Transgenic resistance to *Bamboo mosaic virus* by expression of interfering satellite RNA

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SUMMARY

Plant genetic engineering has broadened the options for plant virus resistance and is mostly based on pathogen-derived resistance. Previously, we have shown that interfering satellite RNA (satRNA) of *Bamboo mosaic virus* (satBaMV) greatly reduces *Bamboo mosaic virus* (BaMV) accumulation and BaMV-induced symptoms in co-inoculated plants. Here, we generated a nonviral source of virus-resistant transgenic *Nicotiana benthamiana* and *Arabidopsis thaliana* by introducing interfering satBaMV. Asymptomatic transgenic *N. benthamiana* lines were highly resistant to BaMV virion and viral RNA infection, and the expression of the transgene BSL6 was higher in asymptomatic than mildly symptomatic lines. In addition, BaMV- and satBaMV-specific small RNAs were detectable only after BaMV challenge, and their levels were associated with genomic viral RNA or satRNA levels. By transcriptomic analysis, the salicylic acid (SA) signalling pathway was not induced in satBaMV transgenic *A. thaliana* in mock conditions, suggesting that two major antiviral mechanisms, RNA silencing and SA-mediated resistance, are not involved directly in transgenic satBaMV-mediated BaMV interference. In contrast, resistance is associated with the level of the interfering satBaMV transgene. We propose satBaMV-mediated BaMV interference in transgenic plants by competition for replicase with BaMV.

INTRODUCTION

Plant viruses cause many important diseases and economic losses by reducing crop quality and yield worldwide. The most efficient way to alleviate the constraints caused by viruses is to use resistant crop cultivars. Since the increase in transgenic approaches about 30 years ago, many virus-resistant cultivars have been generated by genetic engineering. Three strategies exist for the development of transgenic disease-resistant plants: pathogen-derived resistance (PDR), regulation of natural induced host defence and direct interference with pathogen virulence or physiology. PDR is

the most common strategy for the generation of virus-resistant plants (Collinge *et al.*, 2010)

PDR, the concept of introducing functional, or parts of, genes from a pathogen genome into host plants to provide protection, was first proposed by Sanford and Johnston, and illustrated with bacteriophage Q β (Grummet *et al.*, 1986; Sanford and Johnston, 1985). Depending on the viral source introduced into the host, several kinds of resistance are obtained: coat protein (CP)-mediated resistance (CPMR) (Abel *et al.*, 1986; Angenent *et al.*, 1990; Asurmendi *et al.*, 2007; Bau *et al.*, 2003; Lawson *et al.*, 1990; Namba *et al.*, 1991), movement protein-mediated resistance (Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993; Seppanen *et al.*, 1997), replicase-mediated resistance (Anderson *et al.*, 1992; Azadi *et al.*, 2011; Carr *et al.*, 1994; Ehrenfeld *et al.*, 2004; MacFarlane and Davies, 1992; Pinto *et al.*, 1999; Vazquez Rovere *et al.*, 2001) and RNA-mediated virus resistance, including sense and antisense viral RNA (Barker *et al.*, 2001; Powell *et al.*, 1989), inverted repeats (IRs)/hairpin RNA of viral sequences (Fuentes *et al.*, 2006; Hu *et al.*, 2011; Smith *et al.*, 2000; Zhang *et al.*, 2011) and artificial microRNAs (Fahim *et al.*, 2012; Kung *et al.*, 2012; Niu *et al.*, 2006; Qu *et al.*, 2007). CPMR is considered to be the most successful and widely applied strategy (Beachy, 1997). In addition, RNA silencing, one of the major antiviral mechanisms in plants, has become a powerful strategy for transgenic resistance. Transgenic plants containing transgenes derived from viral RNA sequences arranged as IRs yield up to 90% of all transformants resistant to the homologous virus (Smith *et al.*, 2000; Waterhouse and Helliwell, 2003).

In addition to viral sources of transgenic virus resistance, various genes can be introduced into plants. Plant virus resistance genes, such as *N* from tobacco, *Tsi1* from pepper and *Sw-5* from tomato, have been transformed into plants for resistance against viruses such as *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV) and *Tomato spotted wilt virus*, respectively (Shin *et al.*, 2002b; Spassova *et al.*, 2001; Whitham *et al.*, 1996). In addition, the concept of antibodies commonly used in animal systems has been adopted to generate virus-resistant plants by producing single-chain, variable-fragment 'plantibodies' against viral CP and replicase (Boonrod *et al.*, 2004; Gargouri-Bouzid *et al.*, 2006; Prins *et al.*, 2005; Tavladoraki *et al.*, 1993; Zimmermann *et al.*, 1998). An alternative strategy to generate

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transgenic plants resistant to viruses is to constitutively express symptom-attenuating satellite RNAs (satRNAs) in the hosts (Harrison *et al.*, 1987; Taliansky *et al.*, 1998).

SatRNAs are viral parasites that depend on helper viruses for replication and encapsidation, but lack appreciable sequence homology with the helper virus genome (Hu *et al.*, 2009). The replication of satRNAs may affect the pathogenicity of the helper virus and alter symptoms of plant viral disease. For example, the satRNA of CMV (Palukaitis, 1988) and satellites of *Tobacco ring-spot virus* (TobRSV) (Buzayan *et al.*, 1986), *Peanut stunt virus* (Naidu *et al.*, 1992) and *Groundnut rosette virus* (GRV) (Taliansky and Robinson, 1997) can attenuate symptoms. However, some can exacerbate symptoms induced by their helper virus; examples are satC of *Turnip crinkle virus* (Li and Simon, 1990) and satRNA of *Arabidopsis mosaic virus* (Davies and Clark, 1983). The ability of satRNAs to modify virus-induced symptoms is determined by the infected host species, the helper virus strain and satellite isolates (Garcia-Arenal and Palukaitis, 1999). Transgenic plants expressing satRNA can resist or tolerate viral infection. Despite the unknown mechanism behind satRNA-mediated resistance, such resistance was used early against CMV infection (Harrison *et al.*, 1987).

SatRNAs of *Bamboo mosaic virus* (satBaMVs) have been found to be associated with *Bamboo mosaic virus* (BaMV) infection (Lin *et al.*, 1994). They totally depend on BaMV for replication and encapsidation, but share low sequence homology with BaMV, except for its 5' untranslated region (UTR) (Lin *et al.*, 1994). This is the only satRNA known to be dependent on a potexvirus. All satBaMVs are linear RNA molecules of about 836 nucleotides, and contain an open reading frame (ORF) for a protein of 20 kDa (P20) flanked by a 5' UTR of 159 nucleotides and a 3' UTR of 129 nucleotides (Fig. 1A) (Lin and Hsu, 1994). P20 of satBaMV is not required for its replication (Lin *et al.*, 1996), but facilitates the long-distance trafficking of satBaMV in BaMV co-infected plants (Vijaya Palani *et al.*, 2006, 2012). The phosphorylation of P20 down-regulates the formation of a satBaMV–P20 ribonucleoprotein complex (Vijaya Palani *et al.*, 2012). BaMV, a member of the Potexviridae of the Alphavirus-like superfamily, is an approximately 6400-nucleotide, single-stranded, positive-sense RNA with five conserved ORFs flanked by 5' and 3' UTRs of 94 and 142 nucleotides, respectively (Lin and Hsu, 1994; Yang *et al.*, 1997). The five ORFs in the BaMV genome encode replicase, triple gene block proteins (TGBps) and CP, respectively (Fig. 1A).

Among the satBaMV variants isolated from different bamboo hosts, BSL6 has the most alluring property, because it can markedly reduce the accumulation of genomic, subgenomic and minus-strand BaMV RNAs in co-inoculated plants (Hsu *et al.*, 1998, 2006). Co-inoculation of BSL6 also greatly attenuates BaMV-induced symptoms in *Nicotiana benthamiana* and *Chenopodium quinoa* (Hsu *et al.*, 1998). The determinant of the down-regulation of BaMV by BSL6 has been mapped to the hypervariable region, which folds into a conserved apical hairpin stem loop (AHSL)

structure in the 5' UTR of satBaMV (Hsu *et al.*, 2006). The secondary structure of the AHSL and specific nucleotides (C60 and C83) in the internal loops play crucial roles in the down-regulation (Chen *et al.*, 2007). Moreover, the two cytosines at equivalent positions in the AHSL determine the replication competence of BaMV and satBaMV. The down-regulation of BaMV by interfering satBaMV is dosage dependent (Chen *et al.*, 2012).

In Taiwan, more than 90% of bamboo plants with pachymorph rhizomes in the field are infected with BaMV, which results in great economic losses (Lin and Chen, 1991; Lin *et al.*, 1992, 1993a). As a result of the ease of BaMV transmission through vegetative propagation and the lack of effective chemicals or strategies to control or eliminate BaMV in infected bamboos, ways need to be found to prevent viral infection. In this study, we have successfully introduced interfering satBaMV into *N. benthamiana* and *Arabidopsis thaliana*; all transgenic plants showed resistance to BaMV infection. We analysed the resistance to virus infection in four different T₂ homozygous *N. benthamiana* lines. Most of the BSL6 transgenic *N. benthamiana* lines were tolerant to BaMV infection or highly resistant to BaMV viral RNA infection. By the analysis of BaMV- and satBaMV-specific small RNAs (vsRNAs and sat-sRNAs, respectively) and a previous study (Lin *et al.*, 2010), we have demonstrated that RNA silencing is not involved directly in satBaMV-mediated resistance. In addition, by taking advantage of the available genome information of *Arabidopsis* plants, we generated BSL6 transgenic *A. thaliana* showing resistance to BaMV infection. Transcriptome analysis revealed that the salicylic acid (SA)-mediated defence response does not play a major role in BSL6-mediated interference. Instead, the resistance of transgenic lines is positively associated with the transcript level of the transgene, which confirms the dose-dependent satBaMV-mediated interference in BaMV replication (Chen *et al.*, 2012). This technology could be used to generate BaMV-resistant bamboo.

RESULTS

BSL6 transgenic *N. benthamiana* plants showed two phenotypes after BaMV infection

Previously, we have shown that BSL6 satBaMV can greatly reduce BaMV accumulation and attenuate BaMV-induced symptoms in BaMV and BSL6 co-infected plants (Hsu *et al.*, 1998). To determine whether the transgenically expressed BSL6 is able to confer resistance to BaMV infection, we constructed a binary vector by placing full-length BSL6 under the control of a *Cauliflower mosaic virus* (CaMV) 35S promoter and the *Agrobacterium tumefaciens* nopaline synthase (NOS) terminator (Fig. 1B). Leaf discs of *N. benthamiana* were transformed with *A. tumefaciens* harbouring BSL6 placed binary vector, and T₁ transgenic plants were regenerated from these transformed leaf discs. All T₂ progeny of transgenic *N. benthamiana* lines were screened for green

Fig. 1 Generation of BSL6 transgenic *Nicotiana benthamiana*. (A) The genome organization of *Bamboo mosaic virus* (BaMV) and satellite RNA of BaMV (satBaMV). The BaMV genome has five open reading frames (ORFs). ORF1 encodes replicase, which contains three functional domains: methyltransferase, helicase and RNA-dependent RNA polymerase. ORF2–ORF4 are triple block genes that encode proteins for viral movement. ORF5 encodes the coat protein for virus encapsidation. satBaMV encodes a 20-kDa protein. (B) The schematic map of transformed pBaL6 to express full-length BSL6 satBaMV. The full-length BSL6 driven by a *Cauliflower mosaic virus* (CaMV) 35S promoter was transformed into a binary vector. (C) Northern blot analysis of expression of transgene BSL6 in transgenic lines. Total RNA was extracted from inoculated leaves of mock-inoculated BSL6 transgenic plants at 10 days post-inoculation (dpi). In total, 10 µg total RNA was electrophoresed in 1% agarose gel, blotted and hybridized to a full-length satBaMV probe (Lin *et al.*, 1996). (D) Symptoms of BaMV-infected BSL6 transgenic lines and wild-type (wt) *N. benthamiana* systemic leaves at 20 dpi.

fluorescent protein (GFP)-expressing BaMV infection (Lin *et al.*, 2006), and two different phenotypes of infection were found. One group of transgenic lines showed mild symptoms and low GFP expression, and the other showed no symptoms and no GFP signals after infection (Fig. S1, see Supporting Information). Thus, we selected two transgenic lines from each group for further analysis. Polymerase chain reaction (PCR) assay confirmed the presence of transgene BSL6 of 837 bp [excluding the poly(A) tail] in the four lines (lines 1-4, 2-4, 3-2 and 3-3). Northern blot analysis revealed that the transgene satBaMV BSL6 was differentially expressed in these four lines under normal growth conditions (Fig. 1C). Southern blot analysis showed that line 2-4 had only a single copy insertion and lines 1-4, 3-2 and 3-3 had two copies (data not shown).

To further confirm whether BSL6 transgenic plants were resistant to BaMV, we challenged the four transgenic lines and wild-type *N. benthamiana* with 1 µg BaMV-S (Lin *et al.*, 2004) virion. All challenged wild-type plants showed severe mosaic symptoms, BSL6 transgenic lines 1-4 and 2-4 showed delayed and mild symptoms, and lines 3-2 and 3-3 showed no symptoms (Fig. 1D). In wild-type plants, the mosaic symptoms usually appeared in inoculated leaves at 5–6 days post-inoculation (dpi) and in systemic leaves at 8–9 dpi. In transgenic lines 1-4 and 2-4, the mild symptoms usually developed in inoculated leaves at 6–7 dpi and in the fifth upper systemic leaf at 9–10 dpi. Some plants of lines 2-4 even showed no symptoms in the fifth systemic leaves at 10 dpi (Table 1). In mild symptomatic transgenic lines, dot blot analysis revealed a low level of BaMV in inoculated leaves and only in

Table 1 Symptoms and *Bamboo mosaic virus* (BaMV) and satellite RNA of BaMV (satBaMV) accumulation in BaMV-inoculated BSL6 transgenic and wild-type *Nicotiana benthamiana*.

	Plant line	Wild-type	Line 1-4	Line 2-4	Line 3-2	Line 3-3
Inoculated leaves at 10 dpi	No. of plants showing symptoms/no. of plants inoculated	52/52 (100%)	14/14 (100%)	45/45 (100%)	1/22 (4.5%)	2/21 (9%)
	BaMV accumulation level*	+++++	++++	+++	+	+
	satBaMV accumulation level*	—	+++++	++++	++	++
Systemic leaves at 10 dpi	No. of plants showing symptoms/no. of plants inoculated	52/52 (100%)	12/14 (85%)	37/45 (82%)	1/22 (4.5%)	2/21 (9%)
	BaMV accumulation level*	+++++	++++	+++	+	+
	satBaMV accumulation level*	—	+++++	+++	++	++
Systemic leaves at 20 dpi	No. of plants showing symptoms/no. of plants inoculated	52/52 (100%)	14/14 (100%)	45/45 (100%)	1/22 (4.5%)	0/21 (0%)
	BaMV accumulation level*	+++++	++++	+++	+	+
	satBaMV accumulation level*	—	+++++	++++	+++	+++

*The percentage of plants detected with BaMV or satBaMV by dot blot times the intensity of BaMV or satBaMV by dot blot (range, 0–5). dpi, days post-inoculation.

systemic leaves showing symptoms at 10 dpi. Therefore, BSL6 transgenic lines 1-4 and 2-4 were moderately resistant lines showing delayed symptoms when compared with wild-type plants after challenge with BaMV.

Because transgenic lines 3-2 and 3-3 showed no symptoms after BaMV infection, we wondered whether these two lines were immune to BaMV infection. Dot blot analysis revealed a trace amount of BaMV in 90% and 20% of line 3-2 and 3-3 plants in inoculated and fifth systemic leaves at 10 dpi, respectively (data not shown). However, BaMV was undetectable in 15% of these two lines in the sixth systemic leaves at 20 dpi (Table 1). Thus, transgenic lines 3-2 and 3-3 may be highly resistant lines, with the level of BaMV too low to cause symptoms after BaMV infection, which is a consistent phenomenon observed in BaMV and BSL6 co-inoculated *N. benthamiana* (Hsu *et al.*, 1998).

BSL6 transgenic *N. benthamiana* showed resistance to both BaMV viral RNA and virion

Transgenic plants that express the full-length or truncated viral genes show different degrees of resistance to viral RNA and virion (Golemboski *et al.*, 1990; Hemenway *et al.*, 1988; Register and Beachy, 1988). To test whether BSL6 transgenic plants exhibit similar resistance to viral RNA and virion, we inoculated asymptomatic transgenic lines (3-2 and 3-3) with 0.1 µg of viral RNA or 1 µg of virion. Wild-type plants showed delayed symptoms in viral RNA-inoculated relative to virion-inoculated plants. However, asymptomatic transgenic lines remained symptomless even after 30 days of viral RNA or virion challenge.

To monitor BaMV accumulation in BSL6 transgenic plants by viral RNA or virion infection, we conducted time course assays. Inoculated leaves were harvested at 3, 6 and 9 dpi, and the fifth, sixth and seventh systemic leaves were harvested at 12, 15 and 18 dpi, respectively. Enzyme-linked immunosorbent assay (ELISA) with anti-BaMV CP serum showed an extremely low level of BaMV CP in both inoculated and systemic leaves of lines 3-2 and 3-3

challenged with viral RNA during all times of infection (Fig. 2A). Both lines showed about 95% reduction in BaMV accumulation in both inoculated and systemic leaves, and no difference in resistance between these two lines with viral RNA infection. However, resistance to BaMV virion infection was greater for line 3-2 than line 3-3 (Fig. 2B). The CP accumulation levels in the systemic leaves of line 3-2 were about two-fold less than those in leaves of line 3-3 and 5%–20% of the wild-type, although the CP levels in the inoculated leaves of both lines were nearly identical and about 40%–50% of the wild-type level. Thus, BSL6 transgenic plants were resistant to both viral RNA and virion, but different transgenic lines possessed different resistance capacity to virion infection.

To further confirm the resistance, we performed Northern blot analysis to measure BaMV and satBaMV accumulation in BSL6 transgenic and wild-type plants (Fig. 2C). BaMV accumulation was lower in BSL6 transgenic than wild-type plants with both BaMV virion and viral RNA challenge. The satBaMV transgene was barely detected in transgenic lines, but its level was substantial after BaMV virion and viral RNA challenge. The transgene BSL6 was highly amplified with the aid of BaMV, and highly accumulated BSL6 could interfere with the replication of BaMV.

BSL6 was highly expressed in BSL6 transgenic *N. benthamiana* after BaMV challenge

Although the transgene was driven by the constitutively expressed CaMV 35S promoter, a low level of the transgene BSL6 was detected in mock-treated transgenic plants by Northern blot analysis (Figs 1C and 2C). We used real-time PCR to quantify the differential expression level of BSL6 before and after BaMV inoculation. BSL6 expression was about 15–100-fold higher in highly resistant lines than in moderately resistant lines in mock conditions. However, BSL6 accumulation was amplified up to 4000–100 000-fold in these two lines and reached a plateau after BaMV infection (Fig. 3A).

Fig. 2 Accumulation level of *Bamboo mosaic virus* (BaMV) in BSL6 transgenic and wild-type (wt) *Nicotiana benthamiana*. (A, B) Inoculated and systemic leaves of BaMV viral RNA-infected (A) and virion-infected (B) BSL6 transgenic line and wt *N. benthamiana* were harvested at 3, 6, 9 and 12, 15 and 18 days post-inoculation (dpi), respectively, followed by enzyme-linked immunosorbent assay (ELISA) with anti-BaMV coat protein (CP) serum. (C) Total RNA from the inoculated and systemic leaves of BaMV viral RNA and virion-inoculated BSL6 transgenic and wt *N. benthamiana* at 9 and 18 dpi were purified for Northern blot analysis as described in Fig. 1C, except that only 2 µg of total RNA was loaded. A 32 P-CTP-labelled BaMV CP probe and satellite RNA of BaMV (satBaMV) probe were used for BaMV and satBaMV detection, respectively. M, mock-inoculated leaves; RI, inoculated leaves of BaMV viral RNA-infected plants; RS, systemic leaves of BaMV viral RNA-infected plants; VI, inoculated leaves of BaMV virion-infected plants; VS, systemic leaves of BaMV virion-infected plants.

The accumulation of BaMV- and satBaMV-specific small RNAs was associated with their genomic level in BSL6 transgenic *N. benthamiana* after BaMV infection

Not only viruses, but also subviral agents, such as satRNAs and viroids, can trigger post-transcriptional gene silencing to produce small RNAs (Markarian *et al.*, 2004; Wang *et al.*, 2004). Previously, defective interfering RNAs (DI-RNAs) of *Tomato bushy stunt virus* (TBSV) have been found to enhance

the silencing effect by elevating the level of TBSV vsRNAs, thus resulting in saturation of the viral suppressor of RNA silencing and a high level of unbound vsRNAs (Havelda *et al.*, 2005). Furthermore, DI-RNA of *Cymbidium ringspot virus* is a poor target of vsRNAs (Szittyá *et al.*, 2002), but satCMV-derived small RNA can target the CMV 3' UTR and interfere in its replication (Zhu *et al.*, 2011). Thus, we wondered whether RNA silencing is involved in satBaMV-mediated BaMV interference.

Fig. 3 Accumulation of *Bamboo mosaic virus* (BaMV), satellite RNA of BaMV (satBaMV), and BaMV- and satBaMV-specific small RNAs in mock- and BaMV-treated BSL6 transgenic and wild-type (wt) *Nicotiana benthamiana*. Systemic leaves of mock- and BaMV-treated BSL6 transgenic line and wt *N. benthamiana* were harvested at 20 days post-inoculation (dpi). (A) Quantification of real-time polymerase chain reaction (PCR) analysis of satBaMV BSL6 expression. The total RNA of 2 µg was reverse transcribed, followed by real-time PCR. Values are means $\pm$ SE ($n = 3$). (B) Northern blot analysis of BaMV, satBaMV and their small RNA expression. Equal loading of 2 and 6 µg total RNA for viral genomic and small RNA detection in each lane, respectively, was revealed by ethidium bromide staining of the gel. nt, nucleotide.

To determine whether gene silencing, the major antiviral mechanism in plants, is involved in the resistance mechanism of satBaMV-mediated BaMV down-regulation in BSL6 transgenic plants, we detected vsRNAs and sat-sRNAs in BaMV- and mock-infected systemic leaves of transgenic and wild-type plants at 20 dpi. Small RNA Northern blot analysis revealed a substantial level of 21- and 22-nucleotide vsRNAs in BaMV-infected wild-type plants (Fig. 3B). However, 21- and 22-nucleotide vsRNAs levels were lower in moderately resistant plants and barely detectable in highly resistant plants after BaMV challenge, whereas high levels of sat-sRNAs were detected in all transgenic plants. As shown in Fig. 3B, vsRNA and sat-sRNA accumulation was associated with BaMV and satBaMV genome levels. Therefore, gene silencing is not involved in BaMV down-regulation in BSL6 transgenic plants.

Transcriptome analysis of host genes was affected by BSL6 in resistant BSL6 transgenic *A. thaliana*

BaMV and satBaMV cause symptomless infection and BSL6-mediated BaMV interference also occurs in *A. thaliana*, the model

plant for genome analysis (Lin *et al.*, 2010). Therefore, we generated satBaMV transgenic *A. thaliana* for transcriptome analysis to uncover the host genes involved in BSL6-mediated BaMV interference. Because BaMV cannot infect *A. thaliana* systemically (Lin *et al.*, 2010), we collected only inoculated leaves at 7 dpi for further assay. Northern blot analysis revealed greatly reduced BaMV levels in transgenic *A. thaliana* lines 8-3 and 18-4 when compared with wild-type *Col-0* challenged with BaMV (Fig. 4A). Like BSL6 transgenic *N. benthamiana* plants, mock-treated *A. thaliana* showed a barely detectable transgene level, but a high level after BaMV infection.

To uncover the host genes involved in satBaMV-mediated BaMV interference in transgenic plants, we used mock- and BaMV-treated BSL6 transgenic lines 8-3 and 18-4 and *Col-0* *A. thaliana* for microarray assay. Genes were selected if the expression was increased or decreased by two-fold in both lines compared with wild-type *Col-0* before BaMV infection (Tables S1 and S2, see Supporting Information). About 13% and 29% of genes involved in the defence response to biotic stress were up- or down-regulated, respectively, in transgenic lines (Fig. 4B). Some of

Fig. 4 Accumulation of *Bamboo mosaic virus* (BaMV) and satellite RNA of BaMV (satBaMV) in BSL6 transgenic and wild-type (wt) *Arabidopsis thaliana*, and microarray assays of genes with altered expression in BSL6 transgenic *A. thaliana*. (A) Northern blot analysis of BaMV and satBaMV accumulation in leaves of two independent BSL6 transgenic lines and *Col-0 A. thaliana* infected with BaMV at 7 days post-inoculation (dpi). The total RNA of 5 µg for BaMV and satBaMV detection was loaded equally in each lane. A 32 P-CTP-labelled BaMV coat protein (CP) probe and satBaMV probe were used for BaMV and satBaMV detection, respectively. (B) Differential expression of genes in two independent BSL6 transgenic *A. thaliana* lines relative to *Col-0* in mock conditions. Pie charts of up-regulated and down-regulated genes are divided by biological function.

these were reported to be involved in the response to CMV (Zheng *et al.*, 2004) and geminivirus (Ascencio-Ibanez *et al.*, 2008), including *yellow-leaf-specific gene 9* (YLS9, At2g35980) and At3g12830. Of note, genes involved in the SA and jasmonic

acid (JA) biosynthesis and signalling pathways of innate immunity were down-regulated in transgenic *A. thaliana* in the mock condition; examples include *isochorismate synthase 1* (ICS1, At1g74710), *enhanced disease susceptibility 1* (EDS1,

Fig. 5 Genes involved in salicylic acid (SA) and jasmonic acid (JA) pathways were down-regulated in BSL6 transgenic *Arabidopsis thaliana*. Red arrows indicate genes involved in SA and JA biosynthesis and signalling down-regulated in the mock condition. Among them, some genes were up-regulated with *Bamboo mosaic virus* (BaMV) infection (blue arrows). (See main text for definitions of genes.)

At3g48090), *phytoalexin deficient 4* (*PAD4*, At3g52430), *WRKY58* (At3g01080), *allene oxide cyclase 3* (*AOC3*, At3g25780) and *plant defensin 1.2a* (*PDF1.2a*, At5g44420) (Fig. 5). Genes involved in other hormone pathways were not significantly differentially expressed in satBaMV transgenic *A. thaliana*. Thus, satBaMV transgenic *A. thaliana* may have a lower SA level.

Because the marker gene of the SA-mediated defence response, *pathogen-related gene 1* (*PR1*, At2g14610), was up-regulated by BaMV infection in transgenic *A. thaliana* (Fig. 5), we further analysed the expression of the key enzyme gene for SA biosynthesis, *ICS1*, and the SA signalling marker genes, *EDS1* and *PR1*, in wild-type and BSL6 transgenic *N. benthamiana* to confirm the data derived from *A. thaliana* transcriptomic analysis. Levels of *ICS1*, *EDS1* and *PR1* were slightly, but significantly, lower in moderately resistant BSL6 transgenic *N. benthamiana* lines than in wild-type plants, but levels in the resistant BSL6 transgenic *N. benthamiana* lines and the wild-type were similar. However, *EDS1* and *PR1* levels were increased with BaMV infection in both wild-type and all BSL6 transgenic *N. benthamiana*. Notably, the level of *ICS1* was decreased after BaMV infection in wild-type and all BSL6 transgenic *N. benthamiana* (Fig. S2, see Supporting Information). These data demonstrate that SA biosynthesis and the signalling pathway were down-regulated in moderately resistant BSL6 transgenic plants relative to the wild-type. The SA signalling pathway could

be triggered by BaMV infection, but SA biosynthesis is down-regulated by BaMV infection in wild-type and all BSL6 transgenic plants. The SA-mediated defence response may not play major roles in BSL6-mediated interference of BaMV replication in transgenic *N. benthamiana*.

Nonreplicating satRNA transgenic *N. benthamiana* was not resistant to BaMV infection

We found that RNA silencing and SA-mediated antiviral mechanisms were irrelevant to satRNA-mediated BaMV interference. Instead, replication competition was a strong possibility. To test this, we generated BSF4 N58D (N-terminal 1–58-nucleotide deletion in the 5' UTR) transgenic plants to test whether nonreplicating satRNA transgenic plants show interference in BaMV replication. Five BSF4 N58D transgenic lines were selected for inoculation with BaMV, and similar symptoms were observed as in BaMV-infected wild-type plants. Systemic leaves were harvested at 20 dpi for satBaMV and BaMV detection. Northern blot analysis revealed substantial BaMV levels in wild-type and BSF4 N58D transgenic *N. benthamiana* after BaMV inoculation. Unlike L6 transgenic lines, in different BSF4 N58D transgenic lines, the satBaMV level was not induced with BaMV infection, although different satBaMV levels were detected (Fig. 6). From these

Fig. 6 Accumulation of *Bamboo mosaic virus* (BaMV) and satellite RNA of BaMV (satBaMV) in mock- and BaMV-inoculated BSF4 N58D transgenic and wild-type (wt) *Nicotiana benthamiana*. Systemic leaves of mock- and BaMV-inoculated BSF4 N58D transgenic lines and wt *N. benthamiana* were harvested at 20 days post-inoculation (dpi). The expression of BaMV and satBaMV RNAs was analysed by Northern blot. Equal loading of 2 µg of total RNA for viral genomic detection in each lane was revealed by ethidium bromide staining of the gel. A 32 P-CTP-labelled BaMV coat protein (CP) probe and satBaMV probe were used for BaMV and satBaMV detection, respectively.

results, we conclude that nonreplicating satBaMV transgenic plants failed to resist BaMV infection.

DISCUSSION

In this study, we introduced the full-length interfering satRNA BSL6 into *N. benthamiana* and *A. thaliana*, which generated protection against BaMV infection. In BSL6 transgenic *N. benthamiana*, the highly resistant lines conferred resistance to both viral RNA and virion. The expression of the transgene was higher in highly resistant than moderately resistant lines, as shown by Northern blot analysis and real-time PCR, but the level of vsRNAs was lower in highly resistant than in moderately resistant lines after BaMV challenge. Transcriptome analysis of satBaMV transgenic *A. thaliana* revealed down-regulated genes involved in the SA and JA pathways of innate immunity. satBaMV-mediated BaMV interference in transgenic plants may be dose dependent in competing for replicase at the initial stage of infection before cells trigger gene silencing and innate immunity.

SatBaMV-mediated resistance has advantages over CPMR

Previously, several reports have shown that transgenic plants expressing symptom-attenuating satRNA are resistant to their helper virus, such as CMV (Cillo *et al.*, 2004), TobRSV (Gerlach *et al.*, 1987) and GRV (Taliensky *et al.*, 1998). However, no examples of satRNA-mediated resistance have shown that such protection can apply to both virion and viral RNA infection. In this study, we used both BaMV virion and viral RNA to challenge BSL6 transgenic plants. ELISA revealed about 95% reduction in BaMV accumulation on viral RNA infection in highly resistant lines rela-

tive to wild-type plants. Apparently, the asymptomatic, highly resistant satBaMV transgenic plants generated here overcome several major drawbacks of CPMR, the most common way to generate virus-resistant plants (Collinge *et al.*, 2010). CPMR has been shown to be highly resistant to viruses, such as tobamovirus, bromovirus, potexvirus, potyvirus, cucumovirus, tobnavirus and tombusvirus (Abel *et al.*, 1986; Angenent *et al.*, 1990; Bau *et al.*, 2003; Hemenway *et al.*, 1988; Lawson *et al.*, 1990; Lindbo and Dougherty, 1992; Namba *et al.*, 1991; Shin *et al.*, 2002a; Tumer *et al.*, 1987; Vasudevan *et al.*, 2008). However, some CP transgenic plants are not resistant to viral RNA infection; example viruses include TMV and *Alfalfa mosaic virus* (Loesch-Fries *et al.*, 1987; Nelson *et al.*, 1987). Our satBaMV transgenic plants were resistant to both virion and viral RNA, but more to viral RNA (Fig. 2). Moreover, CP plays the role of symptom determinant in some viruses (Bukovinszki *et al.*, 2007; Hu *et al.*, 2011; Knorr and Dawson, 1988; Lan *et al.*, 2010; Yoon *et al.*, 2011). Consequently, CP transgenic plants might develop symptoms and lose viability in agricultural crops (Fuchs and Gonsalves, 1995). Our interfering satBaMV transgene caused no symptoms in transgenic plants. Furthermore, recombination may occur between viral transgene CP and infecting viral RNAs because of their high similarity (Canto and Palukaitis, 2001; Greene and Allison, 1994). Our satBaMV and BaMV share no sequence homology, and so the possibility of recombination is limited. In addition, the transgene satRNA was highly amplified only by the aid of the helper virus. This amplification could be up to 100 000-fold (Figs 2C and 3A), a similar phenomenon to that found in CMV Tfn-satRNA transgenic tomato (Cillo *et al.*, 2004). The transgene may be highly expressed, but silenced; however, sat-sRNAs were undetectable by small RNA Northern blot analysis in our four transgenic lines with mock treatment. Thus, extremely low transgene expression was not

caused by gene silencing, which suggests that transgenic plants would not waste energy expressing the transgene, another advantage of satBaMV-mediated resistance. The alluring characteristics of interfering satBaMV transgenic plants that can attenuate virus-induced symptoms by reducing virus replication, are resistant to both virion and viral RNA infection, and share no sequence homology between the transgene and the helper virus make satRNA transgenic plants a good option for viral disease control.

SatBaMV-mediated silencing is not involved in satBaMV-mediated transgenic resistance against BaMV

Both BaMV and satBaMV contain RNA genomes. Once BaMV and satBaMV enter the cell and start to replicate, the replicating form of double-stranded viral RNA would be sensed by the host dicer-like proteins (DCLs) and cleaved into double-stranded small RNAs of about 21–24 nucleotides (Lin *et al.*, 2010). Moreover, satBaMV transgenic *N. benthamiana* was more resistant to viral RNA than to virion infection (Fig. 2). The transformed highly structured satBaMV may trigger a gene silencing mechanism to diminish incoming BaMV viral RNA. However, the vsRNA accumulation was lower in BSL6 transgenic than in wild-type plants with BaMV infection (Fig. 3B). Small amounts of vsRNAs were also observed in BaMV and BSL6 co-inoculated *N. benthamiana* (Lin *et al.*, 2010). Thus, the accumulation of vsRNAs and sat-sRNAs corresponds to that of BaMV and satBaMV RNAs, respectively. These results indicate that transgene satRNA-mediated resistance to BaMV may not occur via RNA silencing.

The mechanisms of satBaMV-mediated resistance in transgenic plants

Symptom attenuation by satRNAs is often accompanied by reduced accumulation of the helper virus in plants (Hsu *et al.*, 1998; Kaper, 1982). The mechanism of satRNA-mediated symptom attenuation is thought to be caused by the decreased level of elicitor resulting from competition for the replication component between the helper virus and satRNAs, as in CMV (Liu *et al.*, 1997). However, the virus titre is not always reduced in symptom-attenuated plants, and so more than one mechanism may be involved (Moriones *et al.*, 1992). We found BaMV accumulation to be higher in moderately resistant than in asymptomatic transgenic *N. benthamiana*. Therefore, the symptoms caused by BaMV in BSL6 transgenic plants were associated with the level of BaMV. Moreover, the transcript level of the transgene BSL6 was relatively lower in moderately resistant than in asymptomatic transgenic lines with mock treatment (Fig. 3A). Similar results were observed in GRV-satRNA transgenic plants (Taliensky *et al.*, 1998) in which the transgenic lines harbouring relatively low levels of the transgene showed little resistance to GRV infection. Furthermore, BSL6-mediated BaMV interference is dose dependent in BaMV and BSL6

co-inoculated *N. benthamiana* protoplasts (Chen *et al.*, 2012). The interfering BSL6 not only down-regulates the replication of BaMV RNA and noninterfering BSF4 satBaMV in two-satBaMV mixed infection, but also competes more efficiently for replicase than does BSF4 when these two satBaMVs are co-expressed with the BaMV ORF1 in *N. benthamiana* protoplasts (Chen *et al.*, 2012). These results imply that BSL6 competes more effectively for replicase than does BaMV. Moreover, BSF4 N58D transgenic plants expressing nonreplicating satBaMV did not show interference in BaMV replication (Fig. 6). Thus, the basal expression of BSL6 in different transgenic lines determines the ability of the lines to interfere with BaMV infection.

Because an antiviral silencing mechanism is not involved in satBaMV-mediated BaMV resistance (Lin *et al.*, 2010), we wondered whether the innate immune system might play a role. Thus, BaMV-resistant satBaMV transgenic *A. thaliana* was generated to uncover the host genes involved in BSL6-mediated BaMV interference. Affymetrix assay revealed that defence-related genes involved in SA and JA pathways were down-regulated in BSL6 transgenic *A. thaliana* under mock conditions. Unlike the central role of SA in plant fungal and bacterial resistance (Delaney *et al.*, 1994; Shah, 2003), reports of SA efficacy in viral replication are conflicting in different plants (Alamillo *et al.*, 2006; Huang *et al.*, 2005; Mayers *et al.*, 2005; Sekine *et al.*, 2004; Takahashi *et al.*, 2004). In the case of CMV, overaccumulated SA could suppress CMV replication, although the susceptibility of CMV was not altered in SA-deficient NahG transgenic *A. thaliana* (Huang *et al.*, 2005; Sekine *et al.*, 2004). However, activation of the SA pathway enhanced the virulence of *Clover yellow vein virus* (Atsumi *et al.*, 2009). Therefore, the role of SA in plant defence signalling depends on the host–virus combination. From our results, the levels of several genes involved in SA biosynthesis and signalling were reduced in moderately resistant BSL6 transgenic *N. benthamiana*. However, *PR1* and *EDS1*, the marker genes of the SA pathway, were up-regulated after BaMV infection. Thus, SA-mediated host defence genes may not play a crucial role in satBaMV-mediated BaMV interference. In both BSL6 transgenic *N. benthamiana* and *A. thaliana*, the expressed transcripts of satBaMV do not trigger SA innate immunity, but can be greatly amplified and outcompete BaMV for replicase during BaMV infection. Both BaMV and satBaMV double-stranded RNAs are targeted and cleaved into small RNAs by DCLs. These vsRNAs are amplified by cellular RNA-dependent RNA polymerase (RDR) and targeted back to BaMV viral RNAs to generate more secondary vsRNAs, but sat-sRNAs are not (M.R. Liou *et al.*, unpublished data), as reported for CMV satRNA (Wang *et al.*, 2010). The avoidance of RDR-dependent silencing helps satBaMV to achieve preferential replication. Thus, more satRNAs than BaMV can be recruited into the replication complex, resulting in low BaMV accumulation. Our findings confirm that the resistance of BSL6 transgenic plants to BaMV infection depends on the level of the BSL6 transgene in

transgenic lines (Fig. 7), a step before RNA silencing effects and plant innate immunity.

EXPERIMENTAL PROCEDURES

Plasmid construction and generation of BSL6 and BSF4 N58D transgenic plants

Plasmids pBaL6 and pCAMBIAL6 were used for the generation of BSL6 transgenic *N. benthamiana* and *A. thaliana*, respectively. To construct pBaL6 and pCAMBIAL6, the CaMV 35S promoter-driven BSL6 fragment was cut from pCBSL6 (Chen *et al.*, 2012) by *EcoRI* and *PstI*. After polishing, the fragment was ligated into *NheI/SacI*-treated and polished pBin vector (Xiang *et al.*, 1999) and *NcoI/SpeI*-treated and polished pCAMBIA vector (Cambia, Canberra, Australia), respectively. These two plasmids were transformed into *A. tumefaciens* LBA4404 and GV3101 for transformation of *N. benthamiana* and *A. thaliana*, respectively, as described by Topping (1998). Homozygous individuals were selected from T₁ and T₂ progenies on the basis of hygromycin resistance.

To generate nonreplicating BSF4 N58D transgenic *N. benthamiana*, we constructed the plasmid pKBSF4 d1-58. First, the plasmid pCF4 was sub-cloned into the pKn vector [the rbcS terminator of the pKYLX7 vector (An *et al.*, 1985) replaced with a nos terminator] using *HindIII* and *BglII*, and referred to as pKBSF4. Then, the fragment of the 35S promoter and BSF4 1–58 nucleotides were cut out using *HindIII* and *Apal* from pKBSF4, and the 35S promoter fragment derived from the *HindIII/StuI*-treated pCass vector was inserted. pKBSF4 d1-58 was transformed into *A. tumefaciens* C58C1 for *N. benthamiana* transformation. Homozygous individuals were selected from T₁ and T₂ progenies on the basis of kanamycin resistance.

Plant growth and inoculation

Wild-type and homozygous BSL6 and BSF4 N58D transgenic *N. benthamiana* plants were grown in a growth chamber with 16-h light/8-h dark cycles at 25 °C. For each set of experiments, four 4-week-old plants at the four-leaf stage were used for inoculation. Three leaves of each plant were inoculated, and each experiment was repeated at least three times. The methods used for inoculation have been reported previously (Lin *et al.*, 1994, 1996), except when inocula contained 1 µg BaMV-S virion or 0.1 µg viral RNA extracted from the BaMV-S virion (Lin and Chen, 1991).

Arabidopsis thaliana Col-0 and homozygous BSL6 transgenic seeds were sterilized and grown on solid Murashige and Skoog (MS) medium for 8 days, and then transferred to soil and grown in a growth chamber with 22/16 °C day/night temperatures and 16-h light/8-h dark cycles. Typically, for each set of experiments, 12–20 1-month-old *A. thaliana* plants were used for inoculation. Four to five rosette leaves of each plant were mechanically inoculated with 0.1 µg BaMV-S viral RNA per leaf. All inoculated leaves were harvested at 7 dpi for Northern blot analysis.

DNA analysis of transgenic plants

The presence of the transgene was assessed by PCR amplification (McGarvey and Kaper, 1991). Genomic DNA was screened for the BSL6 sequence with the specific reverse primer BS45 (5'-

CATCTTTTAACGCTCTTTATTC-3') and forward primer BS104 (5'-GAAAACTCACCGCAACGA-3'). For Southern blot analysis, 60 µg of genomic DNA were extracted (Nalini *et al.*, 2004) and digested with *HindIII* or *EcoRI*, followed by electroporation, blotting and hybridization with a digoxigenin (Dig)-labelled satBaMV DNA probe following standard protocols (Gold *et al.*, 2003).

Dot blot and Northern blot hybridization

For dot blot and Northern blot analysis, inoculated and systemic leaves were harvested at 10 and 20 dpi. Total RNA was extracted from *N. benthamiana* leaves by the hot phenol–sodium dodecylsulphate (SDS) method (Ausubel *et al.*, 2001). Leaf tissue (0.15 g) was ground into a fine powder in the presence of liquid nitrogen, and 750 µL of RNA extraction buffer [100 mM Tris-HCl, pH 8.0, 4 M LiCl, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 8.2, 1% SDS, 50% phenol], preheated to 80 °C, was added and mixed by vortexing for 5 min. Chloroform–isoamyl alcohol (24 : 1) (150 µL) were added, and the tubes were shaken for 3 min, followed by 15 min of centrifugation at 10 000 g. An equal volume of 4 M LiCl was added to the supernatant, which was kept at –80 °C for 2 h to precipitate RNA. Total RNA was finally resuspended in 30 µL of diethylpyrocarbonate (DEPC)-treated water.

For dot blot analysis, RNA samples were spotted onto Hybond N⁺ nylon membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and immobilized by UV cross-linking. For Northern blot analysis, RNA samples were denatured with glyoxal. Electrophoresis involved 1% agarose gels for the analysis of BaMV and satBaMV RNA. RNA was transferred to Hybond N nylon membrane by the capillary method with 10 × SSC (3 M sodium chloride and 0.3 M sodium citrate, pH 7.0) and immobilized by UV cross-linking. Hybridization was performed as described by Lin *et al.* (1996) with probes labelled with ³²P-CTP. The ³²P-labelled BaMV-specific probe (L probe) was prepared by linearizing pBaHB with *HindIII*, followed by transcription with SP6 RNA polymerase, which is complementary to the 3' ends of positive-strand BaMV RNA (Lin *et al.*, 1993b). The S probe, specific for the detection of positive-strand satBaMV RNA, was transcribed from *EcoRI*-linearized pBSHE using T7 RNA polymerase (Lin *et al.*, 1996).

Real-time PCR analysis

The expression of the BSL6 transgene was assayed by real-time PCR. BSL6 transgenic *N. benthamiana* leaves were harvested for total RNA extraction using TriReagent (Sigma, St. Louis, MO, USA) before or 10 days after BaMV inoculation. Two micrograms of total RNA were reverse transcribed using a SuperScript III kit (Life Technologies, Carlsbad, CA, USA) with poly(T) primer. Real-time PCRs were carried out in triplicate and involved the use of SYBR-Green I Core Reagents (Life Technologies) in a GeneAmp® 9700 Sequence Detection System (Life Technologies) programmed to hold at 50 °C for 2 min and then run at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Specific primers L6-F (5'-CACAAACCGCTTGTAATGA-3') and L6-R (5'-TAATATGGGTGGTGGGTCAATG-3') for transgene BSL6 and 18S-F (5'-TACGCCCGCCCAAA-3') and 18S-R (5'-CACTGGCAGTCCTTCGTGAGT-3') for *N. benthamiana* 18S rRNA, as an internal control, were used for PCRs. PCR results involved the increase in the fluorescence signal of SYBR-Green I dye, and the Ct value for each reaction was determined using ABI Prism 7000 SDS software (Life

Fig. 7 Model of BSL6-mediated interference in *Bamboo mosaic virus* (BaMV) replication in BSL6 transgenic plants. In wild-type plants, BaMV-infected cells will trigger the silencing pathway. When BaMV RNA enters the cell, replicase (Rep) is translated and bound to the 3' untranslated region (UTR), and host factors, such as heat shock protein 90 (Hsp90) (Huang *et al.*, 2012), are recruited to form the replication complex for BaMV replication. Double-stranded RNA (dsRNA) formed during replication is cleaved into 21- and 22-nucleotide BaMV small interfering RNAs (siRNAs) (Lin *et al.*, 2010) by dicer-like proteins (DCLs) and targeted back to viral RNAs for silencing BaMV RNA in infected cells. BaMV siRNAs can be amplified by RNA-dependent RNA polymerase 6 (RDR6) to generate secondary siRNAs for further silencing, and can move into neighbouring cells as silencing signals. In satBaMV BSL6 transgenic plants, the expression of the transgene will down-regulate the salicylic acid (SA) signalling pathway of plant innate immunity. The existing satBaMV BSL6 transcripts, transcribed from the transgene, are highly amplified and outcompete for Rep with BaMV after BaMV infection, although host Hsp90 is recruited by BaMV RNA only (Huang *et al.*, 2012). In addition, BaMV secondary siRNAs are generated through the RDR6 pathway, but satBaMV siRNAs are not. Thus, satBaMV achieves preferential replication. Therefore, the highly amplified satBaMV can mediate resistance to BaMV infection in BSL6 transgenic plants.

Technologies), setting the threshold fluorescence into the exponential phase of the amplifications.

Small RNA Northern blot

For small RNA detection, total RNA was extracted using TriReagent (Sigma). Six micrograms of total RNA were mixed with 1 × loading dye

(Life Technologies) and heated at 95 °C for 5 min. Electrophoresis involved 19% acrylamide/7 M urea gel for the analysis of BaMV and satBaMV small RNAs. RNA was transferred to Hybond N⁺ nylon membrane by the HEP-3 Panther™ Semi Dry Electrophoretic System (Thermo Scientific, Milford, MA, USA) and immobilized by UV cross-linking. Blots were prehybridized at 40 °C with Ultrahyb™ oligo (Life Technologies) for 1 h and probed with alkaline buffer-hydrolysed L and S probes (Lin *et al.*, 1996) at 40 °C

overnight, followed by washing twice with wash buffer (1% SDS and 0.5% SSC) for 30 min each time.

ELISA

To assay transgenic resistance, viral accumulation in transgenic plants was assessed by ELISA after BaMV infection. Leaf tissue (0.5 g) was ground to a fine powder in the presence of liquid nitrogen. In total, 300 µL of 1 × phosphate-buffered saline (PBS) was mixed with the leaf powder vigorously by hand shaking for 30 s, followed by centrifugation at 6000 *g* for 5 min. The supernatant was transferred into a new tube. Protein crude extracts were used for ELISA as described by Lauweryns and Van Lommel (1982), with anti-BaMV CP rabbit serum as the primary antibody and biotinylated goat anti-rabbit serum as the secondary antibody. Horseradish peroxidase-conjugated streptavidin was then conjugated to the secondary antibody, and substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Life Technologies) was added until the colour developed. The stop solution, 2 M sulphuric acid, was added to change the colour from blue to yellow, and the intensity of the colour was measured at 450 nm.

Microarray assay

Mock- and BaMV-inoculated leaves of two independent BSL6 transgenic lines of *Col-0 A. thaliana* were harvested at 7 dpi for RNA purification by TriReagent, followed by microarray assay by an Affymetrix ATH1 array (Affymetrix, Santa Clara, CA, USA). The array data were analysed using GeneSpring GX (Agilent Technologies, Santa Clara, CA, USA).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Symptomless BSL6 transgenic *Nicotiana benthamiana* showed no green fluorescent protein (GFP) signals after *Bamboo mosaic virus* (BaMV)-GFP infection. Symptom development and GFP expression of BSL6 transgenic and wild-type (wt) *N. benthamiana* after BaMV-GFP challenge at 20 days post-inoculation (dpi).

Fig. S2. Expression levels of *PR1*, *EDS1* and *ICS1* in mock- and BaMV-inoculated BSL6 transgenic and wild-type (wt) *N. benthamiana* by qPCR at 20 days post-inoculation (dpi).

Table S1. Genes down-regulated more than two-fold with *Bamboo mosaic virus* (BaMV) infection in BSL6 transgenic *Arabidopsis thaliana* relative to the *Col-0* type.

Table S2. Genes up-regulated more than two-fold with *Bamboo mosaic virus* (BaMV) infection in BSL6 transgenic *Arabidopsis thaliana* relative to the *Col-0* wild type.