Subcellular Dynamics and Role of *Arabidopsis* β -1,3-Glucanases in Cell-to-Cell Movement of Tobamoviruses

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β -1,3-Glucanases (BG) have been implicated in enhancing virus spread by degrading callose at plasmodesmata (Pd). Here, we investigate the role of *Arabidopsis* BG in tobamovirus spread. During *Turnip vein clearing virus* infection, the transcription of two pathogenesis-related (PR)-BG AtBG2 and AtBG3 increased but that of Pd-associated BG AtBG_{pap} did not change. In transgenic plants, AtBG2 was retained in the endoplasmic reticulum (ER) network and was not secreted. As a stress response mediated by salicylic acid, AtBG2 was secreted and appeared as a free extracellular protein localized in the entire apoplast but did not accumulate at Pd sites. At the leading edge of *Tobacco mosaic virus* spread, AtBG2 co-localized with the viral movement protein in the ER-derived bodies, similarly to other ER proteins, but was not secreted to the cell wall. In *atbg2* mutants, callose levels at Pd and virus spread were unaffected. Likewise, AtBG2 overexpression had no effect on virus spread. However, in *atbg_{pap}* mutants, callose at Pd was increased and virus spread was reduced. Our results demonstrate that the constitutive Pd-associated BG but not the stress-regulated extracellular PR-BG are directly involved in regulation of callose at Pd and cell-to-cell transport in *Arabidopsis*, including the spread of viruses.

Plant viruses spread cell to cell by exploiting plasmodesmata (Pd), membranous channels that cross walls of neighboring plant cells, linking their cytoplasm, plasma membrane, and endoplasmic reticulum (ER) (Beachy and Heinlein 2000; Citovsky and Zambryski 2000; Ding et al. 1999; Heinlein and Epel 2004). Pd channels are surrounded by a specialized cell wall sheath which is devoid of cellulose and contains callose, nonesterified pectin, and probably other noncellulosic polysaccharides (Dahiya and Brewin 2000; Heinlein and Epel 2004; Roy et al. 1997; Sutherland et al. 1999). Callose is deposited at the neck region of Pd in the form of an external collar located

at distal ends of the wall sheath surrounding the Pd channel (Bell and Oparka 2011; Fitzgibbon et al. 2010). The amount of callose at Pd was shown to determine the aperture of Pd channels and, thus, control the intercellular transport (Levy and Epel 2009; Rinne and van der Schoot 2003; Zavaliev et al. 2011). Accumulated evidence suggests that the net callose level at Pd is determined by the balance between the activity of callose synthase (CalS) and β -1,3-glucanase (BG), because loss of either of the enzymes affects callose accumulation at Pd and transport through Pd (Guseman et al. 2010; Iglesias and Meins 2000; Levy et al. 2007a; Vaten et al. 2011).

To facilitate cell-to-cell transport, viruses encode one or more proteins designated movement proteins (MP) that target to and purportedly dilate Pd (Beachy and Heinlein 2000; Waigmann and Zambryski 1995). The MP of *Tobacco mosaic virus* (MP^{TMV}) serves as a model for studying mechanisms of viral cell-to-cell spread. Like that of many other plant virus movement proteins, MP^{TMV} binds RNA in a sequence-nonspecific manner (Citovsky et al. 1990, 1992), is associated with inclusion bodies derived from the cortical ER (Haupt et al. 2005; Heinlein et al. 1998; Mas and Beachy 1999; Reichel and Beachy 1998), is present in complexes that also contain viral replicase (rep^{TMV}) (Kawakami et al. 2004), and targets Pd (Epel et al. 1996; Heinlein et al. 1995; Oparka et al. 1995). Microinjection studies, conducted to examine the cell-to-cell trafficking of various fluorescent probes in MP^{TMV} transgenic plants (Deom et al. 1990; Ding et al. 1992; Olesinski et al. 1996; Vaquero et al. 1994; Wolf et al. 1989) and in TMV-infected tissues (Oparka et al. 1997), led to the hypothesis that MP^{TMV} increases the Pd size exclusion limit (SEL, the size of the largest dye capable of moving cell-to-cell), a function also termed “gating”. However, in contrast to microinjection, no gating effect was observed during cell-to-cell diffusion of transiently expressed or photoactivated green fluorescent protein (GFP) in MP^{TMV} transgenic plants (Christensen et al. 2009a and b; Guenoune-Gelbart et al. 2008; Liarzi and Epel 2005). Thus, it was suggested that differences in gating are due to the fact that dye microinjection technique causes the formation of stress callose around Pd, thereby reducing permeability (Liarzi and Epel 2005). It was proposed that MP promoted callose degradation or prevented stress-induced callose synthesis and that, in MP-expressing plants, callose levels around Pd would be lower as a consequence (Guenoune-Gelbart et al. 2008).

The possible link between MP^{TMV} and stress-induced callose accumulation was further investigated by direct measurement of callose around Pd after TMV infection (Guenoune-Gelbart et al. 2008). This study showed that TMV replication in the

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absence of MP^{TMV} resulted in callose accumulation at Pd, whereas callose levels were reduced when replication occurred in the presence of MP^{TMV}. Callose levels were unaffected in plants expressing only MP^{TMV} without virus replication. These data suggested that both replication and MP^{TMV} activity are necessary to reduce callose accumulation. To achieve that, TMV must somehow recruit BG at Pd or, alternatively, inhibit stress-triggered callose synthesis.

Plant BG are cell wall enzymes that catalyze the endo-hydrolysis of β -1,3-glucan (callose). The *Arabidopsis* BG gene family includes fifty members, which can be roughly divided into two subgroups: a large group of developmentally regulated BG and a smaller group of pathogenesis-related (PR)-BG (Doxey et al. 2007; Zavaliev et al. 2011). The first group includes a constitutively expressed glycosylphosphatidylinositol (GPI)-anchored Pd-associated BG, AtBG_{pap}, which localizes to Pd neck region and is functional in controlling intercellular transport by degrading Pd-associated callose (Levy et al. 2007a and b). The PR-BG, or PR-2 family proteins, are regulated by stress conditions and are part of a plant innate immune system strongly induced during pathogen invasion along with other PR genes (Leubner-Metzger and Meins 1999).

Several studies have demonstrated a positive effect of tobacco class I vacuolar PR-BG NtGLA and its homologues on the cell-to-cell spread of TMV and other viruses (Beffa and Meins 1996; Beffa et al. 1996; Bucher et al. 2001; Dorokhov et al. 2012; Iglesias and Meins 2000; Ueki and Citovsky 2002). It was shown that, in NtGLA-silenced plants, there was a delay in local and systemic spread of TMV and *Tobacco necrosis virus* in both resistant (NN) and susceptible (nn) tobacco genotypes (Beffa et al. 1996; Iglesias and Meins 2000). Likewise, the local and systemic spread of *Potato virus X* (Iglesias and Meins 2000) and systemic spread of *Turnip vein clearing virus* (TVCV) (Ueki and Citovsky 2002) were similarly reduced in these plants. Stress-induced callose accumulation in response to wounding, high temperature, and xylanase treatment was markedly increased in NtGLA-deficient tobacco (Iglesias and Meins 2000; Ueki and Citovsky 2002), and this was correlated with reduced BG activity (Beffa et al. 1996; Iglesias and Meins 2000). The NtGLA mutant plants also had reduced Pd SEL, as determined by an inhibition of cell-to-cell movement of the *Cucumber mosaic virus* (CMV) 3a MP-GFP fusion protein and fluorescein isothiocyanate-dextran (Iglesias and Meins 2000).

Based on the above data, a hypothesis has been proposed suggesting that, during TMV spread, the infection-induced PR-BG are recruited to Pd by MP^{TMV} together with rep^{TMV}, which would result in decreased deposition of callose associated with Pd and in increased cell-to-cell viral trafficking (Beffa and Meins 1996; Epel 2009; Guenoun-Gelbart et al. 2008; Iglesias and Meins 2000; Zavaliev et al. 2011). However, the subcellular mechanism of this process is yet unknown. Also, the role of Pd-associated BG (AtBG_{pap}) in virus spread was not determined.

In the present study, we used *Arabidopsis* BG and a compatible host-virus system to further test the above hypothesis. We show that two *Arabidopsis* PR-BG, AtBG2 (PR-2) and AtBG3, are transcriptionally induced during virus infection but the transcription of Pd-localized AtBG_{pap} is unchanged. We demonstrate that AtBG2 has a stress-dependent extracellular localization which does not lead to its accumulation at Pd callose deposits. During TMV spread, AtBG2 remains within the ER and is not delivered to callose deposits at Pd. Finally, in *atbg_{pap}* mutants only, callose levels at Pd were increased and virus spread was reduced. We conclude that the subcellular localization of BG is critical for their function in controlling Pd transport during virus spread. Our findings provide new insight into the mechanism of Pd regulation mediated by BG, as well as reveal the subcellular relationship between virus movement

and defense response, particularly the induction and targeting of PR-BG.

RESULTS

Virus infection induces transcription of PR-BG but not of AtBG_{pap}.

It has variously been reported that PR-BG play a role in cell-to-cell spread of TMV in tobacco (Beffa et al. 1996; Bucher et al. 2001; Dorokhov et al. 2012; Iglesias and Meins 2000). However, the underlying cellular mechanism is unknown. To learn more about the involvement of PR-BG in TMV spread, we turned our efforts to the *Arabidopsis* model, in which a genetic analysis can easily be performed. A BLAST search of *Arabidopsis* sequences was conducted using tobacco NtGLA, which was shown to increase virus spread, in order to identify *Arabidopsis* homologues. Four protein sequences that exhibit over 50% identity to NtGLA were detected: AtBG1 (At3g57270), AtBG2 (At3g57260), AtBG3 (At3g57240), and At4g16260 (Supplementary Fig. S1). All four are predicted secreted extracellular PR proteins shown to be induced during infection with bacterial and fungal pathogens (Dong et al. 1991; Doxey et al. 2007). The most conserved segment among all PR-BG was their catalytic domain, which belongs to the glycosyl hydrolase family 17 (GH17) of enzymes. Real-time polymerase chain reaction (PCR) analysis was performed on plants infected with GFP-tagged TVCV (TVCV-GFP) (Harries et al. 2009), a virus closely related to TMV. We also determined the effect of TVCV-GFP on the mRNA levels of AtBG_{pap} (At5g42100), a Pd-localized BG (Levy et al. 2007a). *Arabidopsis* plants were infected with the purified RNA of TVCV-GFP and gene induction was analyzed using cDNA prepared from the entire inoculated leaves at 0 to 6 days postinoculation (dpi). The infected leaves did not show any detectable symptoms during the time of experiment (not shown). The infection was verified by observation of the inoculated leaves under UV light, which showed discrete TVCV-GFP fluorescent infection sites (Fig. 1A, insert). The expression of both AtBG2 and AtBG3 increased relative to 0 dpi as the infection progressed (Fig. 1A). The relative increase in the expression of At4g16260 was similar between mock and virus infection (Fig. 1A). The mRNA levels of AtBG_{pap} increased only slightly relative to 0 dpi in both mock and virus infections, and remained at the same level throughout the experiment in both treatments (Fig. 1A). The induction of PR-BG was also confirmed in a similar experiment, only with wild-type (WT) CMV using sap from infected *Nicotiana tabacum* leaves as inoculum. The expression of all three PR-BG increased to a much greater extent in response to local CMV infection, reaching a maximum at 2 dpi (Fig. 1B). The latter is consistent with the known stronger induction of salicylic acid (SA)-dependent defense genes during CMV infection in *Arabidopsis* (Lewsey et al. 2010; Singh et al. 2004; Whitham et al. 2003). AtBG1 expression was lower than the detection threshold and the gene could not be calibrated for real-time (RT)-PCR analyses (not shown). Unlike AtBG2, the expression of AtBG3 also was high in mock infection with both viruses (Fig. 1), indicating possible response of this BG to wounding as well. Because AtBG2 showed the strongest and most consistent accumulation in response to virus infection, it was selected for further subcellular analyses.

Transgenically expressed AtBG2 accumulates in the ER and is not secreted to cell wall.

To understand the precise role of AtBG2 in virus movement, we chose a host-virus system in which AtBG2 localization could be followed along with TMV spread in a susceptible plant at the subcellular level. For this purpose, we stably expressed AtBG2 fused to GFP at the C terminus under the 35S

constitutive promoter (35S::AtBG2-GFP) in *N. benthamiana* plants, in which the movement of TMV can be analyzed. Confocal laser-scanning micrography (CLSM) of the transgenic plants show that AtBG2-GFP accumulates in the ER network in healthy cells (Fig. 2A and B). Contrary to expectations, AtBG2-GFP is not detectable in the cell wall, as shown by the observation of extracellular spaces formed on the retraction of the protoplast from the cell wall stained with propidium iodide (PI) in plasmolysed cells (Fig. 2C, asterisk). Fluorescence of AtBG2-GFP in the apoplast could not be detected even at higher excitation intensities (not shown). Weak extracellular accumulation could be observed only in cells of old leaves of transgenic plants showing signs of senescence (not shown). Similar results were obtained in transgenic *Arabidopsis* and *N. tabacum* (NN) transformed with 35S::AtBG2-GFP construct (Supplementary Fig. S2). GFP fusion of AtBG3 also targeted

the ER, similar to AtBG2-GFP (data not shown). We also expressed the AtBG2-GFP fusion under its native promoter in transgenic *N. benthamiana* (data not shown). We observed a low background expression level of AtBG2-GFP in these plants, yet the fusion protein targeted the ER network as in overexpressing plants, indicating that accumulation of AtBG2 in the ER represents its native localization.

SA induces the secretion of AtBG2 in transgenic plants.

The observation that AtBG2-GFP accumulated in the ER and was not secreted was surprising. Thus, we tested the effect of stress signaling on the export of AtBG2 to apoplast. Because SA mediates the stress signaling that controls the accumulation of PR proteins, we analyzed the localization of AtBG2-GFP following treatment with SA. External application by spray of 5 mM SA triggered the secretion of AtBG2-GFP to the cell wall 24 h after treatment (Fig. 2D). In those epidermal cells in which AtBG2-GFP secretion was incomplete, both the secreted (Fig. 2D, arrow) and the ER fraction with secretion vesicles (Fig. 2D, triangle) could be observed. The secreted AtBG2-GFP appeared as continuous fluorescence viewed from the upper cell wall plane, which is the plane where part of the anticlinal cell wall and the ER network can be viewed together. Upon plasmolysis, AtBG2-GFP was detected in the formed extracellular spaces (Fig. 2E and F, asterisks). SA-treated control plants expressing the ER-retained GFP-HDEL (green fluorescent protein with an ER retention signal) (16c plants) (Ruiz et al. 1998) showed no GFP fluorescence in the apoplast (Fig. 2G).

In SA-treated cells, in which AtBG2-GFP was completely secreted, almost no ER fraction was detected and AtBG2-GFP fluorescence appeared only in the apoplast (Fig. 2H and H'). Interestingly, in these cells, the continuous AtBG2-GFP fluorescence in the mid-cell-wall plane was interrupted by fluorescence-free zones stretched along the anticlinal walls (Fig. 2H'). Callose staining with aniline blue confirmed that these zones correspond to Pd/pit fields and showed that AtBG2-GFP is not specifically enriched at those sites (Fig. 2I and I'). This result indicates that, despite its affinity to callose as substrate, the free extracellular AtBG2 is unable to accumulate in cell wall regions that include Pd.

SA-induced secretion of AtBG2-GFP was found to be Golgi dependent, because treatment with Golgi disruptor brefeldin-A (BFA) strongly inhibited the accumulation of AtBG2-GFP in the apoplast (Supplementary Fig. S3). At 5 mM concentration, SA spray induced the appearance of sporadic localized cell death which was visible at 24 h after treatment of *N. benthamiana* leaves (Supplementary Fig. S4). The PI staining, which can also be used to detect nuclei of nonviable cells (Truernit and Haseloff 2008), showed that the region of AtBG2-GFP secretion is found in living cells outside the zone of dead cells of the necrotic lesion. At 2.5 mM SA concentration, the cell death was less conspicuous and, consequently, the secretion of AtBG2-GFP was weaker (not shown). Together, both localization of GFP-HDEL and PI staining show that SA-induced accumulation of AtBG2-GFP in the apoplast is a result of Golgi-dependent secretion taking place in living cells, and is not a result of membrane leakage due to accompanying cell death.

To confirm that the SA-induced increase in AtBG2-GFP accumulation in the apoplast is, indeed, a result of de novo protein accumulation in this compartment, we analyzed the actual protein levels in the secreted and total fractions following SA treatment. Apoplastic fluids (AF) and total tissue proteins were extracted from SA and mock-treated leaves of AtBG2-GFP overexpressing plants at 24 h, and the protein levels were determined in Western blot using α -GFP antibody (Fig. 2J).

Fig. 1. Relative mRNA levels of AtBG2, AtBG3, At4g16260, and AtBG_pap in *Arabidopsis* (Col) following infection with **A**, Turnip vein clearing virus, which expresses green fluorescent protein (TVCV-GFP, shown as TVCV); and **B**, wild-type Cucumber mosaic virus (CMV) at indicated time points (days postinoculation [dpi]). **A**, In TVCV-GFP-infected leaves, AtBG2 and AtBG3 expression was induced by virus infection, while At4g16260 and AtBG_pap RNA levels did not change significantly above mock. **B**, In CMV-infected leaves, AtBG2, AtBG3, and At4g16260 expression was maximum at 2 dpi. AtBG_pap expression was not altered significantly by CMV infection. Data represent average readings relative to 0 dpi $\pm$ standard error from three infected plants at each time point. Three leaves on each plant were infected. *Arabidopsis* actin8 was used as normalizing gene. Insert in A shows the UV images of representative mock- and TVCV-GFP-infected leaves at 6 dpi. CMV infection was confirmed by enzyme-linked immunosorbent assay with anti-Cp^{CMV} antibody (not shown).

The 16c plants were used as a negative control. Compared with mock treatment, AF from the SA-treated leaves contained significantly higher levels of AtBG2-GFP (approximately 64 kDa) (Fig. 2J). In the total protein fraction, the levels of

AtBG2-GFP were similar in both treatments (Fig. 2J). In 16c plants, SA treatment did not alter the localization of GFP-HDEL (Fig. 2J). The weak band of GFP-HDEL in the secreted fraction of SA-treated tissue indicates possible contamination

Fig. 2. Localization and activity of AtBG2-green fluorescent protein (GFP) in transgenic *Nicotiana benthamiana*. **A** and **B**, Single optical slice confocal laser-scanning micrograph through **A**, the mid-cell plane and **B**, a projection of several optical slices of a cell showing AtBG2-GFP localized in cell-to-cell endoplasmic reticulum (ER) connections and throughout the cortical ER. **C**, Plasmolysis coupled with propidium iodide (PI) staining shows no AtBG2-GFP accumulation in the apoplast. **D** to **F**, Salicylic acid (SA) treatment induces the secretion of AtBG2-GFP to apoplast. **D**, In some cells, there is only partial secretion of AtBG2-GFP to apoplast (arrow) with part still retained within the ER, with secretion vesicles (triangle) as viewed at the upper cell wall plane. **E** and **F**, Plasmolysis of SA-treated tissue coupled with **E**, plasma membrane (PM) and **F**, cell wall PI staining shows AtBG2-GFP accumulation in the apoplast. **G**, Plasmolysis and PM staining of SA-treated control plants overexpressing GFP-HDEL (GFP with an ER retention signal) do not show GFP accumulation in the apoplast. **H** and **H'**, In fully secreting cells of SA-treated tissue, the continuous AtBG2-GFP fluorescence in the apoplast is interrupted by plasmodesmata (Pd) sites (arrows). **H**, Projection of 16 optical slices through the entire anticlinal wall; **H'**, the same image in rotation and perspective. **I** and **I'**, Callose staining with aniline blue (shown in red) shows that secreted AtBG2-GFP is not enriched at Pd-callose deposits (arrows). **I**, GFP channel only and **I'**, overlay of both GFP and aniline blue channels. **J**, Western blot of secreted and total tissue proteins of the SA-treated leaves from AtBG2-GFP- and GFP-HDEL-expressing plants using α -GFP antibody. Ponceau staining shows an approximately 22-kDa band, which did not change considerably between treatments, indicating equal loading. **K**, β -1,3-Glucanase (BG) activity (gray bars) of the tissue extracts from the healthy AtBG2-GFP and GFP-HDEL transgenic plants. White bars show total protein content of the extracts. Values represent average of enzyme activity/total protein $\pm$ standard error of three plants from each line. **J**, Asterisk indicates statistical significance of the mean at $P < 0.05$, as determined by Student's t test. The experiment was repeated twice with similar results. Bars = 10 μ m. Arrowhead in **E** and **G** shows the retracted PM. Asterisks in **C**, **E**, **F**, and **G** indicate the extracellular spaces formed after plasmolysis.

from the cellular contents, probably due to cell death induced by SA. The result further confirms that SA-induced accumulation of AtBG2-GFP in the cell wall is a result of its de novo secretion. Low levels of secreted AtBG2-GFP detected in the mock treatment (Fig. 2J) could be a result of cellular stress caused by tissue manipulation.

To find out whether the AtBG2-GFP fusion is enzymatically functional, BG activity was assayed in tissue extracts from

AtBG2-GFP and 16c control transgenic plants using labeled laminarin (β -1,3-glucan) as a substrate. Tissue extracts from AtBG2-GFP plants had higher BG activity compared with control plants expressing GFP-HDEL, indicating that the AtBG2-GFP fusion retains its catalytic function (Fig. 2K). Only a small difference in the activity between 16c and AtBG2-GFP plants was expected, because the ER-localized AtBG2-GFP would be poorly extracted by the BG reaction buffer, which did not

Fig. 3. Confocal laser scanning micrography of leaf section from AtBG2-green fluorescent protein (GFP)-expressing transgenic *Nicotiana benthamiana* infected with *Tobacco mosaic virus*, which lacks coat protein (CP) and expresses movement protein (MP) fused to red fluorescent protein (RFP) (TMV^{ΔCP}-MP-RFP), at 4 days postinoculation. Infection stages indicated by I, II, and III correspond to zones of the infection ring. **A** to **C**, AtBG2-GFP and MP-RFP co-localize in virus-induced endoplasmic reticulum bodies in newly infected cells within zone I. The empty arrow in **A** indicates the direction of virus movement. Cell 0 is the cell in which MP-RFP is as-yet nondetectable. Cell 1 is the first cell in which MP-RFP accumulation can be detected. **A**, Enlargement of the marked area at the wall region (w) in cell 1 shows early formation of MP-RFP/AtBG2-GFP bodies at the cell periphery (arrows). Enlargement of the marked area at the upper cortical focus (c) in cell 1 shows the cortical MP-RFP/AtBG2-GFP body (arrowhead). **D** to **F**, AtBG2-GFP and MP-RFP localization in cells within zone II. **G** to **I**, AtBG2-GFP and MP-RFP localization in cells within zone III. **G** to **I**, Insert shows enlargement of the marked area at the upper cortical focus. **A**, **D**, and **G**, MP-RFP red channel only. **B**, **E**, and **H**, AtBG2-GFP green channel only. **C**, **F**, and **I**, Both green and red channels overlaid. Images in **A** to **C** are overlays with differential interference contrast channel. Bars: **A** to **I** = 10 µm; enlargements in **A** to **C** and **G** to **I** = 5 µm.

include detergents. The high background activity in both plants is probably contributed by the endogenous vacuolar BG which, unlike the ER-localized BG, would be easily extracted by the above method because they were shown to accumulate to high levels in healthy leaf tissues (Ward et al. 1991).

Taken together, the above results show that overexpression of AtBG2 in healthy cells is not a sufficient condition for its secretion to the cell wall. SA induces the secretion and accumulation of the ER-localized AtBG2 in the apoplast. Importantly, when AtBG2 is secreted, it is not enriched at callose deposits at Pd. The fusion of GFP to the C terminus of AtBG2 does not abolish its catalytic function.

In TMV-infected cells, AtBG2 resides in virus-induced ER bodies containing MP and other ER membrane and lumen proteins.

Next, we analyzed the effect of TMV infection on the localization of AtBG2-GFP in epidermal cells of transgenic *N. benthamiana*. Plants were infected with the infectious RNA of a recombinant TMV with its MP fused to the monomeric red fluorescent protein (RFP) and its coat protein (CP), which is dispensable for cell-to-cell movement, deleted (TMV^{ACP}-MP-RFP) (Ashby et al. 2006). This allowed us to track by dual-color CLSM changes in the intracellular localization of both AtBG2-GFP and MP-RFP during viral cell-to-cell spread. Inoculation of *N. benthamiana* with TMV^{ACP}-MP-RFP results in infection sites with the characteristic ring-shaped fluorescence. The ring shape is a result of large amounts of MP at the leading edge of the expanding infection, which subsequently degrades during later stages (Epel et al. 1996; Heinlein et al. 1998; Padgett et al. 1996). At 4 dpi, the subcellular location of AtBG2-GFP and MP-RFP was determined in three areas (zones) of the infection site representing three successive stages in MP-RFP accumulation. In newly infected cells at the leading edge of the infection site (zone I), MP-RFP is first detected as small, weakly fluorescent bodies at both the cellular cortex (not shown) and the cell periphery (Fig. 3A, cell 1 arrows), which possibly indicate Pd sites. Cell 1 indicates the first cell in which MP-RFP fluorescence can be detected (Fig. 3A). Cell 0 indicates an adjacent cell, in which MP-RFP fluorescence is as-yet undetectable (Fig. 3A). The faint peripheral MP-RFP bodies in cell 1 can be seen to co-localize with larger AtBG2-GFP bodies (Fig. 3B and C, arrows). The co-localization occurs also in strongly fluorescent bodies forming at the cortex of cell 1, where there is no cell-to-cell contact and no Pd (Fig. 3A to C, arrowhead). The above observation of cell 1 shows that there is a gradient of MP-RFP accumulation which forms within a single cell along the direction of virus movement, and that the ER reorganization apparently precedes the formation of MP-RFP bodies. These observations indicate that, at the very early stage of infection, there is possibly a time delay between the formation of the ER bodies and MP-RFP accumulation, due to either the time needed for RFP fluorescence maturation or the very low level of MP at this stage (Wright et al. 2007a and b). Consequently, the real infection edge cannot be detected by following the accumulation of the fluorescent-protein-tagged MP during virus spread (Wright et al. 2007a). Therefore, we refer to the infection edge as the one in which strong MP-RFP fluorescence can first be detected. Experiments using transgenic plants expressing either Calnexin-yellow fluorescent protein (YFP) (ER-membranal protein) or GFP-HDEL (ER-luminal protein) showed that MP-RFP co-localizes with AtBG2-GFP in a manner similar to its co-localization with these ER proteins (Supplementary Fig. S5B to G).

Within cells at mid-stages of infection (zone II), there is high accumulation of MP-RFP, which is associated with cytoskeletal structures, including microtubules (Heinlein et al. 1998), and with large amorphous bodies at the cell periphery (Fig. 3D).

Most of the large bodies at the periphery still contain AtBG2-GFP, which is not associated with microtubules (Fig. 3E and F). Little or no accumulation of AtBG2-GFP is detected in tubular ER (cortical region not shown), probably due to the partial disruption of the ER network at this stage (Reichel and Beachy 1998). Within cells at late stages of infection (zone III), MP-RFP and AtBG2-GFP do not co-localize (Fig. 3I). Here, MP-RFP accumulates almost exclusively at Pd sites (Fig. 3G) previously shown to be Pd central cavities (Faulkner et al. 2008). However, AtBG2-GFP accumulates in the restored ER network at both cellular cortex and cell-to-cell connections, similar to uninfected cells (Fig. 3H, insert).

The above results are in agreement with previous observations showing that MP^{TMV} accumulates in virus-induced ER bodies early during infection (Boyko et al. 2000; Gillespie et al. 2002; Hofmann et al. 2009; Mas and Beachy 1999; Sambade et al. 2008). In addition, it is demonstrated that these bodies also contain AtBG2 in a manner similar to the ER luminal and ER membranal proteins.

MP^{TMV}- or AtBG2-containing ER bodies associate with Pd at the edge of the infection spread.

To closely examine the association of MP-RFP- or AtBG2-GFP-containing ER bodies at Pd sites early during infection, the two proteins were imaged along with Pd-associated callose deposits by triple-color CLSM. Callose accumulation around Pd was induced by cutting through the TMV^{ACP}-MP-RFP infection site and wound-induced callose around Pd was identified by aniline blue staining. At the infection edge where MP-RFP can first be detected (cell 1), small MP-RFP or AtBG2-GFP bodies were at first appressed to the cell wall at the vicinity of Pd (Fig. 4A to C, Pd-callose is shown in white). As the infection progressed, these bodies enlarged and appeared as paired fluorescent spots connected by individual Pd in the middle (Fig. 4D to F). Later still, these Pd-associated bodies grew in size and fused laterally at Pd clusters from both sides of the cell (Fig. 4G to I). These results show that, at the infection edge of TMV, both MP-RFP and AtBG2-GFP localize at the ER bodies which are associated with Pd sites.

AtBG2 is not exported to extracellular space during TMV movement.

For AtBG2 to be active in callose degradation at Pd, the protein has to be delivered out of the ER to the extracellularly located callose deposits at the Pd neck region early during infection. If the virus-induced Pd association of AtBG2-GFP is coupled with its export to cell wall, then it would be expected to accumulate also in the apoplast of these cells as a free extracellular protein. Yet, unlike SA treatment (Fig. 2D to F), the extracellular accumulation of AtBG2-GFP could not be observed during TMV^{ACP}-MP-RFP spread. Plasmolysis of the cells from both early and late stages of infection spread (zones I and III, respectively) revealed no AtBG2-GFP accumulation in the formed extracellular spaces (Fig. 5A and B). This result indicates that cell-to-cell spread of TMV does not detectably induce the secretion of AtBG2 to the cell wall.

Because the ER system undergoes structural changes from the very early stages of TMV infection (Figs. 3 and 4), it is possible that these changes interfere with the ability of virus-infected cells to secrete the ER accumulated proteins, including AtBG2. To verify whether virus-infected cells retain the ability to secrete AtBG2-GFP, the TMV^{ACP}-MP-RFP-infected leaves of transgenic plants were treated with SA, and localization of AtBG2-GFP was analyzed. To ensure the delivery of SA to all cells, infected leaves were detached at 3 dpi and the petiole was placed either in water or 2.5 mM SA for 24 h before observation. As a result of the inhibition of virus spread

by SA, the infection sites had blunt edges and an enlarged zone III compared with the control (Fig. 5C and F). A closer examination revealed, that in cells at the leading edge of SA-treated infection site, MP-RFP accumulation was similar to later stages of infection, where MP is associated with enlarged ER bodies and cytoskeleton (Fig. 5G and H). It can be seen that, unlike water control, SA treatment triggered the secretion

of AtBG2-GFP out of the ER, including the virus-induced ER bodies (Fig. 5D and G). In infected 16c control plants, SA treatment did not change the localization of GFP-HDEL compared with control (Fig. 5E and H). This result demonstrates that virus-induced ER reorganization does not interfere with its secretory function and suggests that TMV infection does not significantly induce or suppress the SA response.

Fig. 4. Confocal laser-scanning micrograph showing localization of movement protein fused to red fluorescent protein (MP-RFP), AtBG2-green fluorescent protein (GFP), and callose in cells at the leading edge (zone I) of infection with *Tobacco mosaic virus* (TMV), which lacks coat protein (CP) and expresses movement protein fused to red fluorescent protein (TMV^{ΔCP}-MP-RFP). Transgenic *Nicotiana benthamiana* overexpressing AtBG2-GFP was infected with viral RNA and, at 4 days postinoculation, wound callose at plasmodesmata (Pd) (shown in white) was induced by cutting through the infection site followed by aniline blue staining. **A to C**, In initially infected cell, MP-RFP accumulates in small endoplasmic reticulum bodies appressed to the cell wall, often in the vicinity of Pd (arrows), and co-localizes with AtBG2-GFP. Cell 0 is the one in which MP-RFP is not yet detectable. Cell 1 is the first cell in which MP-RFP accumulation can be detected. **D to F**, Later, bodies appear as double fluorescent spots on both sides of Pd (arrow). **G to I**, Later yet, the bodies enlarge, fuse, and still flank Pd. **A, D**, and **G**, Overlays of MP-RFP red and callose white (pseudo-colored) channels. **B, E**, and **H**, Overlays of AtBG2-GFP green and callose white channels. **C, F**, and **I**, Three channels overlaid. **G to I**, Inserts show enlargements of the area marked by arrows with the callose channel turned off. All arrows indicate also the direction of virus spread. Bars: A to C and G to I = 10 µm and D to F = 5 µm.

Taken together, the above subcellular analysis of TMV-infected cells shows that the association of AtBG2 with MP^{TMV} at virus-induced bodies occurs within the ER at both cortex and Pd sites, but the protein is not delivered to extracellular space of these cells.

Effect of AtBG2 overexpression on TMV spread.

To further test the hypothesis that PR-BG have a positive effect on virus movement, we examined whether TMV^{ACP}-MP-RFP spread is enhanced in transgenic *N. benthamiana* and *N. tabacum* (NN) plants that overexpress AtBG2-GFP. In *N. ben-*

Fig. 5. **A** and **B**, Confocal laser-scanning micrography showing localization of movement protein fused to red fluorescent protein (MP-RFP) and AtBG2-green fluorescent protein (GFP) in plasmolyzed leaf tissue infected with *Tobacco mosaic virus* (TMV), which lacks coat protein (CP) and expresses MP-RFP (TMV^{ACP}-MP-RFP). **A**, In cells at the infection edge (zone I), AtBG2-GFP is associated with virus-induced endoplasmic reticulum (ER) bodies (yellow) but is not detected in the extracellular spaces formed between the retracted protoplast and cell wall. Cell 0 is the one in which MP-RFP is not yet detectable. Cell 1 is the first cell in which MP-RFP accumulation can be seen. **B**, In cells at the late stage of infection (zone III), AtBG2-GFP accumulates in ER, does not co-localize with MP, and is absent from the formed extracellular spaces. Asterisks in **A** and **B** indicate the extracellular spaces formed after plasmolysis and arrowheads point out the retracted plasma membrane. **C** to **H**, Localization of AtBG2-GFP and GFP-HDEL (GFP with an ER retention signal) in cells at the infection front (zone I) following salicylic acid (SA) treatment. Infected leaves expressing either AtBG2-GFP or GFP-HDEL were detached at 3 days postinoculation, and the petiole was placed either in water (–SA) or 2.5 mM SA (+SA) for 24 h. **C**, Water-treated infection site has a larger ring and small central zone. MP-RFP co-localizes with both **D**, AtBG2-GFP and **E**, GFP-HDEL in small ER-derived bodies at the infection edge. **F**, SA-treated infection site has a smaller ring with enlarged central zone. **G** and **H**, At the infection edge, the MP-RFP ER bodies are large, characteristic of later stage of infection. **G**, Most of the AtBG2-GFP is secreted out of the ER bodies (red) and appear as continuous green fluorescence in the cell wall (arrow). **H**, SA has no effect on GFP-HDEL localization. Bars: **A**, **B**, **D**, **E**, **G**, and **H** = 10 µm and **C** and **F** = 500 µm.

thamiana, virus spread was determined by measuring the diameter of fluorescent infection sites at 5 dpi. No significant difference in TMV^{ACP}-MP-RFP spread was observed between AtBG2-GFP and 16c transgenic *N. benthamiana* (Fig. 6). In *N. tabacum* (NN), TMV^{ACP}-MP-RFP was allowed to spread for 4 days at 32°C, the temperature in which hypersensitive response (HR) is suppressed, and then the temperature was switched to 24°C for another 3 days to induce HR formation. Similar to *N. benthamiana*, no significant differences in the area of necrotic lesions formed at 7 dpi were observed between AtBG2-GFP-expressing transgenic and WT *N. tabacum* (NN) plants (Fig. 6). These results show that, even though AtBG2-GFP is abundant and maintains its hydrolytic activity in transgenic plants, it does not increase local TMV spread in either susceptible or resistant genetic backgrounds.

Callose at Pd is increased and virus spread is reduced in *atbg_pap* but not *atbg2* mutants.

In order to determine the effect of BG mutation on callose at Pd and on virus spread, we compared both Pd-callose accumulation and spread of a tobamovirus TVCV in WT and two T-DNA knockout mutants, *atbg2* and *atbg_pap*. The homozy-

Fig. 6. Local spread of Tobacco mosaic virus (TMV), which lacks coat protein (CP) and expresses movement protein fused to red fluorescent protein (MP-RFP) (TMV^{ACP}-MP-RFP) in transgenic *Nicotiana benthamiana* and *N. tabacum* 'Samsun' (NN) overexpressing AtBG2-green fluorescent protein (GFP). Virus spread in *N. benthamiana* leaves was determined by measuring the area of fluorescent infection sites at 5 days postinoculation (dpi). Transgenic plants overexpressing GFP-HDEL (GFP with an ER retention signal) were used as control. Virus spread in *N. tabacum* (NN) was determined in inoculated plants initially incubated at 32°C for 4 days and, subsequently, grown at 24°C for another 3 days (7 dpi). The area of developed necrotic lesions was measured in transgenic and wild-type (WT) plants. A representative fluorescent infection site on *N. benthamiana* and necrotic lesions on *N. tabacum* (NN) leaf are shown in the upper panels. Data are presented as the average area of infection site/necrotic lesion $\pm$ standard error from four (*N. benthamiana*) and six (*N. tabacum*) plants for each line. Two leaves on each plant were infected. Lowercase letters indicate statistical differences of the mean at $P < 0.05$, as determined by Student's *t* test, for each time point. Experiments were repeated twice with similar results. Numbers within columns indicate the number of infection sites measured (*n*). Bars = 0.75 and 5 mm for *N. benthamiana* and *N. tabacum*, respectively.

gous *atbg2* knockout mutants were isolated by PCR screen from *Arabidopsis* T-DNA insertion line WiscDsLoxHs_009_07c (Nishal et al. 2005) disrupted in the AtBG2 coding region. Both genomic DNA and cDNA of the mutants lacked the intact AtBG2 gene sequence (Supplementary Fig. S6). The T-DNA insert was mapped to position 106 of the AtBG2 coding region. The mutant plants had no significant differences in growth and morphology from the WT (not shown). The *atbg_pap* knockout mutants have been described earlier in our lab (Levy et al. 2007a). The two independent knockout alleles of *atbg_pap*, SALK_019116.47.55.x and SAIL_115_G04, were shown to accumulate callose at Pd and have reduced Pd permeability (Levy et al. 2007a). The SAIL_115_G04 mutant, which was more similar to the WT in growth and morphology than to the stronger SALK_019116.47.55.x mutant (Levy et al. 2007a), was used for callose quantification and virus infections. Callose levels at Pd were determined from mature rosette leaves of noninfected WT and *atbg_pap* and *atbg2* mutant plants by the aniline blue staining procedure coupled with CLSM. The intensity of punctate aniline blue fluorescence at Pd sites was quantified from the CLSM images of the abaxial leaf epidermal cells as the measure of callose accumulation at Pd (Fig. 7A). The values of fluorescence intensity were plotted as percentage of WT (Fig. 7A). In *atbg_pap* mutants, callose accumulation at Pd was significantly higher compared with the WT (Fig. 7A). In *atbg2* mutants, however, callose levels at Pd were similar to those of WT plants (Fig. 7A). We then analyzed the local spread of TVCV-GFP in these BG mutants compared with the WT. The plants were inoculated with the RNA of TVCV-GFP and virus spread was analyzed by measuring the area of fluorescent infection sites at 3.5 to 7.5 dpi (Fig. 7B). In *atbg_pap* mutants, virus spread was significantly reduced between 4.5 and 7.5 dpi whereas, in *atbg2* mutants, virus spread was similar to the WT between 5.5 and 7.5 dpi (Fig. 7B). These results show that i) mutation in *atbg_pap* but not in *atbg2* affects both callose accumulation and virus spread and ii) the localization of a BG at Pd is critical for its function as a regulator of Pd transport during virus movement. Even though the transcription of AtBG2 is induced during TVCV infection, it has no effect on callose at Pd and virus spread.

DISCUSSION

Among the many stress responses to pathogens, including viruses, are callose formation, SA synthesis, and such nonspecific responses as the production of various PR proteins (Carr et al. 2010). Among the induced PR proteins are BG, which are involved in callose hydrolysis. Because virus spread is via Pd, callose accumulation at Pd would inhibit virus movement. Thus, the virus must, in some way, control callose accumulation at Pd to allow fast colonization of host tissues. This may be accomplished by suppression of viral-induced callose synthesis or enhancement of its hydrolysis in the cell wall surrounding the Pd neck region. Because PR-BG are induced during stress response, it has been suggested that viruses may exploit this reaction to open Pd and allow unobstructed cell-to-cell spread. Another possibility is that the virus may, in some manner, suppress the stress-induced activity of CalS which is constitutively present (Epel 2009; Guenoune-Gelbart et al. 2008).

This study was conducted to gain a more detailed insight into the role of plant BG, both PR-BG and the constitutively present Pd-associated BG, in virus spread through Pd. Toward this aim, we analyzed the induction, subcellular accumulation, and function of these BG in tobamovirus cell-to-cell movement. Only two PR-BG, AtBG2 and AtBG3, were significantly induced by TVCV (Fig. 1A), a tobamovirus closely related to

TMV (Lartey et al. 1997). AtBG_{pap} is constitutively present and it did not undergo significant induction as a result of TVCV infection (Fig. 1A). PR proteins, including AtBG2, were previously shown to be induced in *Arabidopsis* during compatible interaction with different groups of viruses, including tobamoviruses TVCV, TMV, and *Oilseed rape mosaic virus* (Fig. 1) (Dardick et al. 2000; Love et al. 2005; Wang et al. 2009; Whitham et al. 2003; Yang et al. 2007).

When overexpressed, both AtBG2 and AtBG3 accumulate in the ER and are not secreted to the apoplast in noninfected or nonstressed tissue (Fig. 2A to C). This was surprising considering that both are predicted to be secreted proteins and do not possess any targeting or retention signals at the C terminus. Further examination with AtBG2 showed that this protein is secreted if the tissue is treated with SA (Fig. 2D to F). Previous studies have shown that SA-induced extracellular accumulation of PR proteins in *Arabidopsis* is dependent on the induction of the ER-associated protein-folding and secretion machinery (Jelitto-Van Dooren et al. 1999; Wang et al. 2005). The latter are chaperones and co-chaperones which were shown to be necessary for PR1 secretion in *Arabidopsis* (Wang et al. 2005). Our results support the above data and show that SA induction of AtBG2 is a two-component process involving accumulation in the ER and secretion of the accumulated protein pool into apoplast. The secretion of AtBG2-GFP was observed to be strongest with 5 mM SA. Similarly, strong AtBG2-GFP secretion was observed around symptoms developed in transgenic *Arabidopsis* infected with *Pseudomonas syringae* pv. *tomato* DC3000 and in transgenic *N. benthamiana* infected with *Botrytis cinerea* (R. Zavaliev and B. L. Epel, unpublished data). We also noted that, in SA-treated leaf tissue, large amounts of callose accumulated at Pd sites (R. Zavaliev and B. L. Zavaliev and Epel, unpublished data). SA-induced localized cell death and callose accumulation at Pd were also reported by previous studies (Krasavina et al. 2002; Lee et al.

2005). These latter observations indicate that secretion of PR-BG occurs under conditions of strong immune response when cell death is induced and Pd SEL is expected to decrease due to callose accumulation. It was shown that lower SA concentration (1 mM), which did not induce cell death in tobacco, led to increased Pd SEL of tobacco epidermal cells (Murphy and Carr 2002). Our results with AtBG2 indicate that this positive effect of SA on Pd transport is unlikely to be mediated by extracellular PR-BG, because only higher SA concentrations were required to induce AtBG2 secretion (Fig. 2).

If PR-BG is to gate Pd during TMV spread, then MP^{TMV} must target the protein to Pd and promote its delivery to extracellular Pd-callose deposits. This was found not to be the case. At the leading edge of infection spread, in cells that first showed MP-RFP fluorescence (cell 1), MP-RFP and AtBG2-GFP together with the ER-resident proteins co-localized in bodies that were observed to localize at cellular cortex and at Pd (Figs. 3 and 4, cell 1). However, the functionality of the BG in these Pd-associated bodies is questionable, because plasmolysis of the infected cells from both early and late stages did not show extracellular accumulation of AtBG2-GFP (Fig. 5A and B). The lack of AtBG2 secretion was apparently not a result of changes in the ER structure or function, because SA treatment could promote the secretion of AtBG2-GFP out of virus-modified ER (Fig. 5G). These results, along with data on virus spread in AtBG2-GFP-overexpressing plants (Fig. 6), demonstrate that the ER-localized PR-BG (AtBG2) cannot be functional in controlling Pd transport during TMV spread. It also suggests that the level of defense response activated during compatible infection is not sufficient to trigger secretion of PR-BG to the cell wall. Therefore, it is possible that defense responses, including induction and secretion of PR-BG, are suppressed during successful TMV spread. A recent study showed that the expression of individual TMV proteins (MP^{TMV}, CP^{TMV}, or a combination of both) in a susceptible tobacco

Fig. 7. A, Callose accumulation at plasmodesmata (Pd) and **B**, local spread of TVCV-green fluorescent protein (GFP) in wild-type (WT) and two knockout *Arabidopsis* β -1,3-glucanase (BG) mutants, *atbg_pap* and *atbg2*. **A**, Callose levels at Pd in *atbg_pap* and *atbg2* mutants relative to WT plants. The representative images of aniline-blue-stained leaf epidermis are shown in upper panels. For each line, three plants and three leaves on each plant were used for callose measurements. At least 30 images for each line were analyzed. Values are presented as percentage of average of callose intensity from Pd sites in mutants relative to that in WT plants $\pm$ standard error (SE). Asterisk indicates statistical difference of the mean at $P < 0.0001$, as determined by Student's *t* test. The experiment was repeated twice, with similar results. **B**, Local spread of TVCV-GFP in WT, *atbg_pap*, and *atbg2* mutants quantified by measuring the area of fluorescent infection sites at 3.5 to 7.5 days postinoculation (dpi) from the inoculated adaxial leaf surface. The representative fluorescent infection sites at 6.5 dpi are shown in upper panels. Two plants and three leaves on each plant were inoculated for each time point. Data are presented as the average area of infection sites $\pm$ SE. Lowercase letters indicate statistical differences of the mean at $P < 0.001$, as determined by Student's *t* test for each time point. The experiment was repeated twice with similar results. Numbers within columns indicate number of infection sites measured (*n*). Bars: A = 20 μ m and B = 1 mm.

resulted in PR genes induction and accumulation of reactive oxygen species; however, these responses were significantly reduced when plants expressed the intact virus (Conti et al. 2012). It was also shown that TMV infection in a susceptible *Arabidopsis* ecotype led to the suppression of SA-mediated systemic defense responses by virus-induced degradation of ATAF2, a transcription factor that regulates basal defenses, including PR gene induction (Wang and Culver 2012; Wang et al. 2009). Likewise, our earlier results showed that expression of rep^{TMV} alone in *N. benthamiana* led to the strong defense response manifested by callose accumulation at Pd, which was reduced when the virus movement was complemented by the addition of MP^{TMV} (Guenoune-Gelbart et al. 2008). Based on the present work and the above recent findings, we suggest that the previously observed reduction in Pd-callose during TMV movement is due to suppression of stress-associated callose synthesis at Pd. The latter assumption requires further investigation using mutants compromised in stress-induced callose synthesis.

Consistent with its cellular localization in noninfected and in TMV-infected cells, AtBG2 mutation did not affect the basal callose levels at Pd and the local spread of TVCV in *Arabidopsis* (Fig. 7). This finding led us to the conclusion that AtBG2, although induced during infection, is not involved in callose regulation at Pd and in promoting virus spread in *Arabidopsis*. In contrast, a constitutively expressed BG, AtBG_{pap}, does play such a role, because in *atbg_{pap}* knockout mutants, both Pd permeability (Levy et al. 2007a) and virus spread (Fig. 7B) are compromised. These effects are in agreement with the observed higher accumulation of basal callose levels at Pd in *atbg_{pap}* mutants (Fig. 7A). AtBG_{pap} is a Pd-localized GPI-anchored protein, bound to the external leaflet of the plasma membrane (PM), directly facing the callose deposits at Pd (Levy et al. 2007a). AtBG_{pap} has a constitutive, nearly ubiquitous expression in all tissues (Doxey et al. 2007; Levy et al. 2007a) and it accumulates at Pd of noninfected plants (Levy et al. 2007a). The same constitutive Pd localization was recently demonstrated for GPI-anchored BG from *Populus* spp. (Rinne et al. 2011; van der Schoot et al. 2011). Yet, in contrast to AtBG_{pap}, the tobacco PR-BG, NtGLA, which has a similar effect on Pd SEL and virus spread (Beffa et al. 1996; Bucher et al. 2001; Dorokhov et al. 2012; Iglesias and Meins 2000; Ueki and Citovsky 2002), is an intracellular protein localized to the central vacuole (Shinshi et al. 1988; Sticher et al. 1992b). Interestingly, the NtGLA, like other vacuolar BG, is not secreted to extracellular space in both healthy and infected plants (Keefe et al. 1990; Sticher et al. 1992a; Van den Bulcke et al. 1989). Moreover, in noninfected NtGLA-silenced plants, the Pd SEL was already reduced (Iglesias and Meins 2000), suggesting a role of this PR-BG in a constitutive callose regulation at Pd, a function similar to AtBG_{pap} in *Arabidopsis* (Levy et al. 2007a). Yet, because NtGLA is vacuolar, it is not clear how this BG is delivered to callose deposits at Pd. We show that free extracellular BG (AtBG2) is unable to accumulate at Pd-callose deposits (Fig. 2I and I'), suggesting that it is not the possible extracellular accumulation of vacuolar BG that is functional in Pd regulation. Thus, an alternative secretion pathway must exist for vacuolar BG that would target them to extracellular callose deposits at Pd, apparently through association with the PM.

Conclusion.

As opposed to CalSs, which are regulated at the protein level (Brownfield et al. 2009), the regulation of plant BG occurs at the levels of tissue-specific expression and compartmentalized accumulation (Doxey et al. 2007; Hrmova and Fincher 2009; Leubner-Metzger and Meins 1999). Therefore, the specific

subcellular localization of a BG is critical for its function in different cellular processes. The present work demonstrates that the constitutive control of Pd permeability mediated by Pd-associated GPI-anchored BG, rather than by free extracellular PR-BG, directly affects virus cell-to-cell spread. It also indicates that BG activity at Pd both during compatible virus infection and a strong immune response is unlikely to be increased. We propose that virus-triggered control of callose levels at Pd is mediated by the suppression of stress-associated callose synthesis at Pd. We continue to investigate the role of *Arabidopsis* BG in control of transport through Pd. The upcoming study will focus on the targeting mechanism of different groups of BG to Pd and its modulation by various physiological conditions.

MATERIALS AND METHODS

Plant material and SA treatment.

N. benthamiana and *N. tabacum* 'Samsun' (NN) were grown at 25°C under long-day conditions (16 h of light and 8 h of darkness, total light intensity of 80 $\mu\text{E m}^{-2} \text{s}^{-1}$). *Arabidopsis thaliana* ecotype Columbia (Col) was grown at 22°C under long- or short-day conditions as above. For virus infections, short-day-grown *Arabidopsis* plants were used. All virus-inoculated plants were kept at 25°C. Transgenic *N. benthamiana* expressing the ER luminal marker GFP-HDEL (16c plants) were described by Ruiz and associates (1998). For the ER membranous marker, transgenic *N. benthamiana* expressing *Arabidopsis* Calnexin (At5g07340) fused to YFP was used (D. Guenoune-Gelbart and B. L. Epel, unpublished data). SA (Sigma, St. Louis) was dissolved in dimethyl sulfoxide to 1 M stock solution, and different working concentrations were prepared by dilutions with double-distilled water (DDW) containing 0.01% Tween 20. Unless otherwise stated, SA was sprayed on leaves of *N. benthamiana* 24 h prior to observation or protein extraction.

Viruses and plant inoculations.

For TMV infections, a version of the virus was used which lacks the CP, and the MP is fused to the monomeric RFP (TMV^{ACP}-MP-RFP) (Ashby et al. 2006). For TVCV infections, a full-length virus was used which expresses GFP as free protein (TVCV-GFP) (Harries et al. 2009). Both viral vectors were linearized by *KpnI* and used as DNA templates for in vitro transcription reactions employing the T7 (TMV^{ACP}-MP-RFP) or SP6 (TVCV-GFP) High Yield Message Maker Ampli-Cap Max Kit (Epicentre, Madison, WI, U.S.A.). The obtained viral RNA was directly used as inoculum, and water was used for mock inoculations. For CMV infections, leaf sap of *N. tabacum* 'Xanthi' systemically infected with WT CMV serotype II (Porta et al. 1989) was used. The sap was extracted by homogenizing leaf tissue in a buffer containing 0.5 M sodium phosphate, pH 7, and 0.1% sodium sulfide. The buffer alone was used for mock inoculations. For *Arabidopsis*, 3-week-old short-day-grown plants (approximately eight rosette leaves) were used, and three first fully expanded leaves of a plant were inoculated. CMV infection was confirmed by ELISA with anti-CP^{CMV} antibody (not shown). For *N. benthamiana* and *N. tabacum*, 4-week-old plants were used and the first fully expanded leaf of a plant was inoculated. All plants were rub-inoculated at the adaxial leaf surface using Carborundum.

Real-time PCR.

Real-time PCR with primers for AtBG1 (At3g57270), AtBG2 (At3g57260), AtBG3 (At3g57240), At4g16260, and AtBG_{pap} (At5g42100) (Supplementary Table S1) was performed on TVCV-GFP- and CMV-infected *Arabidopsis* leaves.

Total RNA was extracted from either virus- or mock-inoculated leaves using the SV-total RNA extraction kit (Promega, Madison, WI, U.S.A.) and cDNA was prepared by RT-PCR using Moloney murine leukemia virus RT (Promega). Real-time PCR was performed with the StepOnePlus Real-Time PCR System using SYBRGreen Fast PCR mix (Applied Biosystems, Foster City, CA, U.S.A.). *Arabidopsis* actin (At1g49240) served as the normalizing gene.

Cloning and plant transformation.

For the expression in plants, the AtBG2 (At3g57260) sequence was amplified without its stop codon by PCR with AtBG2 forward primer containing *Xba*I and *Eco*RI sites and the reverse primer containing an *Xba*I site. The AtBG2 gene sequence was cloned into a unique *Xba*I site present in pBinGFP (Sagi et al. 2005), downstream of the 35S promoter, between the omega enhancer sequence and the *sGFP* start codon. *Eco*RI digestion was used to verify AtBG2 orientation in pBIN-AtBG2-GFP construct. *Agrobacterium*-mediated transformation of *N. benthamiana* and *N. tabacum* (NN) plants with pBIN-AtBG2-GFP construct was performed as described (Sagi et al. 2005). For *N. benthamiana*, 6 benzylaminopurine at 1 µg/ml and naphthaleneacetic acid at 0.1 µg/ml were used instead of kinetin and indole-3-acetic acid, and the plants were regenerated in medium containing 3% instead of 1.5% sucrose. A floral dipping method (Clough and Bent 1998) was used for *Arabidopsis* (Col) transformation with pBIN-AtBG2-GFP. In all studies, the T₂ progeny of transgenic plants was used.

Western blotting and enzyme activity assay.

Secreted proteins from *N. benthamiana* leaves were extracted according to Wang and associates (2005), with modifications. Three leaves were vacuum infiltrated with ice-cold AF buffer (100 mM Tris-HCl [pH 7.5], 500 mM sucrose, 10 mM MgCl₂, 10 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM β-mercaptoethanol, and a cocktail of protease inhibitors [Roche, Branchburg, NJ, U.S.A.], gently blotted with tissue paper, arranged in a 20-ml syringe with petioles pointing upward, and centrifuged at 500 × g for 2 min at 4°C. The obtained liquid (approximately 1 ml) was used as the secreted fraction. Total proteins were extracted from 100 mg of leaf tissue homogenized in 300-µl extraction buffer containing 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA, 10% glycerol, 0.5% sodium dodecyl sulfate (SDS), 1% Triton-100, 1 mM PMSF, 2 mM β-mercaptoethanol, and a cocktail of protease inhibitors. The homogenate was incubated on ice for 10 min and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was used as the total protein fraction. Secreted and total proteins were boiled for 5 min in a loading buffer, run on 12% SDS polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with a monoclonal α-GFP antibody (Covance, Princeton, NJ, U.S.A.).

For BG activity, the extract was prepared from 100 mg of leaf tissue homogenized in 300 µl of reaction buffer containing 100 mM sodium acetate (pH 5), 1 mM PMSF, and a cocktail of protease inhibitors (Roche). The homogenate was centrifuged at 14,000 rpm at 4°C for 10 min. The supernatant was assayed for BG activity colorimetrically (Wirth and Wolf 1992) using laminarin azure (Sigma) as a substrate according to Kawai and associates (2006). A 100-µl reaction mixture containing 50 µl of tissue extract and 50 µl of laminarin azure (10 mg/ml final concentration) was incubated at 37°C for 1 h, and the reaction was terminated by the addition of 250 µl of ethanol, incubated at room temperature for 10 min, then centrifuged at 1,500 × g for 10 min, and the absorbance of the supernatant was measured at 595 nm. The reactions were performed in duplicate, and blanks were prepared without the addition of

tissue extracts. Enzymatic activity was expressed in units per milligram of protein as $\Delta A_{595} \text{ min}^{-1} \text{ mg}^{-1}$ of protein. The protein content of the tissue extracts was determined with Bradford protein assay (Bio-Rad, Munich).

atbg2 mutant isolation.

Arabidopsis thaliana (Col) T-DNA insertion line WiscDs-LoxHs009_07C (Nishal et al. 2005), having insertion in the AtBG2 gene, was obtained from the Arabidopsis Biological Resource Center stock. Mutants were initially screened by PCR on the genomic DNA using T-DNA left border (L4) and AtBG2-RP primer set to select those carrying T-DNA. PCR product was cloned and sequenced to confirm the position of T-DNA. Homozygous plants were isolated from T-DNA-positive plants by PCR on genomic DNA and cDNA using primers for the AtBG2 entire coding region. Plants with no PCR product from both genomic DNA and cDNA were assigned as homozygous *atbg2* mutants. Actin8 gene primers were used for positive control in PCR reactions.

Tissue staining, plasmolysis, and callose quantification.

Cell walls were stained by incubating leaf tissue in PI (Sigma) dissolved in H₂O at 1 mg/ml for 5 to 10 min before observation. For plasmolysis, leaf tissue was incubated in 0.75 M mannitol solution for 10 min before observation. Plasma membrane staining was performed with N-[3-triethylammoniumpropyl]-4-[p-diethylaminophenyl]hexatrienyl pyridinium dibromide (FM4-64; Molecular Probes, Eugene, OR, U.S.A.). A 1:500 water-diluted working solution of FM4-64 was prepared from a 10-µg/µl stock solution. Leaf pieces were incubated in the working solution for 0.5 to 1 h before imaging. Pd-associated wound-callose was induced by cutting a 5-by-7-mm leaf tissue sample and incubating for 1 to 2 min in 0.1% Aniline blue (Fluka, Milwaukee, WI, U.S.A.) (dissolved in DDW and 1 M glycine, pH 9.5, at a volume ratio of 2:3) prior to observation. Intact cells adjacent to those at the cut edge were observed. Callose in SA-treated tissue was stained in the same way, except that larger (10-by-10-mm) leaf samples were used and incubation time was 2 h. For Pd-callose quantification, entire leaves were cut at the petiole and fixed immediately in 96% ethanol for approximately 2 h or until completely bleached. After washing in distilled water, the samples were stained in the above Aniline blue solution for 5 h before observation. Callose levels at Pd were quantified based on size of fluorescent particles from threshold CLSM images using an automated particle analysis plug-in in the ImageJ software.

Microscopy.

Confocal fluorescence microscopy was conducted using the Zeiss LSM 510 META confocal microscope. GFP and YFP were excited with a 488-nm argon laser and emission was detected with a 505- to 530-nm band-pass filter. mRFP was excited with a 561-nm DPSS laser and emission was detected with a 572- to 625-nm band-pass filter. Aniline blue was excited with a 405-nm diode laser and emission was detected with a 420- to 480-nm band-pass filter. PI was excited with a 488-nm argon laser and emission was detected with a 585- to 615-nm band-pass filter. FM4-64 was excited with a 561-nm DPSS laser and emission was detected with spectral channel adjusted to 572 to 636 nm. Infection sites produced by fluorescent viruses were observed with stereoscopic fluorescence microscope (MVX10; Olympus, Tokyo). For mRFP, the U-MRFPHQ fluorescence mirror unit, containing a 535- to 555-nm excitation filter, 565-nm dichromatic mirror, and 570- to 625-nm emission filter, was used. For GFP, the U-MGFPHQ fluorescence mirror, containing a 460- to 480-nm excitation filter, 485-nm dichromatic mirror, and 495- to 540-nm emis-

sion filter, was used. The size of infection sites was measured on images using ImageJ software. Images of the whole *Arabidopsis* leaves infected with TVCV-GFP were taken with the CRI Maestro imaging system (Perkin Elmer, Norwalk, CT, U.S.A.) using a 455-nm excitation filter and 515-nm long-path emission filter. The emission scanning range was set to 500 to 620 nm (10-nm step) to collect both GFP (green) and background chlorophyll (red) fluorescence.

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LITERATURE CITED

- Ashby, J., Boutant, E., Seemanpillai, M., Groner, A., Sambade, A., Ritzenthaler, C., and Heinlein, M. 2006. *Tobacco mosaic virus* movement protein functions as a structural microtubule-associated protein. *J. Virol.* 80:8329-8344.
- Beachy, R. N., and Heinlein, M. 2000. Role of P30 in replication and spread of TMV. *Traffic* (Copenhagen, Denmark) 1:540-544.
- Beffa, R., and Meins, F. 1996. Pathogenesis-related functions of plant beta-1,3-glucanases investigated by antisense transformation—A review. *Gene* 179:97-103.
- Beffa, R. S., Hofer, R. M., Thomas, M., and Meins, F., Jr. 1996. Decreased susceptibility to viral disease of beta-1,3-glucanase-deficient plants generated by antisense transformation. *Plant Cell* 8:1001-1011.
- Bell, K., and Oparka, K. 2011. Imaging plasmodesmata. *Protoplasma* 248:9-25.
- Boyko, V., Ferralli, J., Ashby, J., Schellenbaum, P., and Heinlein, M. 2000. Function of microtubules in intercellular transport of plant virus RNA. *Nat. Cell Biol.* 2:826-832.
- Brownfield, L., Doblin, M. S., Fincher, G. B., and Bacic, A. 2009. Biochemical and molecular properties of biosynthetic enzymes for (1,3)-beta-glucans in embryophytes, chlorophytes and rhodophytes. Pages 283-326 in: *Chemistry, Biochemistry and Biology of (1-3)-Beta-Glucans and Related Polysaccharides*, A. Bacic, G. B. Fincher, and B. A. Stone, eds. Academic Press, New York.
- Bucher, G. L., Tarina, C., Heinlein, M., Di Serio, F., Meins, F., Jr., and Iglesias, V. A. 2001. Local expression of enzymatically active class I beta-1, 3-glucanase enhances symptoms of TMV infection in tobacco. *Plant J.* 28:361-369.
- Carr, J. P., Lewsey, M. G., and Palukaitis, P. 2010. Signaling in induced resistance. *Adv. Virus Res.* 76:57-121.
- Christensen, N., Tilsner, J., Bell, K., Hammann, P., Parton, R., Lacomme, C., and Oparka, K. 2009a. The 5' cap of *Tobacco mosaic virus* (TMV) is required for virion attachment to the actin/endoplasmic reticulum network during early infection. *Traffic* (Copenhagen, Denmark) 10:536-551.
- Christensen, N. M., Faulkner, C., and Oparka, K. 2009b. Evidence for unidirectional flow through plasmodesmata. *Plant Physiol.* 150:96-104.
- Citovsky, V., and Zambryski, P. 2000. Systemic transport of RNA in plants. *Trends Plant Sci.* 5:52-54.
- Citovsky, V., Knorr, D., Schuster, G., and Zambryski, P. 1990. The P30 movement protein of *Tobacco mosaic virus* is a single-strand nucleic acid binding protein. *Cell* 60:637-647.
- Citovsky, V., Wong, M. L., Shaw, A. L., Prasad, B. V., and Zambryski, P. 1992. Visualization and characterization of *Tobacco mosaic virus* movement protein binding to single-stranded nucleic acids. *Plant Cell* 4:397-411.
- Clough, S. J., and Bent, A. F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16:735-743.
- Conti, G., Rodriguez, M. C., Manacorda, C. A., and Asurmendi, S. 2012. Transgenic expression of *Tobacco mosaic virus* capsid and movement proteins modulate plant basal defense and biotic stress responses in *Nicotiana tabacum*. *Mol. Plant-Microbe Interact.* 25:1370-1384.
- Dahiya, P., and Brewin, N. J. 2000. Immunogold localization of callose and other cell wall components in pea nodule transfer cells. *Protoplasma* 214:210-218.
- Dardick, C. D., Golem, S., and Culver, J. N. 2000. Susceptibility and symptom development in *Arabidopsis thaliana* to *Tobacco mosaic virus* is influenced by virus cell-to-cell movement. *Mol. Plant-Microbe Interact.* 13:1139-1144.
- Deom, C. M., Schubert, K. R., Wolf, S., Holt, C. A., Lucas, W. J., and Beachy, R. N. 1990. Molecular characterization and biological function of the movement protein of *Tobacco mosaic virus* in transgenic plants. *Proc. Natl. Acad. Sci. U.S.A.* 87:3284-3288.
- Ding, B., Haudenshield, J. S., Hull, R. J., Wolf, S., Beachy, R. N., and Lucas, W. J. 1992. Secondary plasmodesmata are specific sites of localization of the *Tobacco mosaic virus* movement protein in transgenic tobacco plants. *Plant Cell* 4:915-928.
- Ding, B., Itaya, A., and Woo, Y. M. 1999. Plasmodesmata and cell-to-cell communication in plants. *Int. Rev. Cytol. Surv. Cell Biol.* 190:251-316.
- Dong, X., Mindrinos, M., Davis, K. R., and Ausubel, F. M. 1991. Induction of *Arabidopsis* defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. *Plant Cell* 3:61-72.
- Dorokhov, Y. L., Komarova, T. V., Petrunia, I. V., Frolova, O. Y., Pozdyshev, D. V., and Gleba, Y. Y. 2012. Airborne signals from a wounded leaf facilitate viral spreading and induce antibacterial resistance in neighboring plants. *PLoS Pathog.* 8:e1002640.
- Doxey, A. C., Yaish, M. W., Moffatt, B. A., Griffith, M., and McConkey, B. J. 2007. Functional divergence in the *Arabidopsis* beta-1,3-glucanase gene family inferred by phylogenetic reconstruction of expression states. *Mol. Biol. Evol.* 24:1045-1055.
- Epel, B. L. 2009. Plant viruses spread by diffusion on ER-associated movement-protein-rafts through plasmodesmata gated by viral induced host beta-1,3-glucanases. *Semi. Cell Dev. Biol.* 20:1074-1081.
- Epel, B. L., Padgett, H. S., Heinlein, M., and Beachy, R. N. 1996. Plant virus movement protein dynamics probed with a GFP-protein fusion. *Gene* 173:75-79.
- Faulkner, C., Akman, O. E., Bell, K., Jeffree, C., and Oparka, K. 2008. Peeking into pit fields: a multiple twinning model of secondary plasmodesmata formation in tobacco. *Plant Cell* 20:1504-1518.
- Fitzgibbon, J., Bell, K., King, E., and Oparka, K. 2010. Super-resolution imaging of plasmodesmata using three-dimensional structured illumination microscopy. *Plant Physiol.* 153:1453-1463.
- Gillespie, T., Boevink, P., Haupt, S., Roberts, A. G., Toth, R., Valentine, T., Chapman, S., and Oparka, K. J. 2002. Functional analysis of a DNA-shuffled movement protein reveals that microtubules are dispensable for the cell-to-cell movement of *Tobacco mosaic virus*. *Plant Cell* 14:1207-1222.
- Guenoune-Gelbart, D., Elbaum, M., Sagi, G., Levy, A., and Epel, B. L. 2008. *Tobacco mosaic virus* (TMV) replicase and movement protein function synergistically in facilitating TMV spread by lateral diffusion in the plasmodesmal desmotubule of *Nicotiana benthamiana*. *Mol. Plant-Microbe Interact.* 21:335-345.
- Guseman, J. M., Lee, J. S., Bogenschutz, N. L., Peterson, K. M., Virata, R. E., Xie, B., Kanaoka, M. M., Hong, Z., and Torii, K. U. 2010. Dysregulation of cell-to-cell connectivity and stomatal patterning by loss-of-function mutation in *Arabidopsis* CHORUS (GLUCAN SYNTHASE-LIKE 8). *Development* 137:1731-1741.
- Harries, P. A., Park, J. W., Sasaki, N., Ballard, K. D., Maule, A. J., and Nelson, R. S. 2009. Differing requirements for actin and myosin by plant viruses for sustained intercellular movement. *Proc. Natl. Acad. Sci. U.S.A.* 106:17594-17599.
- Haupt, S., Cowan, G. H., Ziegler, A., Roberts, A. G., Oparka, K. J., and Torrance, L. 2005. Two plant-viral movement proteins traffic in the endocytic recycling pathway. *Plant Cell* 17:164-181.
- Heinlein, M., and Epel, B. L. 2004. Macromolecular transport and signaling through plasmodesmata. *Int. Rev. Cytol.* 235:93-164.
- Heinlein, M., Epel, B. L., Padgett, H. S., and Beachy, R. N. 1995. Interaction of tobamovirus movement proteins with the plant cytoskeleton. *Science* 270:1983-1985.
- Heinlein, M., Padgett, H. S., Gens, J. S., Pickard, B. G., Casper, S. J., Epel, B. L., and Beachy, R. N. 1998. Changing patterns of localization of the *Tobacco mosaic virus* movement protein and replicase to the endoplasmic reticulum and microtubules during infection. *Plant Cell* 10:1107-1120.
- Hofmann, C., Niehl, A., Sambade, A., Steinmetz, A., and Heinlein, M. 2009. Inhibition of *Tobacco mosaic virus* movement by expression of an actin-binding protein. *Plant Physiol.* 149:1810-1823.
- Hrmova, M., and Fincher, G. B. 2009. Plant and microbial enzymes involved in the depolymerization of (1,3)-beta-D-glucans and related polysaccharides Pages 119-170 in: *Chemistry, Biochemistry and Biology of (1-3)-Beta-Glucans and Related Polysaccharides*. A. Bacic, G. B. Fincher, and B. A. Stone, eds. Academic Press, New York.
- Iglesias, V. A., and Meins, F., Jr. 2000. Movement of plant viruses is delayed in a beta-1,3-glucanase-deficient mutant showing a reduced plas-

- modest size exclusion limit and enhanced callose deposition. *Plant J.* 21:157-166.
- Jelitto-Van Dooren, E. P., Vidal, S., and Denecke, J. 1999. Anticipating endoplasmic reticulum stress. A novel early response before pathogenesis-related gene induction. *Plant Cell* 11:1935-1944.
- Kawai, R., Igarashi, K., Yoshida, M., Kitaoka, M., and Samejima, M. 2006. Hydrolysis of beta-1,3/1,6-glucan by glycoside hydrolase family 16 endo-1,3(4)-beta-glucanase from the basidiomycete *Phanerochaete chrysosporium*. *Appl. Microbiol. Biotechnol.* 71:898-906.
- Kawakami, S., Watanabe, Y., and Beachy, R. N. 2004. *Tobacco mosaic virus* infection spreads cell to cell as intact replication complexes. *Proc. Natl. Acad. Sci. U.S.A.* 101:6291-6296.
- Keefe, D., Hinz, U., and Meins, F. 1990. The effect of ethylene on the cell-type-specific and intracellular-localization of beta-1,3-glucanase and chitinase in tobacco-leaves. *Planta* 182:43-51.
- Krasavina, M. S., Malysenko, S. I., Ralduzina, G. N., Burmistrova, N. A., and Nosov, A. V. 2002. Can salicylic acid affect the intercellular transport of the *Tobacco mosaic virus* by changing plasmodesmal permeability? *Russ. J. Plant Physiol.* 49:71-77.
- Lartey, R., Ghoshroy, S., Ho, J., and Citovsky, V. 1997. Movement and subcellular localization of a tobamovirus in *Arabidopsis*. *Plant J.* 12:537-545.
- Lee, J. Y., Taoka, K., Yoo, B. C., Ben-Nissan, G., Kim, D. J., and Lucas, W. J. 2005. Plasmodesmal-associated protein kinase in tobacco and *Arabidopsis* recognizes a subset of non-cell-autonomous proteins. *Plant Cell* 17:2817-2831.
- Leubner-Metzger, G., and Meins, F. 1999. Functions and regulation of plant beta-1,3-glucanases (PR-2). Pages 49-76 in: *Pathogenesis-Related Proteins in Plants*. S. K. Datta and S. Muthukrishnan, eds. CRC Press, Boca Raton, FL, U.S.A.
- Levy, A., and Epel, B. L. 2009. Cytology of the (1-3)-beta-glucan (callose) in plasmodesmata and sieve plate pores. Pages 439-463 in: *Chemistry, Biochemistry and Biology of (1-3)-Beta-Glucans and Related Polysaccharides*. A. Bacic, G. B. Fincher, and B. A. Stone, eds. Academic Press, New York.
- Levy, A., Erlanger, M., Rosenthal, M., and Epel, B. L. 2007a. A plasmodesmata-associated beta-1,3-glucanase in *Arabidopsis*. *Plant J.* 49:669-682.
- Levy, A., Guenoun-Gelbart, D., and Epel, B. L. 2007b. Beta-1,3-glucanases: plasmodesmal gate keepers for intracellular communication. *Plant Signal. Behav.* 2:404-407.
- Lewsey, M. G., Murphy, A. M., Maclean, D., Dalchau, N., Westwood, J. H., Macaulay, K., Bennett, M. H., Moulin, M., Hanke, D. E., Powell, G., Smith, A. G., and Carr, J. P. 2010. Disruption of two defensive signaling pathways by a viral RNA silencing suppressor. *Mol. Plant-Microbe Interact.* 23:835-845.
- Liarzi, O., and Epel, B. L. 2005. Development of a quantitative tool for measuring changes in the coefficient of conductivity of plasmodesmata induced by developmental, biotic, and abiotic signals. *Protoplasma* 225:67-76.
- Love, A. J., Yun, B. W., Laval, V., Loake, G. J., and Milner, J. J. 2005. *Cauliflower mosaic virus*, a compatible pathogen of *Arabidopsis*, engages three distinct defense-signaling pathways and activates rapid systemic generation of reactive oxygen species. *Plant Physiol.* 139:935-948.
- Mas, P., and Beachy, R. N. 1999. Replication of *Tobacco mosaic virus* on endoplasmic reticulum and role of the cytoskeleton and virus movement protein in intracellular distribution of viral RNA. *J. Cell Biol.* 147:945-958.
- Murphy, A. M., and Carr, J. P. 2002. Salicylic acid has cell-specific effects on *Tobacco mosaic virus* replication and cell-to-cell movement. *Plant Physiol.* 128:552-563.
- Nishal, B., Tantikanjana, T., and Sundaresan, V. 2005. An inducible targeted tagging system for localized saturation mutagenesis in *Arabidopsis*. *Plant Physiol.* 137:3-12.
- Olesinski, A. A., Almon, E., Navot, N., Perl, A., Galun, E., Lucas, W. J., and Wolf, S. 1996. Tissue specific expression of the *Tobacco mosaic virus* movement protein in transgenic potato plants alters plasmodesmal function and carbohydrate partitioning. *Plant Physiol.* 111:541-550.
- Oparka, K. J., Roberts, A. G., Prior, D. A. M., Chapman, S., Baulcombe, D., and SantaCruz, S. 1995. Imaging the green fluorescent protein in plants—viruses carry the torch. *Protoplasma* 189:133-141.
- Oparka, K. J., Prior, D. A., SantaCruz, S., Padgett, H. S., and Beachy, R. N. 1997. Gating of epidermal plasmodesmata is restricted to the leading edge of expanding infection sites of *Tobacco mosaic virus* (TMV). *Plant J.* 12:781-789.
- Padgett, H. S., Epel, B. L., Kahn, T. W., Heinlein, M., Watanabe, Y., and Beachy, R. N. 1996. Distribution of tobamovirus movement protein in infected cells and implications for cell-to-cell spread of infection. *Plant J.* 10:1079-1088.
- Porta, C., Devergne, J. C., Cardin, L., Briand, J. P., and Van Regenmortel, M. H. 1989. Serotype specificity of monoclonal antibodies to cucumber mosaic virus. *Arch. Virol.* 104:271-285.
- Reichel, C., and Beachy, R. N. 1998. *Tobacco mosaic virus* infection induces severe morphological changes of the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* 95:11169-11174.
- Rinne, P. L., and van der Schoot, C. 2003. Plasmodesmata at the crossroads between development, dormancy, and defense. *Can. J. Bot.* 81:1182-1197.
- Rinne, P. L., Welling, A., Vahala, J., Ripel, L., Ruonala, R., Kangasjarvi, J., and van der Schoot, C. 2011. Chilling of dormant buds hyperinduces FLOWERING LOCUS T and recruits GA-inducible 1,3-beta-glucanases to reopen signal conduits and release dormancy in *Populus*. *Plant Cell* 23:130-146.
- Roy, S., Watada, A. E., and Wergin, W. P. 1997. Characterization of the cell wall microdomain surrounding plasmodesmata in apple fruit. *Plant Physiol.* 114:539-547.
- Ruiz, M. T., Voynet, O., and Baulcombe, D. C. 1998. Initiation and maintenance of virus-induced gene silencing. *Plant Cell* 10:937-946.
- Sagi, G., Katz, A., Guenoun-Gelbart, D., and Epel, B. L. 2005. Class 1 reversibly glycosylated polypeptides are plasmodesmal-associated proteins delivered to plasmodesmata via the Golgi apparatus. *Plant Cell* 17:1788-1800.
- Sambade, A., Brandner, K., Hofmann, C., Seemanpillai, M., Mutterer, J., and Heinlein, M. 2008. Transport of TMV movement protein particles associated with the targeting of RNA to plasmodesmata. *Traffic (Copenhagen, Denmark)* 9:2073-2088.
- Shinshi, H., Wenzler, H., Neuhaus, J. M., Felix, G., Hofsteenge, J., and Meins, F. 1988. Evidence for N-terminal and C-terminal processing of a plant defense-related enzyme—primary structure of tobacco prepro-beta-1,3-glucanase. *Proc. Natl. Acad. Sci. U.S.A.* 85:5541-5545.
- Singh, D. P., Moore, C. A., Gilliland, A., and Carr, J. P. 2004. Activation of multiple antiviral defence mechanisms by salicylic acid. *Mol. Plant Pathol.* 5:57-63.
- Sticher, L., Hinz, U., Meyer, A. D., and Meins, F. 1992a. Intracellular transport and processing of a tobacco vacuolar beta-1,3-glucanase. *Planta* 188:559-565.
- Sticher, L., Hofsteenge, J., Milani, A., Neuhaus, J. M., and Meins, F. Jr. 1992b. Vacuolar chitinases of tobacco: a new class of hydroxyproline-containing proteins. *Science* 257:655-657.
- Sutherland, P., Hallett, L., Redgwell, R., Benhamou, N., and MacRae, E. 1999. Localization of cell wall polysaccharides during kiwifruit (*Actinidia deliciosa*) ripening. *Int. J. Plant Sci.* 160:1099-1109.
- Truernit, E., and Haseloff, J. 2008. A simple way to identify non-viable cells within living plant tissue using confocal microscopy. *Plant Methods* 4:15.
- Ueki, S., and Citovsky, V. 2002. The systemic movement of a tobamovirus is inhibited by a cadmium-ion-induced glycine-rich protein. *Nat. Cell Biol.* 4:478-486.
- Van den Bulcke, M. V., Bauw, G., Castresana, C., Van Montagu, M., and Vandekerckhove, J. 1989. Characterization of vacuolar and extracellular beta(1,3)-glucanases of tobacco: evidence for a strictly compartmentalized plant defense system. *Proc. Natl. Acad. Sci. U.S.A.* 86:2673-2677.
- van der Schoot, C., Paul, L. K., Paul, S. B., and Rinne, P. L. 2011. Plant lipid bodies and cell-cell signaling: a new role for an old organelle? *Plant Signal. Behav.* 6:1732-1738.
- Vaquero, C., Turner, A. P., Demangeat, G., Sanz, A., Serra, M. T., Roberts, K., and Garcia-Luque, I. 1994. The 3a protein from *Cucumber mosaic virus* increases the gating capacity of plasmodesmata in transgenic tobacco plants. *J. Gen. Virol.* 75:3193-3197.
- Vaten, A., Dettmer, J., Wu, S., Stierhof, Y. D., Miyashima, S., Yadav, S. R., Roberts, C. J., Campilho, A., Bulone, V., Lichtenberger, R., Lehesranta, S., Mahonen, A. P., Kim, J. Y., Jokitalo, E., Sauer, N., Scheres, B., Nakajima, K., Carlsbecker, A., Gallagher, K. L., and Helariutta, Y. 2011. Callose biosynthesis regulates symplastic trafficking during root development. *Dev. Cell* 21:1144-1155.
- Wagmann, E., and Zambryski, P. 1995. *Tobacco mosaic virus* movement protein-mediated protein transport between trichome cells. *Plant Cell* 7:2069-2079.
- Wang, D., Weaver, N. D., Kesarwani, M., and Dong, X. 2005. Induction of protein secretory pathway is required for systemic acquired resistance. *Science* 308:1036-1040.
- Wang, X., and Culver, J. N. 2012. DNA binding specificity of ATAF2, a NAC domain transcription factor targeted for degradation by *Tobacco mosaic virus*. *BMC Plant Biol.* 12:157.
- Wang, X., Goregaoker, S. P., and Culver, J. N. 2009. Interaction of the *Tobacco mosaic virus* replicase protein with a NAC domain transcription factor is associated with the suppression of systemic host defenses. *J. Virol.* 83:9720-9730.
- Ward, E. R., Payne, G. B., Moyer, M. B., Williams, S. C., Dincher, S. S.,

- Sharkey, K. C., Beck, J. J., Taylor, H. T., Ahl-Goy, P., Meins, F., and Ryals, J. A. 1991. Differential regulation of beta-1,3-glucanase messenger RNAs in response to pathogen infection. *Plant Physiol.* 96:390-397.
- Whitham, S. A., Quan, S., Chang, H. S., Cooper, B., Estes, B., Zhu, T., Wang, X., and Hou, Y. M. 2003. Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. *Plant J.* 33:271-283.
- Wirth, S. J., and Wolf, G. A. 1992. Micro-plate colourimetric assay for Endo -acting cellulase, xylanase, chitinase, 1,3- β -glucanase and amylase extracted from forest soil horizons. *Soil Biol. Biochem.* 24:511-519.
- Wolf, S., Deom, C. M., Beachy, R. N., and Lucas, W. J. 1989. Movement protein of *Tobacco mosaic virus* modifies plasmodesmatal size exclusion limit. *Science* 246:377-379.
- Wright, K. M., Chapman, S., and Roberts, A. G. 2007a. Plasmodesmal targeting and accumulation of TMV movement protein. *Plant Signal. Behav.* 2:180-181.
- Wright, K. M., Wood, N. T., Roberts, A. G., Chapman, S., Boevink, P., Mackenzie, K. M., and Oparka, K. J. 2007b. Targeting of TMV movement protein to plasmodesmata requires the actin/ER network: evidence from FRAP. *Traffic (Copenhagen, Denmark)* 8:21-31.
- Yang, C., Guo, R., Jie, F., Nettleton, D., Peng, J., Carr, T., Yeakley, J. M., Fan, J. B., and Whitham, S. A. 2007. Spatial analysis of *Arabidopsis thaliana* gene expression in response to *Turnip mosaic virus* infection. *Mol. Plant-Microbe Interact.* 20:358-370.
- Zavaliev, R., Ueki, S., Epel, B. L., and Citovsky, V. 2011. Biology of callose (beta-1,3-glucan) turnover at plasmodesmata. *Protoplasma* 248:117-130.