 28 °C with supplementary lighting of 16 h per day. HR formation was scored over a 2 week period post-inoculation as either positive or negative for necrotic lesions. The cowpea cultivar Zaojiang No.1 was used for all of the HR induction assays. Trypan blue (0.3%) was used to stain the dead cells, and 0.01% DAB was used to visualize the H_2O_2 burst at 36 h post-infiltration (hpi). Total RNA from the infiltrated leaves at 36 hpi was analyzed for the expression of *PR1a* and *Chitinase* using semi-quantitative RT-PCR.

To determine the CMV component required for elicitation of the hypersensitive response in cowpea, we transiently expressed each protein of the necrosis-inducing CMV-Fny strain in the epidermal cells of cowpea leaves via agro-infiltration. Infiltration of the 2a recombinant cDNA construct into the leaves of cowpea plants resulted in the appearance of cell death in the infiltrated areas within 4 days (Fig. 1B). No such response was observed in equivalent areas infiltrated with 1a, 2b, MP and CP (Fig. 1B), suggesting that 2a alone is sufficient for the induction of cell death in cowpea. The transient expression of GFP failed to produce necrotic lesions in cowpea, indicating that neither agrobacterium nor the infiltration process causes cell death in this assay (Fig. 1B). To determine whether the necrosis induced by the expression of the CMV 2a protein resulted from the hypersensitive response, Trypan blue was used to stain the dead cells selectively in the infiltrated tissues. The cells stained blue in the 2a-infiltrated leaves (Fig. 1D), whereas Trypan blue was not taken up in the GFP-infiltrated leaves (Fig. 1C). DAB was also used to detect the H_2O_2 burst during HR, revealing that H_2O_2 accumulated only in the cells of the 2a-infiltrated leaves (Fig. 1F), whereas H_2O_2 was not detected in the cells of the GFP-infiltrated leaves (Fig. 1E). The 2a-infiltrated leaves were also analyzed for the expression of defense response markers. A semi-quantitative RT-PCR analysis revealed a strong induction of *PR1a* and *Chitinase* gene expression in the 2a-infiltrated leaves (Fig. 1G). In contrast, induction was not detected in the GFP-infiltrated leaves (Fig. 1G).

In a previous study, Kim and Palukaitis (1997) identified two amino acids, Phe-631 and Ala-641, of the Fny 2a protein as determinants of CMV-mediated HR in cowpea. However, Karasawa et al. (1999) showed that a single amino acid, Phe-631, of the 2a protein determines HR in cowpea. To test the effects of these mutants on HR induction using a nonviral expression assay, mutations were introduced into either or both of the amino acids (positions 631 and 641) of the 2a replicase of CMV-Fny. Each mutant construct (Fig. 2A) was agro-infiltrated into the leaves of cowpea plants. The Ala-641 to Ser-641 (A641S) mutant elicited a cell death response that was indistinguishable from that induced by the infiltration of wild-type Fny 2a. However, the Phe-631 to Tyr-631 (F631Y) mutant did not induce cell death (Fig. 2B), nor did the double mutant Phe-631 to Tyr-631 and Ala-641 to Ser-641 (F631Y & A641S) (Fig. 2B). Our results for $2a^{A641S}$ and $2a^{F631Y\&A641S}$ are consistent with the earlier study by Kim and Palukaitis (1997), though the phenotype of $2a^{F631Y}$ differed from their report. We have previously inoculated the RNA transcripts of $RNA2^{F631Y}$ with RNA1 and RNA2 of CMV-Fny into cowpea and found that the $2a^{F631Y}$ mutant could induce enlarged necrotic local leaf lesions (Tao et al., 2003). To determine whether other CMV-encoded proteins affect the HR response induced by $2a^{F631Y}$, $2a^{Wt}$ or its site mutants were co-infiltrated with all of the other CMV proteins (1a + 2b + MP + CP). Even in the presence of all of the other CMV proteins, $2a^{F631Y}$ still did not induce the HR in cowpea (Fig. 2C). The reason(s) for the difference between