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Resistance to cassava mosaic disease in transgenic cassava expressing antisense RNAs targeting virus replication genes

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Summary

African cassava mosaic virus (ACMV) is a major contributor to cassava mosaic disease (CMD), the economically most important and devastating disease of cassava in Africa. We have developed transgenic cassava plants with increased ACMV resistance using improved antisense RNA technology by targeting the viral mRNAs of *Rep* (*AC1*), *TrAP* (*AC2*) and *REn* (*AC3*). Viral DNA replication assays in detached leaves demonstrated that replication of two ACMV isolates was strongly reduced or inhibited in most transgenic lines. After ACMV infection of plants using biolistic inoculation, several lines remained symptomless at lower infection pressure (100 ng viral DNA/plant). Symptom development was reduced and attenuated even at higher DNA doses. Transgenic ACMV-resistant plants had significantly reduced viral DNA accumulation in their infected leaves. Short sense and antisense RNAs specific to *AC1* were identified in transgenic lines expressing *AC1* antisense RNA, suggesting that the short RNAs mediate interference by post-transcriptional gene silencing. Our results demonstrate that resistance to ACMV infection of cassava can be achieved with high efficacy by expressing antisense RNAs against viral mRNAs encoding essential non-structural proteins, providing a new tool to combat CMD in Africa.

Introduction

Cassava mosaic disease (CMD) can result in massive economic losses and can destabilize food security in Africa, as evidenced during the severe CMD epidemics in the 1990s (Legg and Thresh, 2003). In 2002, the total cassava losses in Africa due to CMD were estimated to be 19.6%–27.8% of actual production of 97 million tons (FAO, 2003). CMD is caused by either African cassava mosaic virus (ACMV) alone or by virulent recombinants from different cassava geminiviruses and synergisms among these geminiviruses. For example, EACMV-UG1, a recombinant between a Ugandan isolate of ACMV and the closely related virus East African cassava mosaic virus (EACMV), causes severe symptoms and has been linked to the recent pandemic (Gibson *et al*., 1996; Zhou *et al*., 1997; Pita *et al*., 2001). This event underscores the importance of developing geminivirus-resistant cassava by either conventional breeding or genetic engineering. If possible, resistance should be effective against several or all ACMV strains. Although some tolerant cultivars exist, it has been difficult to produce ACMV-resistant cassava by conventional breeding because of the high heterozygosity and strong inbreeding depression of many elite varieties or farmer-preferred landraces. In addition, the polygenic character of the CMD resistance trait and rapid evolution of geminiviruses in the field also impede conventional breeding efforts (Zhou *et al*., 1997; Akano *et al*., 2002; Legg and Thresh, 2003). Transgene-mediated approaches could offer new resistance traits/genes that currently do not exist within the cassava germplasm pool.

A number of strategies to interfere with ACMV DNA replication and virus proliferation by genetic engineering have been reported, mostly using *Nicotiana benthamiana* as a model system. Until recently, cassava was recalcitrant to most molecular approaches and genetic strategies, mostly because of difficulties in genetic transformation (reviewed in

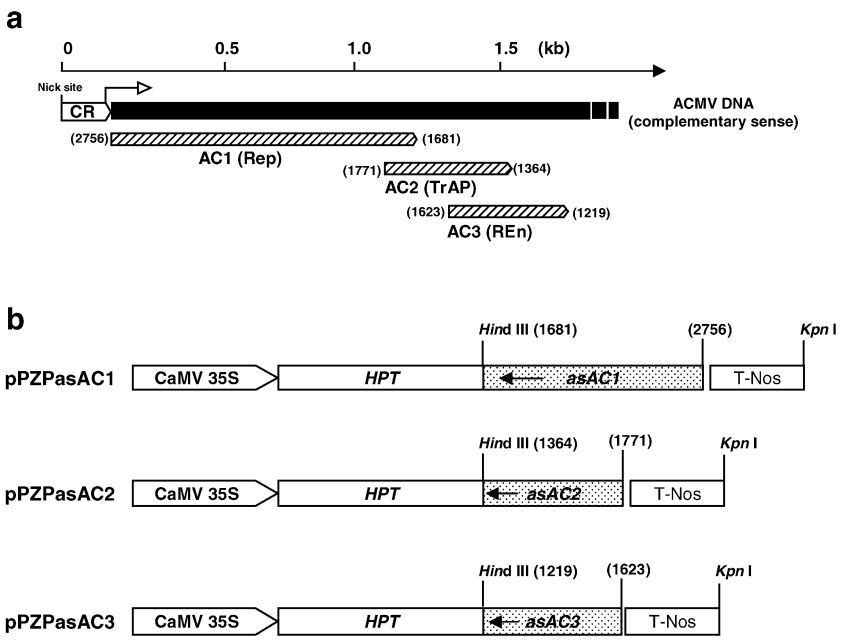


Figure 1 Genomic arrangement of three overlapping genes encoded on the complementary sense strand of African cassava mosaic virus (ACMV) DNA-A (a) and antisense RNA expression constructs (b). The antisense genes of *AC1*, *AC2* and *AC3* were separately cloned as fusions to the 3′ untranslated region (3′UTR) of the hygromycin phosphotransferase (*HPT*) gene. The fusion gene was under transcriptional control of the cauliflower mosaic virus (CaMV) 35S promoter and contained the nos polyadenylation signal (T-Nos). Each expression cassette was integrated in the T-DNA region of the binary vector pPZP100. The positions of the cloned DNA fragments in the genome of the ACMV isolate originating from Kenya (ACMV-KE) are indicated in parentheses (nucleotide positions follow GenBank J02057).

Taylor *et al*., 2004). Several interference strategies have targeted the viral genes required for DNA replication and gene expression. For example, the expression of a full-length *AC1* transgene and the Rep protein with a mutated NTP-binding domain could interfere with DNA accumulation during ACMV infection (Hong and Stanley, 1996; Sangare *et al*., 1999; Chatterji *et al*., 2001). The accumulation of defective interfering DNA of ACMV in transgenic *N. benthamiana* also resulted in reduced susceptibility to infection with closely related strains of ACMV (Stanley *et al*., 1990; Frischmuth and Stanley, 1991). In some cases, resistance was clearly dependent on the expression of the protein; in others, it was probably caused by post-transcriptional gene silencing (PTGS; Hong and Stanley, 1996; Sangare *et al*., 1999). The expression of full-length or truncated ACMV proteins in cassava, however, may have a deleterious effect on plant development as these proteins are known to interact with plant proteins that function in the control of gene expression and defence (Hanley-Bowdoin *et al*., 1999; Kong *et al*., 2000). Constitutive expression of viral proteins might also reduce the acceptance of ACMV-resistant transgenic crops by regulators and consumers. In addition, as most of the experimental approaches discussed above were developed for *N. benthamiana*, ACMV resistance strategies ultimately need to be tested in cassava plants to confirm their reproducibility in the original virus host.

ACMV encodes three non-structural proteins, Rep (AC1), TrAP (AC2) and REn (AC3), on the complementary sense strand of DNA-A. They have key roles in viral replication and transcriptional regulation (Hanley-Bowdoin *et al*., 1999; Figure 1a). Here, we report that increased ACMV resistance can be achieved by the expression of viral antisense RNAs (asRNAs). To ensure the efficient expression of the asRNAs, we used an improved expression strategy (Bejarano and Lichtenstein, 1994) by inserting the full coding sequences of *Rep*, *REn* and *TrAP* separately, in antisense orientation, into the 3′ untranslated region (3′UTR) of a hygromycin phosphotransferase gene (*HPT*) under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Figure 1b). This linkage to the selectable marker increases the probability that asRNA is produced in the regenerated transformants, and thus provides a better success rate, while reducing the workload associated with the handling, regeneration and analysis of transformants that lack integration of the gene of interest.

Results

Construction of transgenic cassava plants expressing viral asRNAs of *AC1*, *AC2* and *AC3*

Transgenic cassava plants were regenerated via embryogenesis from hygromycin-resistant embryogenic suspension-cultured cells obtained after *Agrobacterium*-mediated transformation. These included 25 lines for antisense *AC1* (*asAC1*), 41 lines for antisense *AC2* (*asAC2*) and 34 lines for antisense *AC3* (*asAC3*). Ten per cent of transgenic plant lines showed mutant phenotypes, such as abnormal leaves, stunted growth and fasciated stems. These phenomena have been frequently observed in cassava transformation using

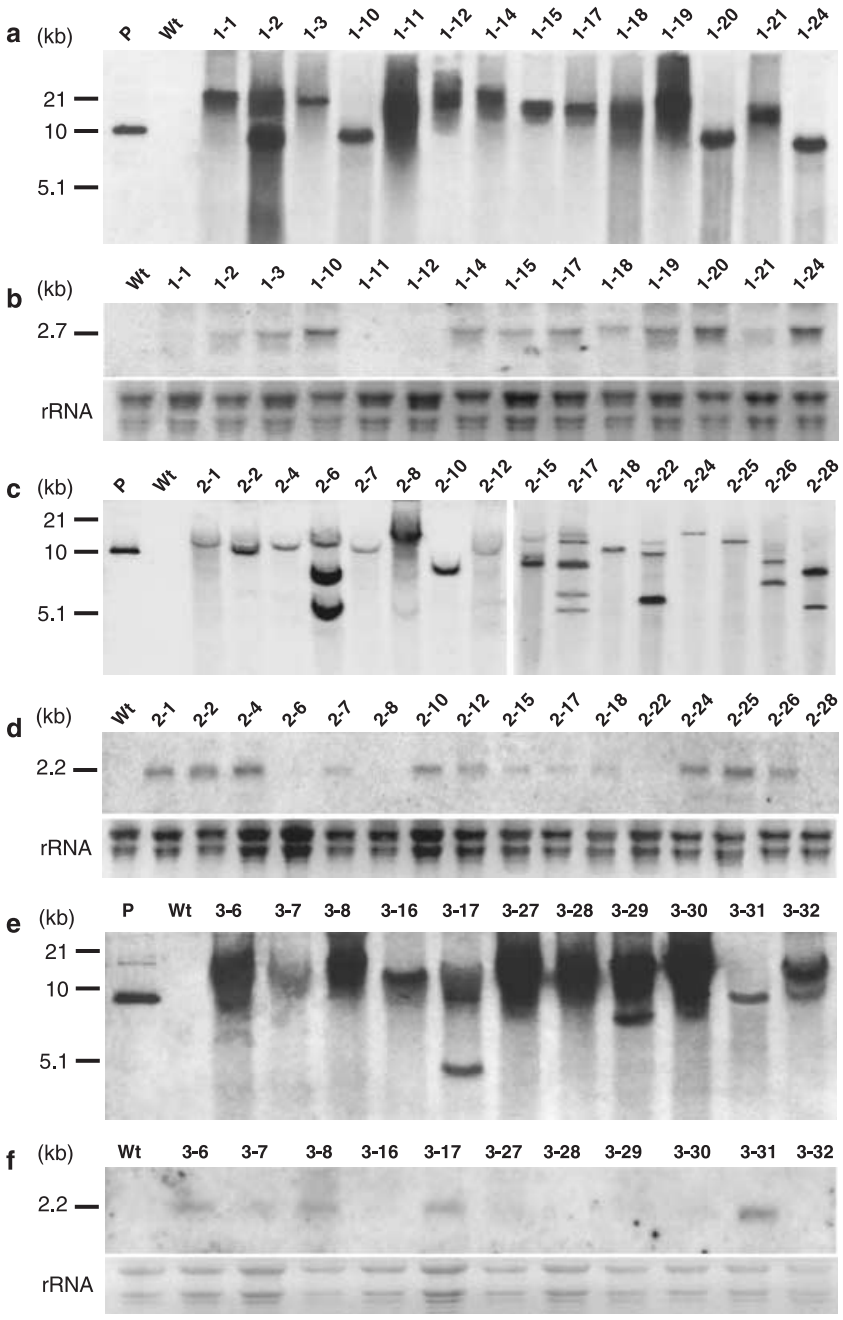


Figure 2 Southern and Northern blot analyses of transgenic cassava lines for transgene integration and expression. Transgene integration patterns in *asAC1* lines (a), *asAC2* lines (c) and *asAC3* lines (e) detected in *Kpn*I-digested genomic DNA by corresponding DIG-labelled probes of *AC1*, *AC2* and *AC3*. Expression profiles of *asAC1* lines (b), *asAC2* lines (d) and *asAC3* lines (f) in leaves using antisense probes of *AC1*, *AC2* and *AC3*, respectively. P, control plasmid digested with *Kpn*I; Wt, wild-type control; *asAC1* lines indicated by 1-*x*, *asAC2* by 2-*x* and *asAC3* by 3-*x*.

friable embryogenic callus (Raemakers *et al*., 2001; Taylor *et al*., 2004). After screening by polymerase chain reaction (PCR) and phenotype comparison of *in vitro* plants (data not shown), we only selected plant lines that were PCR positive and had a normal phenotype for further analysis. We selected 14 *asAC1*, 16 *asAC2* and 11 *asAC3* lines for further analysis to confirm the integration of the three viral asRNA genes by DNA blot analysis using related DIG-labelled probes for *AC1*, *AC2* and *AC3* (Figure 2a,c,e).

The expression of *asAC1*, *asAC2* and *asAC3* RNAs was detectable by reverse transcriptase-polymerase chain reaction (RT-PCR) in all plant lines (data not shown). RNAs of expected length could also be detected in most plant lines by RNA blot analysis (Figure 2b,d,f). The levels of the viral asRNAs were inversely correlated with the transgene insertion number. In most cases, in plants with a single copy, transgene RNAs were expressed at higher levels than in plants with multicopy insertions. For example, *asAC2* lines 2-1, 2-2, 2-4 and 2-10 each

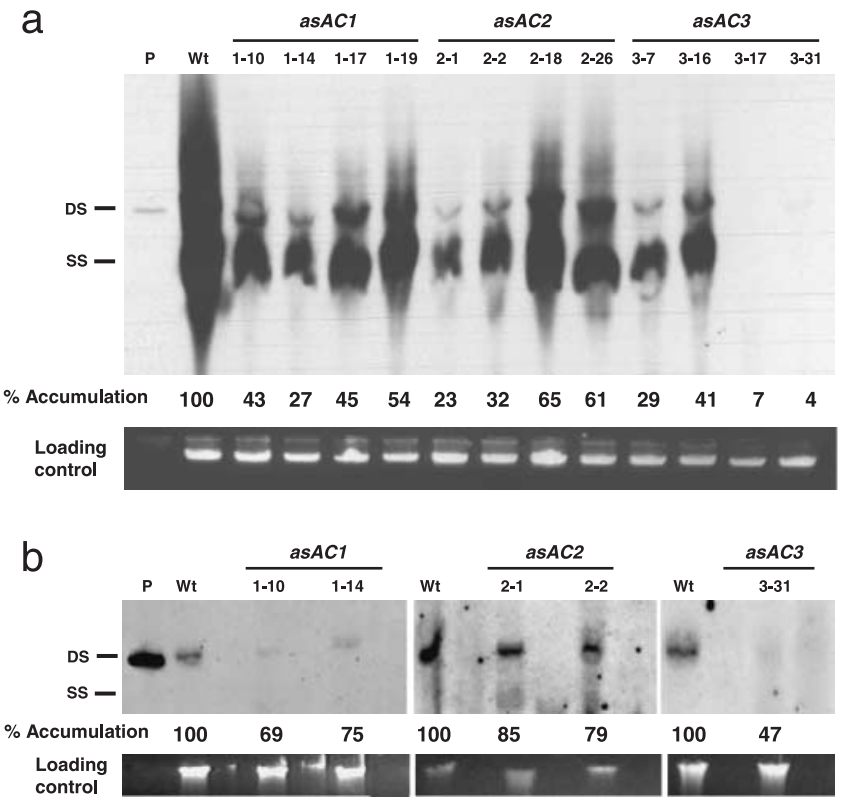


Figure 3 Decreased viral DNA accumulation after biolistic inoculation of leaf discs with African cassava mosaic virus isolates originating from Cameroon (ACMV-CM) (a) and Kenya (ACMV-KE) (b). For technical details, see 'Experimental procedures'. P, control plasmid digested with *Pst*I for ACMV-CM and *Mlu*I for ACMV-KE; Wt, wild-type control; *asAC1* lines indicated by 1-*x*, *asAC2* by 2-*x* and *asAC3* by 3-*x*. The positions of viral single-stranded (SS) and double-stranded (DS) DNA forms are indicated.

contained a single transgene insertion, and their expression of *asAC2* RNAs was higher than in lines 2-6, 2-8, 2-22 and 2-28, which each contained multiple insertions of *asAC2* (Figure 2c,d). This result would be consistent with increased silencing of the transgenes in these lines, a phenomenon that has been frequently reported for plants with multicopy insertions of transgenes (Assaad *et al*., 1993; Que and Jorgensen, 1998; Lechtenberg *et al*., 2003).

Reduced virus accumulation in leaf discs of *in vitro* plants

In order to select the most promising ACMV-resistant plants for further propagation and testing, we first analysed viral DNA replication and accumulation in ACMV-inoculated leaf discs from selected *in vitro* transgenic plant lines, as described by Zhang and Gruissem (2003). Tests were performed with ACMV isolates originating from Cameroon (ACMV-CM; Figure 3a) and Kenya (ACMV-KE; Figure 3b). For ACMV-CM, viral DNA accumulation was strongly decreased in all tested *asAC1* (46%–73%), *asAC2* (35%–77%) and *asAC3* (59%–96%) lines, with two *asAC3* lines showing an almost complete absence of viral DNA (Figure 3a, lines 3-17 and 3-31). For ACMV-KE, all *asAC1* lines showed reduced DNA accumulation (by about 30%), while the group of *asAC2* and *asAC3* lines contained lines with more than 50% reduction (Figure 3b). Of the 12 lines tested with both ACMV strains, lines 1-14, 1-17, 2-1, 2-2 and 3-31 showed the most consistent decrease in viral DNA accumulation. All of these lines strongly expressed the respective asRNAs (Figure 2). This suggests an inverse correlation between asRNA expression and viral DNA accumulation, as previously shown by Bendahmane and Gronenborn (1997).

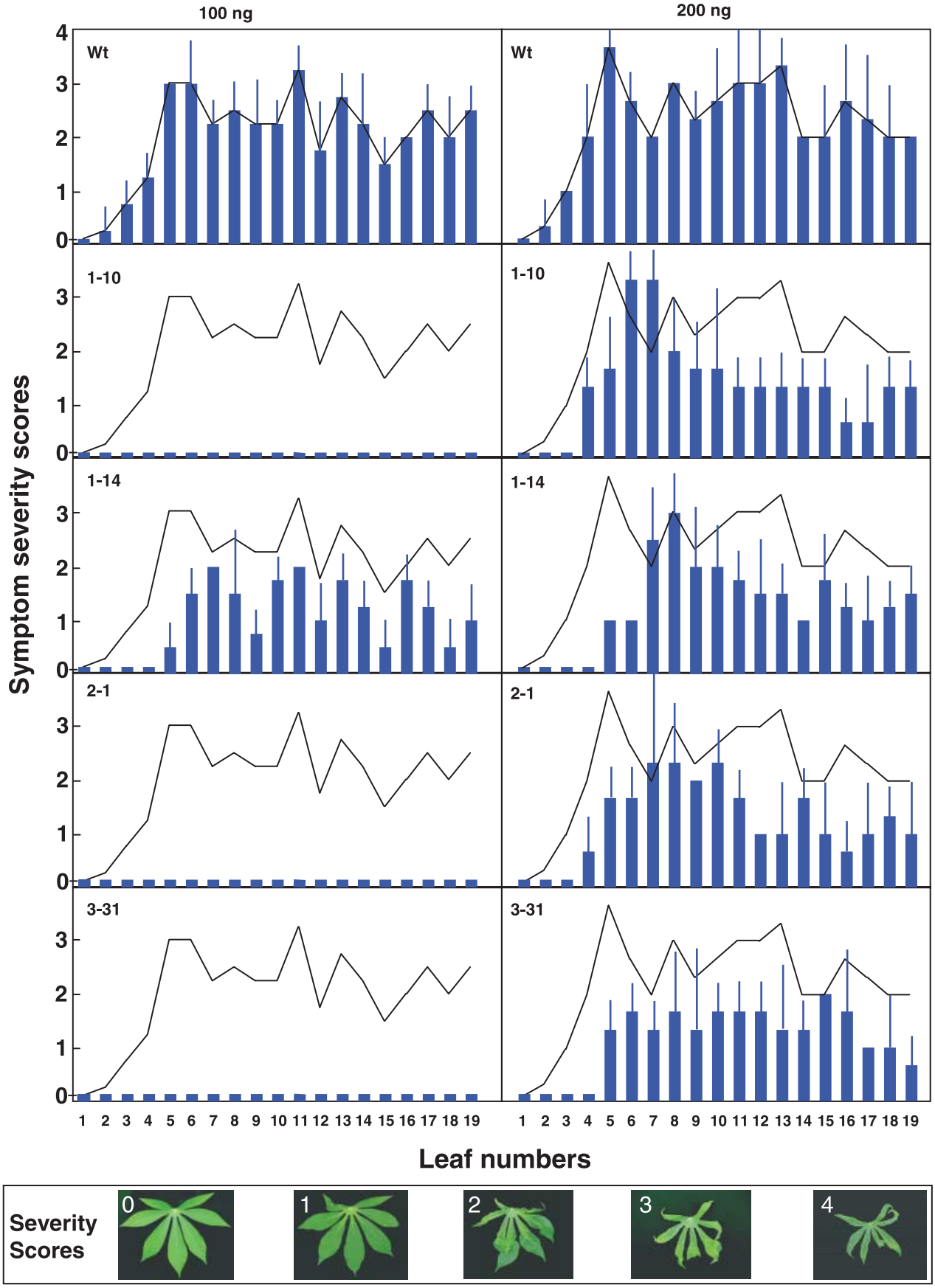
ACMV-resistant cassava plants show delayed and attenuated CMD symptoms and viral load pressure-dependent levels of resistance

We optimized several parameters for ACMV infection by biolistic inoculation of cassava plants under our glasshouse conditions. Parameters included age and plant size, location of inoculation and amount of delivered DNA. In our optimized procedure, 4-week-old and at least 20 cm tall cassava plants generated from stem cuttings were bombarded at the apex, including immature leaves and apical young stem and meristem. One hundred nanograms of input viral DNA was sufficient for a 100% infection rate with wild-type cassava plants. Typical mosaic symptoms appeared on new emerging young leaves 6 days after inoculation. Symptoms included mosaic bleaching of leaves, leaf deformation and decrease in

Figure 4 Resistance of transgenic cassava plants to African cassava mosaic virus (ACMV) infection. Transgenic cassava plants expressing antisense RNAs were infected with 100 ng of DNA per plant from a Nigerian ACMV strain (ACMV-NOg) by biolistic inoculation. Non-transformed plants (a, f) showed typical disease symptoms of virus infection. Similar symptoms were observed in a transgenic plant line with low resistance (2-26, c), whereas highly resistant transgenic plant lines (3-31 and 2-1) were free of disease symptoms (b, e and h) and a partially resistant line (1-14) displayed attenuated disease symptoms (d and g).

leaf size (Figure 4a,f). Different leaves that emerged after inoculation usually showed a range of symptom severity. Symptoms were evaluated according to a standard scale (Fauquet and Fargette, 1990) from 0 (asymptomatic) to 4 (severe mosaic and distortion of leaves with a 50%–80% decrease in size). On this scale, a score of 1 represents very light symptoms, whereas a score of 2 represents mild symptoms (Figure 5).

Two sets of experiments were conducted in selected plant lines. One group of transgenic lines was challenged with 100 ng viral DNA per plant, and another group with 200 ng viral DNA per plant. As shown in Figures 4 and 5, when transgenic plant lines were inoculated with 100 ng viral DNA per plant, several lines (e.g. 1-10, 2-1 and 3-31) showed no symptoms on newly emerging leaves (Figures 4b,e,h and 5, left panel). Compared with wild-type plants (Figures 4a,f and 5, left panel), the remainder of the tested lines (e.g. 1-14) developed symptoms at a much reduced level of severity (approximately 50%) and with a delayed appearance (new leaf 2 of wild-type plants compared with leaf 5 or 6 in transgenic lines; Figure 5, left panel). Symptoms in these transgenic lines included reduced chlorosis close to the main veins, but not extended mosaic formation, leaf deformation or decrease in leaf size (Figure 4d,g), suggesting that the agronomic performance of these plants might be unimpaired in the presence of the virus. Attenuation of symptom development and a decrease in symptom severity were also observed when the inoculation pressure was raised to 200 ng input viral DNA per plant (Figure 5, right panel). The transgenic lines displayed symptoms after 9–12 days in emerging leaf 4 or 5, compared with control plants in which symptoms appeared on leaf 2, 6 days post-inoculation (Figure 5). Lines performing best when charged with 100 ng viral DNA also showed the mildest symptoms when inoculated with 200 ng viral DNA (Figure 5, right panel), indicating that resistance is viral load pressure dependent. Under our experimental viral load pressure, even among the transgenic lines with the lowest resistance (e.g. 2-26), each plant showed reduced symptoms on most of the newly emerging leaves, including a significant number of leaves that were completely symptomless. Of the total number of leaves from three infected 2-26 plants, if we disregard the first two leaves, nine of 51 leaves showed no symptoms and several leaves showed strongly reduced symptoms, while all leaves of wild-type plants showed strong


100 ng
200 ng
Wt
Wt
1-10
1-10
1-14
1-14
2-1
2-1
3-31
3-31
Symptom severity scores
0 1 2 3 4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Leaf numbers
Severity Scores
0
1
2
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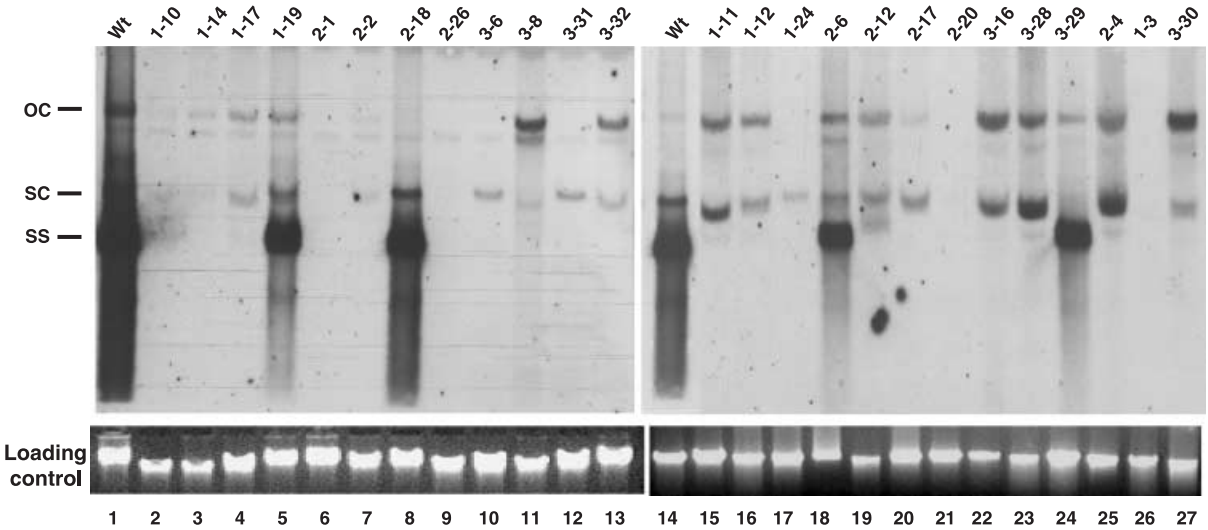

Figure 6 Southern blot analysis of African cassava mosaic virus (ACMV) replication in wild-type and transgenic plants showing reduced viral DNA accumulation in resistant transgenic lines. Five micrograms of total DNA extracted from the leaves collected from the top of infected plants per line was loaded for gel electrophoresis for DNA blotting. Viral DNA was detected using a DIG-labelled *AV1* probe. Viral single-stranded (SS), supercoiled (SC) and open-circular (OC) DNA forms are indicated.

symptoms (data not shown). Between individual plants of a given wild-type or transgenic genotype, leaves at comparable positions showed variations in symptom severity.

Accumulation of ACMV DNA is decreased in infected transgenic cassava plants

Viral DNA was extracted from wild-type and transgenic plants inoculated with 200 ng DNA per plant. The accumulation of ACMV DNA was decreased in most of the infected transgenic lines compared with wild-type plants (Figure 6). In lines with the most clearly attenuated symptoms (1-10, 1-14, 2-1, 2-2, 2-20 and 3-31; Figure 4), only very low levels of viral DNA could be detected, confirming the correlation between symptom development and the accumulation of ACMV DNA (Figure 6).

Accumulation of small interfering RNA (siRNA) in ACMV-infected transgenic lines

Virus resistance in plants can be induced by the simultaneous expression of sense RNAs and asRNAs, and is associated with the accumulation of small RNAs (Waterhouse *et al*., 1998, 2001; Hamilton and Baulcombe, 1999). In the described transgenic cassava plants, such small RNAs may be generated by processes similar to unintended PTGS induction by sense transgenes (Chellappan *et al*., 2004a), or by the formation of double-stranded RNA (dsRNA) between transcripts from an antisense *AC* transgene integrated in the cassava genome and a sense gene of the virus, and subsequent action of a DICER-like enzyme to produce siRNA in a sequence-specific manner. We probed for the presence of small dsRNAs in infected and uninfected untransformed control and two *asAC1* lines (1-10 and 1-19). No small RNAs could be detected in uninfected plants, indicating that no pre-induced PTGS effects are active in the tested transgenic lines. However, small RNAs of about 24 nt, homologous to sense and antisense *AC1* sequences, were detectable in infected wild-type and transgenic plants (Figure 7). The ACMV-resistant line 1-10 produced more siRNA than the non-resistant line 1-19 and wild-type plants (Figure 7, lanes 3, 5, 7). The presence of *AC1*-specific small RNAs in ACMV-infected cassava has been reported recently (Chellappan *et al*., 2004b), and can be interpreted as a natural antiviral defence response that is, however, overcome by the virus in wild-type plants. The mode of generation of these small RNAs is unclear. Further study

Figure 5 Cassava mosaic disease (CMD) symptom development in transgenic and non-transformed cassava plants infected with an African cassava mosaic virus isolate originating from Nigeria (ACMV-NOg) at two different inoculation pressures (left panels, 100 ng; right panels, 200 ng). Average symptom scores (bars) on leaves 1–19 for four plants per transgenic genotype are shown in comparison with the trend line (black line) of scores for non-transformed plants (Wt) at the same infection pressure. Three examples of transgenic lines *asAC1* (1-10), *asAC2* (2-1) and *asAC3* (3-31) that display strong resistance and one (*asAC1* line 1-14) with moderate resistance are shown. The bottom panel illustrates representative leaves showing different degrees of symptoms for the evaluation of symptom severity scores (0, no symptoms; 4, severe symptoms).

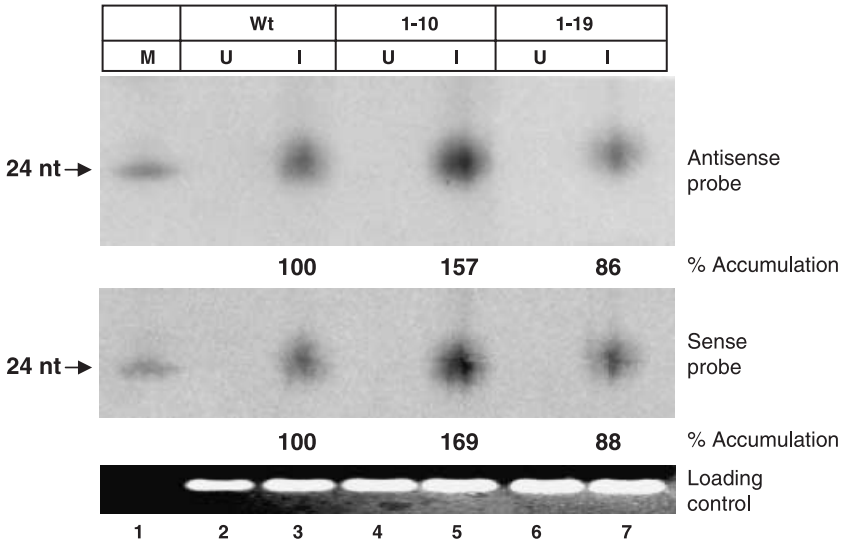

Figure 7 RNA gel blot analysis of *AC1*-specific small interfering RNAs (siRNAs) in two selected *asAC1* transgenic plant lines, 1-10 and 1-19, and in wild-type plants (Wt). Without infection, no siRNAs were detected (U, lane 2, 4, 6). In contrast, siRNAs were detected in all infected plants (I, lane 3, 5, 7) using both antisense and sense probes. Two 24 nt oligonucleotides synthesized on the basis of sequences from the antisense and sense DNA of *AC1* from African cassava mosaic virus (ACMV) DNA-A served as size markers (lane 1, M). Equal loading of low molecular weight RNA was verified by ethidium bromide staining. Detection was with α-^{32}P-labelled riboprobes specific to both sense and antisense sequences of *AC1*.

using probes specific to different regions of *AC1* may discriminate the origin of these small RNAs.

Discussion

Recent reports have demonstrated the feasibility of engineering resistance to plant virus infection by the constitutive expression of viral proteins as intact or mutant forms, or by the induction of PTGS (reviewed by Dasgupta *et al*., 2003; Goldbach *et al*., 2003). For most examples, however, only laboratory-scale evaluation of resistance has been reported using model species, such as *N. benthamiana*. For agronomic applications, engineered resistance must not only afford sufficient protection to disease symptom development, but also must be hereditable over many generations. In addition, engineering resistance should provide protection against the many sequence variants that make up the viral quasispecies in natural environments. Of the available strategies, protein expression is often effective against related viruses, but may pose problems with acceptance because of possible interactions with other viruses or restrictions in the current regulatory system (Goldbach *et al*., 2003). Experimental approaches based on gene silencing have been shown to be very efficient in conferring resistance; however, concerns about the long-term stability of PTGS in natural settings have been increasing (Prins, 2003). Continuous expression of a PTGS trigger may cause nonspecific effects (Van Houdt *et al*., 2003), or result in transcriptional silencing of the triggering gene with a concomitant loss of resistance (Aufsatz *et al*., 2002; Fojtova *et al*., 2003; Zilberman *et al*., 2004). Stable expression of viral asRNA could provide a useful and effective alternative because it does not require the expression of a functional viral gene product. In addition, small differences in viral DNA sequences that occur in natural virus populations should not significantly affect RNA–RNA base pairing between long molecules. If asRNAs induce PTGS after annealing with virus-derived RNAs, the effective dsRNAs would be present only transiently after virus infection, therefore reducing the possibility of either nonspecific effects or transcriptional silencing of the asRNA genes. Reports published to date have shown that the expression of asRNAs of specific genes from tomato golden mosaic virus (TGMV), tomato yellow leaf curl virus (TYLCV), bean golden mosaic virus (BGMV) and cotton leaf curl virus (CLCuV) in transgenic plants results in partial or full resistance to related geminiviruses (Day *et al*., 1991; Bejarano and Lichtenstein, 1994; Bendahmane and Gronenborn, 1997; Aragão *et al*., 1998; Asad *et al*., 2003).

We have engineered cassava plants with increased ACMV resistance using improved asRNA technology. To select transgenic plants stably expressing asRNA, we constructed transgenes in which the DNA for the viral asRNA gene was fused to the 3'UTR of the *HPT* gene (Figure 1b; Bejarano and Lichtenstein, 1994). The ACMV genes targeted in our approach act early in infection and have important functions in virus–host interaction, as well as in the regulation of viral DNA replication and gene expression (Hanley-Bowdoin *et al*., 1999). We expected that interference with these functions would reduce virus reproduction and shift the balance between viral proliferation and plant defence in favour of defence. In most of our selected cassava lines, asRNAs of the expected size were clearly detectable by Northern blotting (Figure 2b,d,f).

The efficacy of asRNA expression against ACMV DNA replication was analysed using three different ACMV strains and two different assay systems. In order to test a large number of independent lines, we first employed a transient assay system by inoculating isolated leaf discs with infectious viral DNA by particle bombardment. This assay can reveal differences in resistance between cassava genotypes and also genotype-specific plant–virus interactions that result in differential accumulation of the various forms of viral DNA (Zhang and Gruissem, 2003). In the transgenic asRNA-expressing lines, we found a general decrease in the accumulation of viral DNA in all assay systems (Figure 3). Lines expressing *asAC1* RNA showed the most consistent decrease in viral DNA accumulation, and *asAC3* lines displayed the highest levels of resistance. In two of the *asAC3* lines, viral DNA synthesis appeared to be fully suppressed (Figure 3). The decrease in viral DNA replication varied between 35% and 96% and equally affected all ACMV DNA forms. We observed no difference in response to inoculation with ACMV-KE, which is the source for the asRNA genes, and ACMV-CM (Figure 3a,b). The homology in the asRNA regions between these two viruses is 96.7% for *AC1*, 94.9% for *AC2* and 92.8% for *AC3*. Together, the results suggest that our strategy using long asRNA constructs is robust against at least some sequence variation. Although our leaf disc assay mainly measures processes within and in close vicinity to the bombarded cells, bombardment of cassava apices on intact plants allowed the analysis of transgene effects on systemic infection. In this case, we used a Nigerian ACMV strain (ACMV-NOg), because this virus develops more pronounced symptoms on the plant after infection (Liu *et al*., 1997). This strain shows 97.3%, 97.8% and 97.3% homology to the *asAC1*, *asAC2* and *asAC3* transgenes, respectively. Depending on the transgene and infection pressure, we observed complete or partial resistance to infection. Several transgenic lines were fully resistant at an infection pressure of 100 ng viral DNA per plant, which resulted in reproducible infections of wild-type plants (Figures 4 and 5). The transgenic lines that showed reduced viral DNA replication in the leaf disc assay also had the highest resistance after whole-plant inoculation, confirming the direct relevance of the leaf disc assay (Zhang and Gruissem, 2003). At an increased viral load pressure of 200 ng viral DNA per plant, even the most resistant plants became infected, but still showed significantly delayed and reduced symptoms in newly developing leaves (Figure 5) and decreased accumulation of viral DNAs (Figure 6). In particular, single-stranded DNA (ssDNA) was absent or hardly detectable in high-resistance lines (Figure 6).

In the leaf disc assay, the relative proportion of the viral DNA forms varied between viral strains, but not between transgenic and non-transgenic plants. The differential accumulation of viral DNA forms in systemically infected transgenic plants suggests an alteration in the viral propagation mode in these plants compared with non-transgenic plants. The dsDNA forms are replication intermediates and templates for the transcription of viral genes. ssDNA represents the stably packaged form of the viral genome, which is transmitted by whiteflies, but may also be transported systemically (Duffus, 1987; Hull, 2002). The lack of viral ssDNA in transgenic plants suggests the absence of viral particles, even in symptomatic leaves. Thus, it is likely that virus-infected transgenic cassava plants will be a poor source of virus particles and therefore may reduce further spreading of ACMV by the whitefly vector, even though the plants may show symptoms and contain some viral dsDNA.

The infection of plants by particle bombardment delivers thousands of DNA molecules into cells. This experimental situation is certainly very different from a natural infection by viruliferous whiteflies. The high viral load pressure might be more difficult to control by the PTGS system, even in the transgenic *asRNA* plants, because the large viral DNA reservoir could continuously produce low levels of viral transcripts. The fact that several of our transgenic cassava *asRNA* lines show significantly increased resistance to ACMV infection is therefore very encouraging, but further field test experiments will be necessary to confirm resistance against the virus pressure of epidemic zones in Central and Eastern Africa.

Several molecular mechanisms may be involved in the observed resistance to ACMV in transgenic cassava. These include a pre-formed PTGS response or various post-infection mechanisms. By linking the viral asRNA to the selectable marker RNA, we avoided the production of transgenic plants in which the asRNA gene was post-transcriptionally silenced. The absence of small dsRNAs in uninfected transgenic plants confirmed the absence of a premeditated PTGS process. Therefore, interactions between asRNA and sense RNA or ssDNA to block translation, enhance post-infection PTGS or even inhibit replication are more likely causes for the resistance. The observed changes in the accumulation of viral DNA forms were not transgene specific because they occurred with all three transgenes. However, it should be noted that the different *asRNA* genes may target the same sense RNA, as the *asRNA* genes partially overlap (Figure 1a). Furthermore, some of the viral proteins are known to interact, such as Rep and REn (Settlage *et al*., 2001), and a decrease in either protein of the complex may affect virus DNA replication similarly. It has also been reported that non-transformed

cassava plants can recover from severe infection, but still express milder symptoms on subsequent leaves. Recovery is associated with an increased appearance of siRNA that is directed primarily to the downstream sequence of the *AC1* gene that overlaps with the upstream sequence of the *AC2* gene (Chellappan *et al*., 2004b). The presence of siRNA suggests that PTGS is an active plant defence against ACMV infection. We observed a fluctuation in the severity of symptoms between the leaves of infected plants (Figures 4 and 5). Symptom reduction indicates activity of the plant defence by PTGS, while the reappearance of more severe symptoms on successive leaves may be caused by possible counteractions of the ACMV PTGS suppressor AC2 (Voinnet *et al*., 1999). Symptom fluctuation occurred in transformed and non-transformed plants, but its precise timing and degree varied between individual plants (data not shown). Infected transgenic plants with increased resistance usually developed some leaves without any symptoms, a feature never observed with non-transformed lines. We observed an accumulation of short, 24 nt, siRNA-like molecules in infected transgenic and non-transgenic plants, and the highest siRNA level was found in the resistant transgenic lines (Figure 7, lanes 1–10). This is consistent with an enhanced interference of ACMV replication by an siRNA-mediated PTGS mechanism; however, further analyses of siRNA levels and kinetics of siRNA accumulation are necessary to clarify this point.

Together, our results demonstrate that the resistance of cassava against ACMV infection can be achieved by stable expression of asRNAs. To establish the economic viability of the interference approach, it will now be necessary to test the resistant plant lines under natural infection conditions.

Experimental procedures

Plant material

Cassava (*Manihot esculenta* Crantz) TMS60444 plants, provided by IITA (International Institute of Tropical Agriculture, Ibadan, Nigeria), were grown in the glasshouse under a 28 °C/25 °C day/night rhythm and a 16 h/8 h photoperiod. Shoot cultures were maintained on CBM [MS medium (Murashige and Skoog, 1962), supplemented with 2% sucrose and 2 μM $CuSO_4$, solidified with 0.3% Gelrite, pH 5.8] at 25 °C under a 16 h photoperiod (90 μmol/m^2/s) and subcultured at 4 week intervals. The conditions for the induction and maintenance of cassava somatic embryos and embryogenic suspensions were as described by Zhang *et al*. (2000). *In vitro* transgenic plants were maintained in the same conditions as wild-type plants. Transgenic plants used for ACMV infection tests were grown at a 28 °C/25 °C day/night rhythm with a 16 h/8 h photoperiod in a controlled-environment chamber.

Bacterial strains, plasmids and virus isolates

Escherichia coli strain XL-1-blue was used for the construction and preparation of plasmids, and *Agrobacterium tumefaciens* strain LBA4404 carrying the following binary vectors was used for the transformation of cassava. Three binary vectors, pPZPasAC1, pPZPasAC2 and pPZPasAC3, harbouring full-length viral antisense genes of *AC1*, *AC2* and *AC3* from ACMV-KE, respectively, were constructed. Each viral antisense gene was linked to the 3′UTR of an *HPT* gene, which was driven by the constitutive CaMV 35S promoter (Figure 1b). To construct the three binary vectors, firstly the *Eco*RI fragment from pDH51 (Pietrzak *et al*., 1986), containing CaMV 35S promoter and terminator, was inserted into the *Eco*RI restriction site of the polylinker of pPZP100 (Hajdukiewicz *et al*., 1994). The complete antisense (as) sequence of the AC1, AC2 or AC3 gene of ACMV-KE was generated by PCR and cloned between the *Xba*I and *Hin*dIII site of paRNA14 to give rise to vector pasAC1, pasAC2 or pasAC3. Then, the *Eco*RV-*Kpn*I fragment containing the antisense sequence, *HPT* and partial 35S promoter of pasAC1, pasAC2 or pasAC3 was inserted between the *Eco*RV site in the 35S promoter and the *Kpn*I site close to the right border of the modified pPZP100.

Infectious clones of ACMV-KE, ACMV-NOg and ACMV-CM were used for transient replication assays and for the infection of cassava plants (Klinkenberg *et al*., 1989; Liu *et al*., 1997; Fondong *et al*., 2000). The GenBank accession numbers (shown in parentheses) of the published cassava geminivirus DNA-A and DNA-B sequences used in this paper are as follows: ACMV-KE (J02057, J02058), ACMV-NOg (AJ427910, AJ427911) and ACMV-CM (AF112352, AF112353).

Plant transformation

Transformation of cassava by *Agrobacterium*-mediated gene transfer was performed as described by Zhang *et al*. (2000). Transgenic plants were recovered from transformed suspension cells via embryogenesis and maintained on CBM as shoot cultures.

Molecular analysis of transgenic cassava plants

Genomic DNA was extracted from freeze-dried plant material according to Soni and Murray (1994). Total RNA was isolated from leaves of *in vitro*-grown plants using the RNeasy plant mini kit (Qiagen, Hombrechtikon, Switzerland). PCR, RT-PCR,

Southern and Northern analyses were carried out following standard protocols (Sambrook *et al*., 1989). Aliquots of 5 μg of genomic DNA were digested with or without corresponding restriction enzymes for Southern analysis, while, for Northern analysis, 15 μg of total RNA was used. The hybridization probes specific to the antisense genes of *AC1*, *AC2* and *AC3* were DIG-dUTP-labelled by PCR using a PCR DIG probe synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions.

Transient viral replication assay

The analysis of transient viral replication in leaf discs from different plant lines was conducted as described by Zhang and Gruissem (2003). Two ACMV isolates, ACMV-KE and ACMV-CM, were used in the assay. The identity of the individual viral DNA forms was verified by digestion with specific nucleases. To distinguish between input DNA (dam-methylated) and *de novo* synthesized viral DNA (not methylated), the restriction enzyme *Dpn*I (active only when DNA is methylated) was also used to digest the samples.

Infection of cassava plants, symptom assessment and virus detection

Four-week-old cassava plants from transgenic and wild-type lines were inoculated with ACMV-NOg by biolistic-mediated DNA delivery. After a test series to determine the minimum inoculation pressure for successful infection of cassava plants, each plant was bombarded with 0.0625 mg of gold particles coated with 100 or 200 ng of ACMV-NOg DNA by two bombardments. A minimum of six plants per transgenic line was inoculated. Wild-type TMS60444 plants were used as a control.

Disease symptom severity on fully expanded leaves was recorded for a minimum of 8 weeks on a scale of 0–4 (0, no symptom; 1, faint mosaic; 2, yellow mosaic, malformation, 5%–10% size reduction; 3, severe mosaic, distortion, up to 50% size reduction; 4, severe mosaic, severe distortion, leaf reduced to veins with 50%–80% size reduction) (Figure 5). This scale follows that described by Fauquet and Fargette (1990). Symptom development was assessed every week. Generally, the symptoms appeared on new leaves 6 days post-inoculation (dpi). We recorded the symptoms after 10 dpi to 60 dpi, when each plant had developed more than 20 new leaves. Five micrograms of undigested cellular DNA, isolated from the pool of young leaves on the top of the infected plants, was used for the analysis of viral DNA accumulation with an *AV1* probe in Southern analysis.

Detection of short RNAs in selected transgenic cassava lines

Total RNA was extracted from 1 g of freeze-dried young leaves using the trizol (Invitrogen, Basel, Switzerland) method. Small RNAs were purified from total RNA using columns from the Midi RNeasy kit (Qiagen). The flow-through fraction containing the small RNA was precipitated with isopropanol. The pellet was dissolved in DEPC(diethyl pyrocarbonate)-treated water and the concentration was measured by a spectrophotometer and ethidium bromide staining.

Fifteen micrograms of small RNA was separated on a 20% polyacrylamide–8 M urea gel and electrotransferred to Hybond-N+ membrane (Amersham Biosciences, Otelfingen, Switzerland) in a semi-dry transfer cell (Trans-Blot SD, Bio-Rad, Reinach BL, Switzerland) overnight at 10 V. The membrane was then UV cross-linked and probed with specific ssRNA probes. Riboprobes were obtained by *in vitro* transcription using T7 and T3 RNA polymerase and (α-^{32}P)-UTP. Riboprobes corresponded to the sense and antisense sequences of *AC1* from ACMV-KE. In order to improve the signal, the riboprobes were hydrolysed with 300 μL of alkaline buffer (80 mM sodium bicarbonate, 120 mM sodium carbonate) at 60 °C for 2.5 h, according to the formula reported by Hamilton and Baulcombe (1999). The riboprobe solution was then neutralized by the addition of 20 μL of 3 M sodium acetate, pH 5.2. The membrane was pre-hybridized for 1 h at 40 °C with OligoHyb solution (Ambion, Cambridgeshire, UK) before the addition of the labelled riboprobe solution. Hybridization was performed at 40 °C for 24 h with rotary shaking in a hybridization oven. The membrane was washed twice with 2 × SSC/0.2% sodium dodecylsulphate (SDS) solution for 20 min at 50 °C. Oligonucleotides from within the antisense and sense sequences of *AC1* from ACMV-KE DNA-A were used as size standards.

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