Brachypodium distachyon line Bd3-1 resistance is elicited by the barley stripe mosaic virus triple gene block 1 movement protein

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Barley stripe mosaic virus North Dakota 18 (ND18), Beijing (BJ), Xinjiang (XJ), Type (TY) and CV21 strains are unable to infect the Brachypodium distachyon Bd3-1 inbred line, which harbours a resistance gene designated Bsr1, but the Norwich (NW) strain is virulent on Bd3-1. Analysis of ND18 and NW genomic RNA reassortants and RNAβ mutants demonstrates that two amino acids within the helicase motif of the triple gene block 1 (TGB1) movement protein have major effects on their Bd3-1 phenotypes. Resistance to ND18 correlates with an arginine residue at TGB1 position 390 (R390) and a threonine at position 392 (T392), whereas the virulent NW strain contains lysines (K) at both positions. ND18 TGB1 R390K (NDTGB1R390K) and ND TGB1T392K single substitutions, and an NDTGB1R390K,T392K double mutation resulted in systemic infections of Bd3-1. Reciprocal NDTGB1 substitutions into NW TGB1 (NWTGB1K390R and NWTGB1K392T) failed to affect virulence, implying that K390 and K392 compensate for each other. In contrast, an NWTGB1K390R,K392T double mutant exhibited limited vascular movement in Bd3-1, but developed prominent necrotic streaks that spread from secondary leaf veins. This phenotype, combined with the appearance of necrotic spots in certain ND18 mutants, and necrosis and rapid wilting of Bd3-1 plants after BJ strain (BJTGB1K390,T392) inoculations, show that Bd3-1 Bsr1 resistance is elicited by the TGB1 protein and suggest that it involves a hypersensitive response.

INTRODUCTION

Barley stripe mosaic virus (BSMV) exhibits enormous phenotypic variation that depends both on the virus strain and on the infected host variety (Petty et al., 1994). BSMV was one of the first viruses for which extensive strain mutants were collected and, by 1965, more than 25 strains had been described that elicited different symptom phenotypes on several cereals and some dicots (McKinney & Greeley, 1965). Over the past half century, BSMV has been shown to occur naturally in field infections of several cereals, in addition to barley, and has been transmitted experimentally to many wild grasses and several dicot species (Jackson & Lane, 1981), as well as some tropical monocots (Renner et al., 2009). Disease phenotypes in these hosts range from mild stunting and mosaic symptoms to severe chlorosis, necrotic local lesions and systemic lethal syndromes, as well as developmental anomalies. Reverse genetic analyses of the BSMV Type and North Dakota 18 (ND18) strains has revealed that most virus-induced phenotypes examined map to variations in the γb pathogenesis gene and the γα replicase subunit (Donald & Jackson, 1994; Santos & Edwards, 2003; Jackson et al., 1991a, b; Weiland & Edwards, 1996).

In contrast, very little is known about host gene effects on BSMV disease phenotypes (Edwards et al., 2006) because modern genomics applications have not been developed for cereals until relatively recently. However, we have found that several BSMV strains cause different infection phenotypes on purple false brome [Brachypodium distachyon (L.) P. L. 1810] and other host species, and that these phenotypes can be elicited by the TGB1 protein, as described above. Here, we extend these previous findings to other BSMV strains and host species, and to a broader range of TGB1 mutations, to demonstrate that the Bd3-1 resistance gene differs from the Bsr1 gene targeted by the TGB1 protein from BSMV Type and ND18.
Beauv], a wild grass that has been developed as a model host for cereal genomics (Brljajcic et al., 2011; Garvin et al., 2008; Opanowicz et al., 2008; Vogel & Bragg, 2009). Among several B. distachyon lines tested, the BSMV ND18 strain is able to infect line Bd21, and infected plants exhibit mild to intense mosaic symptoms, stunting and failure to set seeds, and contain large amounts of virus (Cui et al., 2012). In contrast, the Bd3-1 line is resistant to ND18 and harbours a temperature-sensitive resistance (R) gene designated barley stripe resistance 1 (Bsr1) (Cui et al., 2012). We have used Bd21 × Bd3-1 F2 crosses and an F2 recombinant inbred line (RIL) population resulting from a Bd3-1 × Bd21 cross to investigate the genetics of Bsr1 and have shown that Bsr1 is a single dominant R gene (Cui et al., 2012) that differs from BSMV R genes in barley and oats that are generally recessive (Edwards et al., 2006). Hence, the Bsr1 R gene represents a departure from the R genes that have originated during cereal cultivation. We also have generated an F2 RIL genetic linkage map to locate Bsr1 within a 23 kb region at the top of chromosome (Chr) 3 and our initial analyses indicated that the mapped region of Bd21 contains five ORFs, including a putative R gene (Cui et al., 2012). However, more recently released sequence information has shown that the Bd3-1 strain has undergone a deletion event, and retains the putative R gene, but not the other four ORFs (http://genomesonline.org/cgi-bin/GOLD/bin/GOLDCards.cgi?gold stamp=Gr00035).

Moreover, our genomics analyses reveal that both Bd21 (Vogel et al., 2010) and Bd3-1 harbour related R alleles within the mapped region of Chr 3, but that the candidate R alleles vary in their sizes and in their predicted functions. The putative Bd3-1 R gene specifies a protein with N-terminal coiled-coil (CC) and NBS (also called NB-ARC) domains (van Ooijen et al., 2008) and a C-terminal 'leucine-rich repeat' (LRR) domain that is structurally similar to several dominant R genes that specify resistance to viral, bacterial and fungal hosts (Gururani et al., 2012; Kang et al., 2005; Maule et al., 2007; Moffett, 2009; Whitham et al., 1994). In contrast, the Bd21 R allele lacks the LRR domain that is thought to be involved in protein–protein interactions and probably represents a non-functional allele. Because the Bd3-1 R gene appears to have a functional motif, our analyses suggest that the CC-NBS-LRR allele corresponds to Bsr1.

Our recent results have shown that the BSMV Norwich (NW) strain is able to circumvent Bd3-1 resistance, and that NW infections of Bd3-1 and Bd21 result in nearly identical phenotypes. Here, we report genome-reassortant experiments demonstrating that the major NW virulence determinant resides within RNAβ, which encodes the coat protein (CP) and three overlapping triple gene block (TGB) movement proteins designated TGB1, TGB2 and TGB3 (Jackson et al., 2009; Verchot-Lubicz et al., 2010). We have constructed ND18 RNAβ (NW RNAβ) and NW RNAβ chimeras and have shown that a sequence block containing two critical amino acid residues within the helicase motif of Norwich TGB1 (NW TGB1) affects the ability to infect Bd3-1. Among the infection phenotypes observed, those of some TGB1 mutants containing site-specific mutations of the two TGB1 amino acid residues (positions 390 and 392) elicit a striking necrosis on leaves emerging 5–7 days post-inoculation (p.i.), but these leaves appear to accumulate very low levels of virus. We provide a model whereby the ND TGB1 protein interacts with the Bd3-1 Bsr1 protein to elicit a hypersensitive response (HR) that constrains ND18 infections, whereas the NW TGB1 and Bsr1 proteins fail to interact and elicit resistance, hence permitting NW to establish systemic infections on Bd3-1.

RESULTS

BSMV ND18 and NW phenotypes differ in the B. distachyon line Bd3-1

Previously constructed biologically active ND18 cDNA clones have been reported (Petty et al., 1989), and NW RNA cloning and sequencing are described in the supplementary Methods (available in JGV Online). Comparisons of the deduced ND18 RNA (NDRNA) and Norwich RNA (NW RNA) sequences reveal several minor differences in their coding regions, and these are shown in the codon maps of RNAs α, β and γ (illustrated in Fig. S1). The infectivity of in vitro transcripts synthesized from linearized NW (pT7ND, pT7βND and pT7γND) and ND18 (pT7αND, pT7βND and pT7γND) plasmids were tested by mechanically inoculating barley, and the B. distachyon lines Bd21-3, Bd21 and Bd3-1 (Fig. 1). All inoculated barley varieties developed typical systemic mosaic symptoms by about 5–7 days p.i. (Fig. 1a), and the NW and ND18 strains were also virulent on lines Bd21-3 and Bd21, which normally developed systemic mosaic symptoms on emerging leaves by 7 days p.i. accompanied by high levels of viral RNA in leaf extracts (Fig. 1b, c). In contrast, Bd3-1 plants exhibited a differential phenotype when infected with NW and ND18 (Fig. 1d). The NW strain was virulent on Bd3-1; however, as shown previously, the ND18 strain was unable to infect Bd3-1, which contains the Bsr1 gene (Cui et al., 2012), and inoculated plants failed to exhibit signs of infection and detectable amounts of BSMV RNAs did not accumulate (Fig. 1d).

TGB1 determinants affect Bd3-1 resistance

For more detailed comparisons, ND18 and NW α, β and γ genomic RNA (gRNA) reassortants, and a series of RNAβ chimeras and site-specific mutants were used to delineate the region responsible for NW virulence on B. distachyon line Bd3-1. ND18 and NW α, β and γ in vitro transcripts were mixed in all possible combinations and inoculated into barley (data not shown), and the B. distachyon Bd3-1 and Bd21 lines (Fig. 2). Again, Bd3-1 plants inoculated with ND18 did not develop symptoms, but with wild-type (wt) NW or transcript reassortants containing NW RNAβ, Bd3-1 exhibited fully susceptible phenotypes (Fig. 2a). In
these cases, the uninoculated emerging leaves developed strong mosaic symptoms and accumulated high levels of BSMV RNA (Fig. 2a). Occasionally, leaves of Bd3-1 plants inoculated with NWRNA<sub>c</sub> reassortants developed a mild mottling phenotype with small amounts of necrosis, but this phenotype was not reproducible, and the leaves did not contain CP or viral RNA (data not shown). Therefore, we believe that these inconsistencies result from unidentified environmental stresses and/or unspecific biotrophic interactions (Cui et al., 2012). In summary, the results demonstrate that NDRNA<sub>a</sub> encodes the primary determinants that elicit Bd3-1 resistance, and that NDRNA<sub>b</sub> and NDRNA<sub>c</sub> have negligible contributions to the resistance phenotype.

To further delineate the RNA<sub>b</sub> region responsible for Bd3-1 resistance to ND18, chimeric pT7<sub>b</sub> clones were constructed by restriction fragment substitutions between the pT7<sub>b</sub>ND and pT7<sub>b</sub>Mock strains. The results indicate that Bd3-1 is resistant to ND18 and susceptible to ND18 RNA<sub>b</sub>, which is in agreement with previous findings (Cui et al., 2012). In contrast, Bd3-1 is susceptible to ND18 RNA<sub>a</sub> and ND18 RNA<sub>c</sub>, which suggests that these RNAs are not required for Bd3-1 resistance.

These results suggest that the RNA<sub>b</sub> region of BSMV is critical for Bd3-1 resistance, and that the resistance phenotype is not simply due to the absence of specific viral proteins. Further studies are needed to identify the specific RNA<sub>b</sub> region responsible for Bd3-1 resistance and to understand the molecular mechanisms underlying this resistance.

**Fig. 1.** Symptoms and RT-PCR detection of BSMV ND18 and NW strain infections on barley and the B. distachyon lines Bd21-3, Bd21 and Bd3-1. (a) Appearance of emerging uninoculated leaves of barley plants at 7 days p.i. (top), and RT-PCR detection (bottom) of BSMV RNA<sub>c</sub> with specific primers BS-10 and BS-32 (see Table S1). Note the upper uninoculated leaves of the BSMV-inoculated barley have typical chlorotic stripes and mosaic symptoms. (b, c) Typical systemic BSMV ND18 and NW symptoms (top) and RT-PCR (bottom) at 14 days p.i. on emerging leaves of mock-inoculated or BSMV-inoculated B. distachyon line Bd21-3 (b) or line Bd21 (c). (d) Differential virulence of BSMV strains on B. distachyon line Bd3-1. The ND18 strain is unable to infect Bd3-1 and elicits resistance in which visible symptoms or accumulation of BSMV RNA are not evident on leaves emerging after inoculation, whereas NW is virulent and inoculated plants develop systemic infections on the emerging leaves, which accumulate high levels of BSMV RNA, similar to Bd21 infections with the ND18 and NW strains.

**Fig. 2.** Identification of ND18 and NW BSMV gRNA requirements for infection of B. distachyon Bd3-1 and Bd21 lines. ND18 and NW strain α, β and γ wtRNAs were transcribed in vitro and inoculated in all possible combinations into Bd3-1 (a) and Bd21 (b) seedlings. Top: phenotype on emerging leaves of inoculated plants. Bottom: RT-PCR detection of BSMV RNA in leaves. Note: all ND18 RNA (NDRNA) and NW (NWRNA) combinations were able to infect barley (data not shown) and Bd21, but only combinations containing NWRNA<sub>b</sub> elicited a systemic mosaic infection phenotype on Bd3-1 leaves. Leaves showing systemic symptoms also accumulated high levels of virus as assessed by RT-PCR to detect BSMV RNA or ELISA to evaluate CP accumulation (data not shown), whereas uninfected plants failed to exhibit detectable amounts of BSMV RNA or CP.
and pT7/NWTGB1 plasmids (see supplementary Methods). A unique PstI restriction site common to both plasmids is located approximately halfway along each cDNA insert near the middle of the TGB1 ORF (Fig. 3a). This site was used to construct complementary chimeric clones (βND/NW-PS-Ec and βNW/NDP-Ec) containing about equal amounts of pT7/βND and pT7/βNW. The βND/NW-PS-Ec plasmid contains 5′ ND18 and 3′ ND sequences, whereas βNW/NDP-Ec consists of 5′ NW and 3′ ND18 sequences (Fig. 3a). By 7–14 days p.i., inocula containing the chimeric RNAβ derivatives and NW/NRAz and NW/NRAy transcribed from pT7/βND and pT7/βNW elicited systemic infections on barley (data not shown) and Bd21, where positive RT-PCR and ELISA results were obtained (Fig. 3b). In contrast, the respective susceptible and resistant infection phenotypes of the βND/NW-PS-Ec and the βNW/NDP-Ec chimeras clearly revealed that the 3′ half of NW/NRAβ contains a determinant responsible for eliciting Bd3-1 resistance (Table 1). Site-specific mutagenesis was used to produce single mutants (βND/TGB1R390K, βND/TGB1T392K and βNW/TGB1I404E) containing NW/TGB1 variant codons, and complementary mutants (βNW/TGB1K390R, βNW/TGB1K392T and βNW/TGB1E404G) with NW/TGB1 residues (Fig. 4; see Supplementary Methods and Table S2 for construction of site-specific mutants). Double mutants (βND/TGB1R390K,T392K and βNW/TGB1K390R,K392T) with substitutions in both positions were also constructed.

All of the site-specific mutants developed systemic infections on barley and Bd21 (data not shown), but the Bd3-1 phenotype varied (Fig. 4). The NW/NRAβI404E and NW/NRAyE404G mutations had the same Bd3-1 phenotype as those of the parental NW/NRAβ and NW/NRAy combinations (Fig. 4, lanes 2, 3, 7 and 8). However, lysine substitutions to generate the βND/TGB1R390K and βND/TGB1T392K single mutants and the βND/TGB1I404E double mutant each elicited visible Bd3-1 symptoms, but the disease phenotypes had distinct differences (Fig. 4, lanes 4–6). The βND/TGB1I404E and βND/TGB1I404K,T392K mutants elicited visible mosaic symptoms on Bd3-1, and the infected plants accumulated substantial amounts of BSMV (Fig. 4, lanes 5 and 6). In contrast, emerging leaves of βND/TGB1I404E-inoculated plants developed a barely discernible mottling with very low CP accumulation, accompanied by variable numbers of dispersed necrotic spots (Fig. 4, lane 4). These data suggest that the K392 residue in NW/TGB1 is involved in virulence on Bd3-1 and that both K residues in the βND/TGB1I404K,T392K double mutant act synergistically to increase virus accumulation. Our interpretation of these results is that the NDTGB1I404K double mutant is able to mediate limited systemic movement in Bd3-1, and that the mutant protein elicits a Bsr1-associated HR in the invaded tissue.

The complementary NW/TGB1 mutants (βNW/TGB1K390R, βNW/TGB1K392T and βNW/TGB1K390R,K392T) RNAs (Fig. 4, Table 1) were co-inoculated with NW/NRAz and NW/NRAy. The single substitution βNW/TGB1K390R and βNW/TGB1K392T mutant RNA combinations produced visible systemic infections on Bd3-1 accompanied by high levels of virus accumulation (Fig. 4, lanes 9 and 10). Only Bd3-1 plants inoculated with the βNW/TGB1K390R,K392T double mutant combination RNAs failed to develop a distinctive mosaic, but the emerging leaves instead exhibited very faint mottling symptoms coupled with a characteristic necrosis that appeared to originate from the parallel veins and spread along the veins (Fig. 4, lane 11). To explore the phenotypic effects of the NW/TGB1I404E,K392T mutant in greater detail, the time course of Bd3-1 infections was followed over a 2 week period (Fig. 5). In these infections, necrosis emanating from the vascular tissue continued to spread during leaf emergence and, by 14 days post-inoculation, the leaves often died. Taken together, the ND/TGB1 and NW/TGB1 mutant effects provide evidence that background elements in the TGB1 proteins can affect BSMV movement and Bsr1 resistance sufficiently to elicit variable Bd3-1 phenotypes.

The NW/TGB1 residue K392 is a major Bsr1 resistance-breaking determinant and residue K390 has a synergistic role

Comparisons of the PstI–EcoRI fragment sequences from the ND18 and NW strains revealed differences at TGB1 positions 390, 392 and 404, consisting of lysine (K), arginine (R), threonine (T), glycine (G) and glutamic acid (E) residues (Table 1).
Fig. 3. Mapping of infection determinants of RNAβ chimeras on B. distachyon line Bd3-1. (a) Schematic representation of \( \beta_{\text{ND}} \) RNAβ \( (pT7/\beta_{\text{ND}}) \) and \( \beta_{\text{NW}} \) RNAβ \( (pT7/\beta_{\text{NW}}) \) cDNA clones and chimeric RNAβ recombinant clones. RNAβ plasmid designations are shown on the left, illustrations of the RNAβ plasmid chimeras are in the centre, and the Bd3-1 phenotype elicited by the different derivatives is indicated on the right. The blue pentagon in the schematic drawing represents the T7 promoter sequence used for \textit{in vitro} transcription of the BSMV gRNAs, the \( \beta_{\text{ND}} \) sequence is illustrated by open white rectangles and the \( \beta_{\text{NW}} \) sequence is depicted by grey rectangles. The approximate locations of \( \text{PstI} \) and \( \text{EcoRI} \) restriction sites used for chimeric RNAβ engineering are shown on the plasmid diagrams and the \( \beta_{\text{ND}} \) (white) and \( \beta_{\text{NW}} \) (grey) regions from which the sequence blocks originated are illustrated in the chimeras. (b) Symptoms and ELISA and RT-PCR detection of the infection phenotypes elicited after inoculation of \( pT7/\beta_{\text{ND}} \) and \( pT7/\beta_{\text{NW}} \) wtRNA transcripts and RNAβ chimeras to Bd3-1 (top) and Bd21 (bottom). By 14 days p.i., all of the recombinant derivatives developed systemic infections with visible mosaic symptoms on Bd21, and the ELISA and RT-PCR bands below the leaves confirmed that the chimeras were infectious. On Bd3-1, the \( \beta_{\text{ND}}\beta_{\text{NW}}\beta_{\text{Ps-3}} \) and \( \beta_{\text{ND}}\beta_{\text{NW}}\beta_{\text{Ec-3}} \) recombinant inoculations resulted in visible mosaic symptoms similar to NW strain infections on Bd3-1, whereas \( \beta_{\text{ND}}\beta_{\text{NW}}\beta_{\text{Ec-3}} \)-inoculated Bd3-1 plants developed a mild mottling phenotype on emerging leaves accompanied by very low intensities of the ELISA and RT-PCR bands. In addition to the mottling appearance on \( \beta_{\text{ND}}\beta_{\text{NW}}\beta_{\text{Ec-3}} \), Bd3-1-inoculated leaves photographed at 5 days p.i. necrotic spots were often interspersed randomly along the leaf blade. In contrast, the \( \beta_{\text{NW}}\beta_{\text{ND}}\beta_{\text{Ps-3}} \) and \( \beta_{\text{NW}}\beta_{\text{ND}}\beta_{\text{Ps-3}} \) chimeras failed to infect Bd3-1.
### Table 1. Comparison of nucleotide and amino acid sequence and substitutions in the TGB1 helicase regions of the ND18 and NW strains

<table>
<thead>
<tr>
<th>BSMV strain</th>
<th>Amino acid at TGB1 position</th>
<th>Bd3-1 phenotype*</th>
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<td></td>
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<td>ND18</td>
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*R, Resistant Bd3-1 reaction; S, susceptible Bd3-1 reaction; →, change in Bd3-1 phenotype.
†Site-specific changes in the ND1TGB1 protein.
‡Site-specific changes in the NW1TGB1 protein.
Alanine and serine substitutions of \textit{ND}TGB1 residues R\textsuperscript{390} and T\textsuperscript{392} affect Bd3-1 infection phenotype

Sequence comparisons of \textit{ND}TGB1 revealed a putative internal N-myristoylation-like site (data not shown) encompassing the residue G\textsuperscript{388}GRDTV\textsuperscript{393} that might potentially function during infection if post-translational TGB1 clavages generated fragments with N-terminal glycine (G) residues (Gordon \textit{et al.}, 1991; Traverso \textit{et al.}, 2008). Because the G\textsuperscript{388} and G\textsuperscript{389} residues are critical components of the putative internal N-myristoylation site, we introduced site-specific alanine (A) or serine (S) substitutions to create the $\beta_{ND\text{TGB1G388AG389A}}$, $\beta_{ND\text{TGB1R390AR392A}}$, $\beta_{ND\text{TGB1R390SR392S}}$, $\beta_{ND\text{TGB1T392A}}$, and $\beta_{ND\text{TGB1T392S}}$ mutants (see supplementary Methods). The results obtained after co-inoculation of Bd3-1 and Bd21 with the \textit{ND}RNA mutants, and \textit{ND}RNAs A and $\gamma$ revealed that the $\beta_{ND\text{TGB1G388AG389A}}$ mutation did not alter the ND18 phenotype on either \textit{Bd3-1} line (Fig. 6). These results suggest that myristoylation following possible post-translational cleavage of TGB1 does not have a substantial role in eliciting Bd3-1 resistance. However, the $\beta_{ND\text{TGB1R390AR392A}}$, $\beta_{ND\text{TGB1R390SR392S}}$, $\beta_{ND\text{TGB1T392A}}$ and $\beta_{ND\text{TGB1T392S}}$ substitution mutants exhibited fully susceptible phenotypes on both Bd3-1 and Bd21 (Fig. 6). The latter results reiterate our previous suggestion that both the R\textsuperscript{390} and T\textsuperscript{392} residues in \textit{ND}TGB1 are essential for elicitation of \textit{Bsr1} resistance to ND18.

**TGB1 background effects have roles in Bd3-1 phenotypic variation**

To evaluate the virulence of additional BSMV strains, Bd3-1 and Bd21 seedlings were inoculated with leaf sap from barley plants inoculated with the BSMV ND18, NW, BJ, XJ, TY and CV21 strains (Fig. 7a, b). At 14 days p.i., all of the Bd21 plants inoculated with these strains were fully susceptible (Fig. 7b). However, the XJ-, TY- and CV21-inoculated Bd3-1 plants exhibited resistance similar to that of ND18, in which no visible phenotype appeared (Fig. 7a). Moreover, inspection of the XJ, TY and CV21 TGB1 sequences revealed that these strains contain R and T residues in positions corresponding to those of the ND18 R\textsuperscript{390} and T\textsuperscript{392} residues (Fig. 7c). Thus, these results indicate that the XJ, TY and CV21 strains elicit Bd3-1 \textit{Bsr1} resistance gene functions similar to those elicited by ND18.

The BJ strain developed an entirely different phenotype from those of XJ, TY and CV21 that consisted of necrotic stripes on emerging leaves that appeared within 5 days p.i. The necrosis quickly spread along the veins and progressed to wilting and death of the plants by 14 days p.i. Sequence comparisons of BJ and the site-specific mutants revealed that these strains contain R and T residues at the positions corresponding to those of the ND18, in which no visible phenotype appeared (Fig. 7c). Moreover, the $\beta_{ND\text{TGB1R390K}}$ mutant, which develops a modulated systemic response consisting of necrotic spots, and the BJ strain (\textit{bj}TGB1K390,T392) have the same TGB1 amino acids at this position (Fig. 7c). In addition, all of the strains and mutants that elicited necrosis contained a TGB1T392 residue, but varied in their K\textsuperscript{390} or R\textsuperscript{390} profiles. This observation again points to the importance of T392 in eliciting \textit{Bsr1} resistance in Bd3-1.

**DISCUSSION**

This study extends our previous findings showing that the \textit{B. distachyon} inbred line Bd3-1 harbours a gene (\textit{Bsr1}) conditioning resistance to BSMV ND18 (Cui \textit{et al.}, 2012).
Our results show that, in contrast to ND18, the NW strain circumvents Bd3-1 resistance and is virulent on Bd3-1 (Cui et al., 2012; Yuan et al., 2011). We have mapped the resistance determinant to RNAβ, and have shown that the NW and ND18 α and γ gRNAs have only minor contributions to Bd3-1 resistance. More detailed mapping reveals that the major sequence conditioning Bd3-1 resistance resides within a unique PstI–EcoRI fragment of NDRNAβ.

The 136 aa sequence within this fragment is located in the C-terminal half of the 58 kDa TGB1 protein, and consists of only three amino acid variations at positions 390, 392 and 404. Site-specific amino acid substitutions of ND18 and NW at position 404 failed to affect the respective phenotypes, but single exchanges of the NW TGB1 390 or 392 residues into ND TGB1 (ND TGB1R390K) or (ND TGB1T392K) or a double mutant (ND TGB1R390K,T392K) resulted in the ability to infect Bd3-1. However, incorporation of single ND TGB1 residues into the NW TGB1 protein (NW TGB1K390R and NW TGB1K392T) produced virus derivatives that were able to infect Bd3-1. These results imply that the K390 and K392 residues in NW TGB1 can compensate for each other to circumvent Bd3-1 resistance. In addition, alanine and serine substitutions of either of the R390 or T392 residues into ND TGB1 enables the mutants to infect Bd3-1. Moreover, comparisons of XJ, TY and CV21 TGB1 sequences are in agreement with the conclusion that TGB1 positions 390 and 392 are critical residues affecting Bd3-1 Bsr1 resistance, and that analysis of such residues in uncharacterized BSMV strains may provide a potential tool to predict Bsr1 resistance interactions.

What might be the nature of such interactions? Our current studies show that amino acids involved in avirulent ND18 and virulent NW infections of Bd3-1 are located...
Fig. 7. Differential virulence of BSMV isolates on *B. distachyon* line Bd3-1. (a) Bd3-1 seedlings inoculated with leaf sap from barley infected with the ND18, NW, BJ, XJ, TY and CV21 strains of BSMV (top). Note: photographs were taken of inoculated plants at 14 days p.i., except for Bd3-1 plants inoculated with the BJ isolate, which were photographed at 6 days p.i. In contrast to the NW virulent control, BJ-inoculated Bd3-1 leaves showed the first signs of necrosis at ~4 days p.i., the necrosis spread rapidly after the plants began to wilt, and by 14 days p.i. the inoculated plants had died and dried out completely. ND18, XJ, TY and CV21-inoculated Bd3-1 plants normally failed to develop signs of infection or accumulate CP (middle) or BSMV RNA (bottom) on Bd3-1. (b) Systemic infection response and CP and RNA accumulation in Bd21 plants inoculated with the BSMV strains. (c) Comparisons of the amino acid sequences surrounding the TGB1 virulence motif of different BSMV isolates. Numbers represent the amino acid positions of the ND18 and NW TGB1 proteins.

within the helicase domain of the TGB1 protein. Previous studies indicate that TGB1 is a multifunctional protein that participates in a number of movement-related events. Moreover, the helicase domain, which contains seven motifs that are conserved in several virus genera (Morozov & Solovyev, 2003; Verchot-Lubicz et al., 2010), has several biochemical functions, all of which are required for cell-to-cell movement (Jackson et al., 2009). Amongst these functions are protein–protein interactions (Leshchiner et al., 2006; Lim et al., 2008), RNA binding (Donald et al., 1997; Leshchiner et al., 2006; Lim et al., 2008) and RNA helicase (Kalinina et al., 2002) activities. We have previously shown that ND TGB1 homologous binding is compromised by site-specific amino acid substitutions in helicase motifs I (residue 259) and II (residues 339 and 340), and that disrupting these interactions interferes with cell-to-cell movement of ND18 (Lim et al., 2009). However, the TGB1390 and TGB1392 mutations do not compromise critical TGB1 movement functions, but do affect the Bd3-1 disease phenotype. Hence, the TGB1390 and TGB1392 amino acids appear to specifically affect Bd3-1 *Bsr1* R gene activities.

A plausible mechanism for Bd3-1 resistance to ND18 is that the ND TGB1 and Bd3-1 *Bsr1* proteins engage in interactions that activate the *Bsr1* resistance pathway, and that lysine, serine and alanine substitutions incorporated at ND TGB1 residues 390 and 392 disrupt these interactions. The results also suggest that the ability of the NW strain to circumvent Bd3-1 resistance is a consequence of the absence of NW TGB1–*Bsr1* interactions. Such a mechanism is consistent with the quadratic check model classically found in plant pathogen ‘gene-for-gene’ interactions (Flor, 1971). In the case of BSMV–*B. distachyon* interactions, we posit that, in addition to its other activities, the ND TGB1 protein is an elicitor that functions by interacting physically with the Bd3-1 *Bsr1* protein to culminate in a resistance response. Moreover, we hypothesize that ND TGB1 does not interact with the non-functional Bd21 *Bsr1* allelic protein, and hence ND18 is virulent on Bd21. Conversely, the NW TGB1 protein is unable to interact with either of the Bd3-1 or Bd21 *Bsr1* allelic proteins, and hence the NW strain is virulent on both hosts.

Our data also provide indirect evidence supporting the hypothesis that Bd3-1 resistance involves an HR typical of responses often associated with dominant R genes (Gururani et al., 2012). This evidence is based on the unveiling of a necrotic response by site-specific substitutions in ND TGB1 and NW TGB1 mutants. The clearest example that an HR may be involved in resistance is the spreading necrosis occurring after Bd3-1 inoculation with the βND TGB1K390R,K392T mutant, and to a lesser extent the necrotic spots exhibited in Bd3-1 leaves inoculated with the βND TGB1R390K mutant. Variable necrosis associated with disease phenotypes of these mutants and lower abundances of BSMV RNA and CP in emerging leaves indicates that these phenotypes are elicited by TGB1 interactions, but that the mutants compromise the Bd3-1 resistance response sufficiently to permit limited...
vacular movement of BSMV to emerging secondary leaves while maintaining Bsr1 HR activities. The necrosis associated with these two mutants (β<sub>NW</sub>TGB1<sub>K390R,K392T</sub> and β<sub>ND</sub>TGB1<sub>K390R</sub>), coupled with the rapid wilting and death of Bd3-1 plants inoculated with the BJ strain (genotype= BJ TGB1<sub>K390,T392</sub>) RNAs, and the low levels of CP and viral RNA accumulation in these infections lends additional credence for an HR elicited by Bsr1. Our premise is that the Bsr1 protein functions in part via eliciting an HR in infected tissue that is typical of classical R genes functioning in plant disease interactions (Gassmann & Bhattacharjee, 2012; Heath, 2000). Under this scenario, the Bd3-1 Bsr1 protein interacts with the ND<sub>TGB1</sub> protein, but not with the NW<sub>TGB1</sub> protein, to elicit a resistance signalling cascade that constrains ND18 infections to the initially infected cell foci.

We have recently cloned both candidate Bsr1 alleles, and our preliminary results indicate that the ND<sub>TGB1</sub> protein is able to bind directly to the Bd3-1 Bsr1 protein, but not to the Bd21 Bsr1 allelic protein (L. Yan & D. Li, unpublished data). However, as predicted in the model above, NW<sub>TGB1</sub> is unable to bind to either of the R proteins. Moreover, we have also accumulated biological evidence demonstrating that gene-specific TGB1–Bsr1 allelic combinations result in specific HR activities that reflect the BSMV–B. distachyon disease phenotypes. These results, which we plan to describe in a forthcoming manuscript, combined with the data presented above, provide a convincing scenario whereby Bd3-1 resistance involves an HR whose elicitation requires ND<sub>TGB1</sub>–Bd3-1 Bsr1 binding activities.

**METHODS**

Construction of full-length infectious Type (TY) and North Dakota 18 (ND18) BSMV cDNA clones and the origins of the strains have been described previously (Petty et al., 1988, 1989). The Norwich (NW) strain (provided by Dr R. Hull, John Innes Centre, Norwich, UK) was originally isolated as an isolate from the Rothamsted strain, and its physico-chemical and biological properties were first described by Lane (1974). Seed transmission of the CV21 strain was studied in North Dakota by R. G. Timian (Timian, 1974), and this strain was apparently called the ‘moderate strain LQ’ (Dr M. C. Edwards, personal communication) in descriptions of the biological properties of BSMV strains collected before 1965 (McKinney & Greetley, 1965). The two Chinese strains, Beijing (BJ) (Sun et al., 2007) and Xinjiang (XJ) (Xie et al., 2011), along with the CV21 strain, were kindly provided by Professors Xianchao Sun (College of Plant Protection, South-west University, China) and Bingsheng Qiu (Institute of Microbiology, Chinese Academy of Sciences) and Xinjiang (XJ) (Xie et al., 2011), along with the CV21 strain, were kindly provided by Professors Xianchao Sun (College of Plant Protection, South-west University, China) and Bingsheng Qiu (Institute of Microbiology, Chinese Academy of Sciences) for providing BSMV Beijing, Xinjiang and CV21 strains. The CV21 strain originated from North Dakota and had been stored in dried leaves in the Chinese Academy of Sciences for about 30 years and we presume that the strain was provided by the late Dr Roland Timian. This work was supported by the National Natural Science Foundation of China (31270184 and 31210103902) and the National Basic Research Program (973 program, no. 2009CB118306), The Innovative Project of SKLAB (2012SKLAB01-7) to D. L. and Z. L., the Project for Extramural Scientists of SKLAB (2012SKLAB06-02) and a United States Department of Agriculture competitive grant (CSREES-2008-35319-19225) to A.O.J.

**ACKNOWLEDGEMENTS**

We thank Dr Roger Hull (John Innes Centre, Norwich, UK) for the NW strain of BSMV. We also thank Professors Xianchao Sun (College of Plant Protection, South-west University, China) and Bingsheng Qiu (Institute of Microbiology, Chinese Academy of Sciences) for providing BSMV Beijing, Xinjiang and CV21 strains. The CV21 strain originated from North Dakota and had been stored in dried leaves in the Chinese Academy of Sciences for about 30 years and we presume that the strain was provided by the late Dr Roland Timian. This work was supported by the National Natural Science Foundation of China (31270184 and 31210103902) and the National Basic Research Program (973 program, no. 2009CB118306), The Innovative Project of SKLAB (2012SKLAB01-7) to D. L. and Z. L., the Project for Extramural Scientists of SKLAB (2012SKLAB06-02) and a United States Department of Agriculture competitive grant (CSREES-2008-35319-19225) to A.O.J.

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Phytopathology 18, 163–177.


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