A single amino acid change in a geminiviral Rep protein differentiates between triggering a plant defence response and initiating viral DNA replication

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We have devised an in planta system for functional analysis of the replication-associated protein (Rep) of African cassava mosaic virus (ACMV). Using this assay and PCR-based random mutagenesis, we have identified an ACMV Rep mutant that failed to trigger the hypersensitive response (HR), but had an enhanced ability to initiate DNA replication. The mutant Rep–green fluorescent protein (GFP) fusion protein was localized to the nucleus. Sequence analysis showed that the mutated Rep gene had three nucleotide changes (A6→T, T375→G and G852→A); only the A6→T transversion resulted in an amino acid substitution (Arg to Ser), which is at the second residue in the 358 amino acid ACMV Rep protein. Our results indicate that a single amino acid can alter the differential ability of ACMV Rep to trigger the host-mediated HR defence mechanism and to initiate viral DNA replication. The implications of this finding are discussed in the context of plant–virus interactions.

African cassava mosaic virus (ACMV) is an economically important geminivirus in the genus Begomovirus, family Geminiviridae. The bipartite ACMV genome consists of two circular single-stranded DNA components that share an almost identical sequence of approximately 200 nucleotides that contains the origin of replication and encodes eight putative proteins (Stanley, 1983; Stanley & Gay, 1983). Of these, the replication-associated protein (Rep) is essential for the initiation of rolling circle replication (RCR) and is involved in the modulation of gene expression (Etessami et al., 1991; Haley et al., 1992; Hong & Stanley, 1995; Saunders et al., 1991). ACMV Rep also plays an important role in host–virus interactions as it triggers the hypersensitive response (HR) although the question remains as to whether this is a classical virulence factor/R-gene interaction (Dixon et al., 1994; Greenberg, 1997). Expression of Rep in Nicotiana benthamiana causes rapid cell death and a systemic burst of H2O2 production (Hong et al., 2003; van Wezel et al., 2002a). Thus, while Rep is vital for viral DNA replication, Rep also acts as an elicitor of the HR which is detrimental to the virus life cycle. Such a functional dichotomy creates a paradox for the establishment of a successful viral infection. Indeed, almost all viral proteins required for virus infection, including encapsidation (Berzal-Herranz et al., 1995; Saito et al., 1987), virus movement (Chu et al., 1999; Garrido-Ramirez et al., 2000; Malcuit et al., 1999; Mushi et al., 1989) and replication (Erickson et al., 1999; Kim & Palukaitis, 1997), can be recognized by plants and elicit HR. However, viruses have evolved to encode functions that can counteract the HR defence mechanism. For instance, the HR induced by the nuclear shuttle protein of tomato leaf curl New Delhi virus is inhibited by the transcriptional activator protein of the same virus (Hussain et al., 2007).

Viruses could survive the HR by producing modified proteins which still possess the functions essential for the viral life cycle but are not recognized by the plant defence mechanism. To test this hypothesis, we used an artificial system to produce ACMV Rep mutants to investigate their functions in inducing HR and/or initiating viral DNA replication in transgenic N. benthamiana line pOri-2. This line contains a direct repeat of the ACMV origin of replication (ori) flanking a non-viral DNA fragment including the β-glucuronidase (GUS) coding sequence and a polyadenylation region, in which initiation of trans-replication occurs in the presence of the ACMV Rep protein (Hong et al., 2003).

Error-prone PCR amplification and the primers PP50 and PP77 (van Wezel et al., 2002a) were used to mutate the
ACMV Rep gene, cloned into a modified potato virus X vector (PVX/GFP) (van Wezel et al., 2001). The cloning strategy was designed to produce in-frame fusions of each mutated Rep coding sequence and the green fluorescent protein (GFP) gene. A C-terminal GFP tag had no effect on the ability of ACMV Rep to initiate DNA replication and to trigger the HR, while allowing direct visualization of Rep-GFP expression and cellular localization.

By screening plants inoculated with PVX/Rep–GFP RNA transcripts produced from SpeI-linearized recombinant plasmids we identified a mutant Rep, designated Rep*, which did not induce HR (Fig. 1). Expression of Rep*–GFP from PVX/Rep*–GFP produced only chlorotic lesions on the inoculated leaves of both non-transgenic N. benthamiana and pOri-2 plants (Fig. 1b). These plants survived and developed only mild systemic symptoms which resembled the phenotype of plants inoculated with PVX/mRep–GFP, containing an untranslatable Rep gene (designated mRep). In contrast, localized infection by PVX/Rep–GFP triggered a typical HR and led to extensive cell death at 3–7 days post-inoculation (p.i.) (Fig. 1a); systemic expression of Rep–GFP resulted in the collapse of the whole plant at 10–14 days p.i. Expression of Rep–GFP (Fig. 1c) and Rep*–GFP (Fig. 1d) and their associated HR or chlorotic lesions were also visible as GFP fluorescence under long-wavelength UV (365 nm) light. Both fusion proteins were localized to the nuclei (data not shown).

PCR detection using primers P1 and P2 (Hong et al., 2003) (Fig. 2a) demonstrated that Rep–GFP or Rep*–GFP expressed from PVX/Rep–GFP or PVX/Rep*–GFP, respectively, could mobilize the episomal replicon in pOri-2 plants to produce the diagnostic 1.64 kb DNA fragment, which could only be generated from the circular molecules (Fig. 2). These results showed that Rep*–GFP retained the ability to initiate DNA replication. In three separate experiments, semiquantitative PCR assays (Fig. 2b–e) showed that the level of the circular replicon in pOri-2 plants infected with PVX/Rep*–GFP (Fig. 2b–e, lane 5) was higher than in those infected with PVX/Rep–GFP (Fig. 2b–e, lane 3). This was estimated by measuring the intensity of the 1.64 kb amplicon produced after 20 (Fig. 2b) or 25 (Fig. 2c) cycles of PCR, on an agarose gel stained with 0.5 μg ethidium bromide ml⁻¹. There was no difference in the level of endogenous 18S rRNA gene among all treated samples, although the rRNA signal could be saturated after a greater number of PCR cycles. No replicon-specific PCR product was observed for mock-inoculated plants or for plants infected with PVX/GFP or PVX/mRep–GFP (Fig. 2b–e, lanes 1, 2 and 4, respectively). The finding was further confirmed by real-time quantitative PCR using primers P3 (5’-CAAGATCAAAACTAGTCCCTCAG-3’) and P4 (5’-CAGTGGATCCACATTGGCAAG-3’) (Fig. 2a). The average amount of episomal DNA replicon from five DNA samples extracted from pOri-2 infected with PVX/Rep*–GFP (Fig. 2f, bar 5) was three- to fourfold more than that in plants infected with PVX/Rep–GFP (Fig. 2f, bar 3), while none was detected in controls.

Expression of the ACMV Rep gene in pOri-2 was verified further by RT-PCR (Fig. 3). Primers PP77 and PP82 (van Wezel et al., 2002b) amplified the predicted fragment of approximately 1.3 kb, representing the entire Rep gene and part of the PVX genome immediately upstream of the inserted Rep sequence. This confirmed that Rep RNA was stably maintained in the recombinant viruses during systemic infection (Fig. 3a). No Rep-specific RNA was

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**Fig. 1.** Induction of local responses to ACMV Rep in transgenic pOri-2 plants. (a) Necrotic lesions on leaves inoculated with PVX/Rep–GFP. (b) Chlorotic lesions on leaves inoculated with PVX/Rep*–GFP. Inset panels show areas of necrosis and chlorosis at higher magnification. (c, d) Correlation between transient expression of Rep– and Rep*–GFP and host responses to PVX/Rep–GFP (c) or to PVX/Rep*–GFP (d). Leaves were photographed 12 days p.i. under white light (a, b), or through a yellow filter under long-wavelength UV light (c, d). In (c) and (d), tissues expressing GFP fusion proteins showed green fluorescence, while non-GFP-expressing regions appeared red due to chlorophyll fluorescence. Dead tissues (c) appeared bright or yellow in the centre of necrotic lesions.
detected in mock-inoculated plants or in plants infected with PVX/GFP. However, all PVX vectors were infectious, since a 430 bp fragment indicating PVX RNA was detected (Fig. 3b) using primers PP269 (Zeng et al., 2008) and PP373 (5'-GTATGCTGTTCGTTGTGATCTCTGTTAG-3'). As controls, a 185 bp 18S rRNA fragment was detected using primers PP271 and PP272 (Zeng et al., 2008) in all RNA samples (Fig. 3c). These data were consistent with Western blot analyses showing that PVX coat protein (approx. 27 kDa) accumulated to similar levels in each infection (Fig. 3d). Moreover, Rep–GFP and Rep*–GFP (approx. 67 kDa), produced by PVX/Rep–GFP or PVX/Rep*–GFP, and free GFP (approx. 26.9 kDa), expressed from PVX/GFP, were detectable by immunoblotting using GFP antiserum (Fig. 3e).

The different host responses to viral infection and the different capacities of Rep–GFP or Rep*–GFP to initiate DNA replication suggested that one or more nucleotide change had occurred in the Rep gene, resulting in an amino acid substitution(s) in Rep. To characterize the mutation(s), the complete sequences of the Rep gene in PVX/Rep*–GFP, PVX/Rep–GFP and PVX/mRep–GFP were determined. Direct sequencing revealed that three nucleotide changes at positions 6 (A6→T), 375 (T375→G) and 852 (G852→A) had been introduced into the open reading frame for Rep*. However, only the A6→T transversion led to substitution of the second amino acid arginine (R) with a serine (S) in the Rep* mutant. Both the T375→G transversion and the G852→A transition were silent mutations. In the mRep gene, the start codon was converted to stop codon TAG, consistent with the fact that no Rep was detected in PVX/mRep–GFP infection (Fig. 3e, lane 4).

These results show that viral expression of Rep–GFP was responsible for induction of the HR defence response and for initiation of replicon replication which is consistent with RCR, as demonstrated in a similar trans-replication system (Morilla et al., 2006). Rep*–GFP is an HR-induction ‘loss-of-function’ mutant which also increases initiation of the episomal DNA replication. It is unlikely that Rep-induced cell death affected the level of episomal replication.
DNA replication. At the time of sampling, there were comparable levels of viral RNA, coat protein and, most significantly, Rep–GFP fusion proteins (Fig. 3) in plants infected with PVX/Rep–GFP or PVX/Rep*–GFP. It should be noted that the nucleotide change of T375A was not neutral for AC4 and caused a valine to glycine change. However, the Rep-overlapping AC4 gene is not involved in DNA replication and the induction of HR (Selth et al., 2004; van Wezel et al., 2002a). Nevertheless, to attribute the observed phenotype to Rep* unequivocally, we introduced just the A6A change into the wild-type Rep to produce PVX/Rep(R2S)–GFP. Infection of pOri-2 plants with PVX/Rep(R2S)–GFP did not induce cell death. PCR analysis of the 1.64 kb DNA amplicon (Fig. 3f) and direct detection of the circular replicon by Southern blot with probes specific to the transgene sequences (Fig. 3g) indicated that Rep(R2S)–GFP mobilized the episomal replicon and the DNA level increased compared with that triggered by Rep–GFP. Thus, the elevated level of Rep*-initiated episomal DNA replication probably arose because the R2S change enhanced the Rep* activity to initiate RCR. The single amino acid (R2S) mutation responsible for the functional change in Rep* also suggests that the differential functions of ACMV Rep can be separated.
Functions of geminiviral Reps can be classified into two broad categories which contribute differently to the virus life cycle (Gutierrez, 2000; Hanley-Bowdoin et al., 1999; Laufs et al., 1995a). First, the oligomeric Rep protein plays an essential role in viral DNA replication (Desbiez et al., 1995; Fontes et al., 1994; Laufs et al., 1995b; Orozco & Hanley-Bowdoin, 1998; Pant et al., 2001). Rep cleaves the viral genome at the replication initiation site (TAATATT)AC and also recirculates progeny ssDNA during RCR (Stanley, 1995). However, Rep is not a DNA polymerase and geminiviruses rely on and reprogramme the host cell machinery to replicate their genomes within the nuclei of fully differentiated cells which are otherwise inactive in DNA replication. This process is mainly mediated by Rep (Ach et al., 1997; Egelkrout et al., 2001; Kong et al., 2000; Nagar et al., 1995). Moreover, efficient viral DNA replication also depends on interactions between Rep and the virus-encoded replication-enhancing protein and coat protein (Malik et al., 2004; Settlage et al., 2005), as well as with several other host factors (Bagewadi et al., 2004; Castillo et al., 2003; Kong & Hanley-Bowdoin, 2002; Luque et al., 2002; Settlage et al., 2001; Singh et al., 2007; Xie et al., 1999, 1995). Second, Rep of ACMV and related begomoviruses can elicit HR (Hong et al., 2005; Nagar et al., 2000; Xie et al., 1999, 1995). Rep and its homologue in tomato yellow leaf curl virus can also interact with the plant cell machinery to elicit the hypersensitive cell death response (described in the next section). We speculate that a Rep* mutant could be elucidated. We speculate that a Rep* mutant could be elucidated. We speculate that a Rep* mutant could be elucidated. We speculate that a Rep* mutant could be elucidated. We speculate that a Rep* mutant could be elucidated.

The biological relevance of our findings with respect to natural ACMV infection, in which the production of Rep is tightly controlled to an extremely small amount, remains to be elucidated. We speculate that a Rep* mutant could also occur naturally during ACMV infection of plants to produce a Rep derivative which can avoid recognition by the host defence. This idea can be tested by the generation of a site-directed ACMV mutant with Rep(R2S) and by examination of its effect on bona fide ACMV replication. On the other hand, due to the N-terminal Met excision (NME) by methionine aminopeptidase (Giglione et al., 2004), it is likely that the Rep* mutant will lack Met 1 and will most probably become acetylated at its now N-terminal serine. A survey of the geminiviral Rep proteins in the database reveals that only Rep of Indian cassava mosaic virus possesses a serine at position 2, and most geminiviral Reps have Ala or Thr at this position and are likely to undergo NME to remove Met 1 (Meinnel et al., 2005). Therefore, an amino acid alteration at position 2 to one of the non-bulky residues could cause a more drastic modification of the Rep protein; this may impact its specific recognition of the origin of replication that is needed to initiate RCR and may also influence Rep to trigger HR or other pathways that are toxic to the cell.

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