Characterization of the hypersensitive response-like cell death phenomenon induced by targeting antiviral lectin griffithsin to the secretory pathway

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Summary
Griffithsin (GRFT) is an antiviral lectin, originally derived from a red alga, Griffithsia sp (Mori et al., 2005). GRFT is one of the most potent HIV-1 entry inhibitors yet described, and hence of great interest for use in HIV-1 prophylaxis and therapy (Lusvarghi and Bewley, 2016; O’Keefe et al., 2009). We developed an efficient and scalable manufacturing method for recombinant GRFT using a tobamovirus-based gene expression system in Nicotiana benthamiana and have shown that the plant-produced GRFT product has a favourable preclinical safety and efficacy profile, supporting its development as a topical microbicide to prevent human immunodeficiency virus (HIV). Targeting GRFT to the apoplast for production in Nicotiana benthamiana resulted in necrotic symptoms associated with a hypersensitive response (HR)-like cell death, accompanied by H2O2 generation and increased PR1 expression. Mannose-binding lectins surfactant protein D (SP-D), cytochalasin-N (CV-N) and human mannose-binding lectin (hMBL) also induce salicylic acid (SA)-dependent HR-like cell death in N. benthamiana, and this effect is mediated by the lectin’s glycan binding activity. We found that secreted GRFT interacts with an endogenous glycoprotein, α-xyllosidase (XYL1), which is involved in cell wall organization. The necrotic effect could be mitigated by overexpression of Arabidopsis XYL1, and by co-expression of SA-degrading enzyme NahG, providing strategies for enhancing expression of oligomannose-binding lectins in plants.

Introduction
Griffithsin (GRFT) is a lectin originally isolated from a red alga, Griffithsia sp (Mori et al., 2005). GRFT is one of the most potent HIV-1 entry inhibitors yet described, and hence of great interest for use in HIV-1 prophylaxis and therapy (Lusvarghi and Bewley, 2016; O’Keefe et al., 2009). The GRFT homodimer has six carbohydrate-binding sites, three located at each of the opposite ends of the double-prism homodimer (Moulaei et al., 2010). GRFT binds to high-mannose N-glycans on the HIV viral envelope spike gp120 and inhibits the viral envelope structural transitions which are essential for viral entry, thus preventing HIV infection of T cells (Mori et al., 2005). GRFT also inhibits cell-associated HIV-1 infection of dendritic cells (DC) by inhibiting the transfer of the virus from DC-SIGN to uninfected T cells. Replacement of an aspartic acid residue in each of the glycan binding sites abrogates GRFT’s ability to bind oligomannose glycosylated proteins and eliminates its viral neutralization activity (Xue et al., 2012). Therefore, the GRFT activity is dependent on its ability to bind multiple mannose N-glycans.

In preliminary studies, we found that targeting GRFT to the secretory pathway induced a strong necrotic phenotype reminiscent of the hypersensitive reaction (HR), a resistance response induced by interaction between a plant resistance gene (R gene) and an avirulence factor of a pathogen (Avr gene). HR is a type of programmed cell death that accompanies defence reactions such as generation of reactive oxygen species (ROS) including H2O2, the accumulation of plant hormones such as salicylic acid (SA), jasmonic acid (JA) or ethylene, and the expression of pathogenesis-related (PR) genes (Hammond-Kosack and Jones, 1997; Nurnberger et al., 2004). The HR signals extend to noninfected cells and lead to systemic acquired resistance that is effective against a broad-spectrum of pathogens (Durrant and Dong, 2004). In most HR, SA signalling is involved and SA accumulation shows a strong correlation with generation of ROS (Durrant and Dong, 2004).
In this study, we expressed GRFT with subcellular localization signals using the magnICON replicon-based expression system (Girtich et al., 2006) to avoid any potentially confounding pathogenesis signalling that involve the tobamovirus coat protein, but also to achieve expression levels comparable to the recombinant TMV vector system we used previously (O’Keefe et al., 2009, 2010). Our preliminary data showed that GRFT fused with an apoplast localization signal caused severe laminar necrosis, despite low accumulation levels of apoplast-targeted GRFT compared with cytosol-localized GRFT. Here, we analysed the mechanism whereby GRFT induces HR-like cell death in *N. benthamiana*. We show GRFT-induced HR-like cell death is SA-dependent. Analysis of expression of the GRFT mutant showed six carbohydrate-binding sites per GRFT dimer were required for the induction of HR-like cell death and provided data supporting our hypothesis that a specific interaction between GRFT and an apoplast-located endogenous glycoprotein, XYL1, initiates the HR response. Molecular characterization of the protein interactions and physiological response to targeting GRFT expression to the apoplast helped us identify strategies to dampen the necrosis response and rescue expression of GRFT- and similar engineered lectin fusions in the plant secretory pathway.

**Results**

**Effect of SA accumulation on GRFT protein expression**

Griffithsin localized in the cytosol (GRFT-Cyt) expressed by magnICON showed a higher yield relative to GRFT fused with an apoplast subcellular localization signal (Figure S1a). Accumulation levels of GRFT localized in apoplast (GRFT-Apo) were markedly lower. Targeting GRFT expression to the apoplast caused necrosis in *N. benthamiana*, which was not observed in the plants expressing GRFT in cytosol (Figure S1d). As another group had shown that overexpression of plant lectin CaMBL1 caused salicylic acid (SA)-dependent HR-like cell death in *N. benthamiana* (Hwang and Hwang, 2011), we hypothesized that a specific interaction between GRFT and an apoplast-located endogenous glycoprotein, XYL1, initiates the HR response. Molecular characterization of the protein interactions and physiological response to targeting GRFT expression to the apoplast helped us identify strategies to dampen the necrosis response and rescue expression of GRFT- and similar engineered lectin fusions in the plant secretory pathway.

**PR1 is a marker for salicylic acid-mediated plant defence.** PR1’s induction indicated the possibility that SA accumulation was involved in GRFT-Apo’s induction of HR-like cell death. To test this hypothesis, we used NahG, a protein from *Pseudomonas syringae* which degrades SA (Brodersen et al., 2005). We cloned NahG into a PVX replicon vector (Figure S2, Girtich et al., 2006). Concomitant analyses of PR1 expression and H$_2$O$_2$ levels demonstrated that control plants (transiently expressing GRFT alone) supported high levels of PR1 gene expression and H$_2$O$_2$ generation (Figure 1a,b). Conversely, plants that were inoculated with PVX-NahG and magnICON-GRFT exhibited low levels of PR1 gene expression and we could not detect H$_2$O$_2$ generation in such plants (Figure 1a,b). Therefore, GRFT-Apo-induced HR-like cell death required SA accumulation, and the inhibition of SA accumulation by NahG suppressed the symptoms.

Our results to this point suggest that expression of NahG suppressed HR-related cell death and consequently may allow the co-expressed GRFT protein to accumulate to higher levels in the healthier tissue. To validate this hypothesis, functional GRFT protein levels were measured using an HIV-1 gp120-binding enzyme-linked immunosorbent (ELISA) and total GRFT amounts were assessed by Western blotting (O’Keefe et al., 2009). The ELISA and Western blot analysis showed that both functional and gross expression levels are substantially increased by NahG expression (Figure 1c,d). This result suggests that overexpressing NahG in plants may enhance GRFT production and that a NahG transgenic host may facilitate transient expression of secreted GRFT products.

**GRFT carbohydrate-binding domains induce HR**

GRFT’s activity is due to its specific binding of high-mannose N-glycans. Mutations at the carbohydrate-binding site in GRFT disrupt its ability to bind gp120 and inhibit HIV infection (Moulaei et al., 2010). Based on this feature, we hypothesized that GRFT’s high mannose binding activity is involved in induction of necrosis. To determine whether the carbohydrate-binding sites in GRFT are required for the induction of HR-like cell death, we created a mutated GRFT (GRFT$^{lec}$), which contains the amino acid substitutions D30N, D70N and D112N (Brodersen et al., 2005). We cloned NahG into a PVX replicon vector (Figure S2, Giritch et al., 2006). Based on this feature, we hypothesized that a specific interaction between GRFT and an apoplast-located endogenous glycoprotein, XYL1, initiates the HR response. Molecular characterization of the protein interactions and physiological response to targeting GRFT expression to the apoplast helped us identify strategies to dampen the necrosis response and rescue expression of GRFT- and similar engineered lectin fusions in the plant secretory pathway.

**Purified GRFT can induce HR**

To determine whether the presence of GRFT protein is sufficient to induce HR-like cell death, and to rule out any confounding effect of viral replication on the GRFT-mediated necrotic phenotype, we infiltrated purified GRFT or GRFT$^{lec}$-protein into 2-week-old *N. benthamiana* plants using vacuum infiltration. 5 days after GRFT protein infiltration, the *N. benthamiana* plants showed necrotic symptoms (Figure S5a). GRFT$^{lec}$-protein or buffer-infiltrated plants did not show any symptoms. We detected
H2O2 signal and cell death only in the GRFT-infiltrated plants. PR1 gene expression level was also increased in the GRFT-infiltrated plants (Figure S5b). These results show that GRFT protein with intact lectin activity alone is sufficient to induce HR-like cell death even in the absence of any interaction with a viral factor in the magnICON vector.

Figure 1 NahG expression suppressed GRFT-induced cell death in Nicotiana benthamiana. (a) Necrosis induction, H2O2 generation and enhancement of PR1 gene expression by GRFT were inhibited by NahG expression in N. benthamiana. Detection of cell death and H2O2 generation in the inoculated plant leaves. Dead cells were stained by Evans blue, and H2O2 was detected using the H2O2-sensitive fluorescent probe H2DCFDA. (b) Analysis of PR1 expression levels by real-time RT-PCR. The PR1 mRNA levels relative to the actin mRNA levels were shown. PVX-empty + magnICON-empty, PVX-NahG + magnICON-empty, PVX-empty + magnICON-GRFT and PVX-NahG + magnICON-GRFT were vacuum agro-infiltrated into N. benthamiana. Leaves were harvested 5 days after infiltration and analysed. **P < 0.01 asterisks indicate significant difference [one-way ANOVA with Bonferroni’s multiple comparison test (n = 3)] between inoculated plants group. Error bars represent standard errors of the means. (c) Measurement of GRFT accumulation levels by ELISA. HIV gp-120 was bound to the wells of a 96-well plate and subsequently incubated with total protein from the plants agro-infiltrated with PVX-empty + magnICON-GRFT or PVX-NahG + magnICON-GRFT. After visualized by HRP-labelled anti-GRFT antibody, OD was measured by absorbance at 450 nm. **P < 0.01, asterisks indicate significant difference [Student’s t-test (n = 3)] between PVX-empty + magnICON-GRFT and PVX-NahG + magnICON-GRFT inoculated plants. Error bars represent standard errors of the means. (d) Measurement of GRFT accumulation levels by Western blotting. Anti-GRFT (rabbit) and anti-rabbit HRP were used for detection. CBB stain indicates loaded protein amounts are equal.

Figure 2 The loss of carbohydrate-binding sites in GRFT inhibits GRFT-induced cell death in N. benthamiana. magnICON-mutated GRFT (GRFTlec-), which contains the substitutions D30A, D70A and D112A in carbohydrate-binding sites, was agro-infiltrated into N. benthamiana. (a) Detection of cell death and H2O2 was performed using H2DCFDA and Evans blue in magnICON-GRFTlec- infiltrated N. benthamiana 5 days after infiltration. Cell death and H2O2 were not detected in magnICON-GRFTlec- infiltrated plants. (b) Analysis of PR1 expression levels by real-time RT-PCR. The PR1 mRNA levels relative to the actin mRNA levels were shown. Empty magnICON vector, magnICON-GRFT or magnICON-GRFTlec-, was vacuum agro-infiltrated into N. benthamiana. Leaves were harvested 5 days after infiltration and analysed. **P < 0.01, asterisks indicate significant difference [one-way ANOVA with Bonferroni’s multiple comparison test (n = 3)] between inoculated plants group. Error bars represent standard errors of the means.
Other mannose binding lectins also induce HR when targeted to the apoplast

To test whether other mannose-binding lectins induce HR-like cell death in *N. benthamiana* as observed in GRFT-Apo expression, we focused on surfactant protein D (SP-D, Hasegawa et al., 2015), cyanovirin-N (CV-N, Gao et al., 2010; Xiong et al., 2010), human mannose-binding lectin (hMBL, Ibernon et al., 2014) and galectin-9 (Gal-9, Arikawa et al., 2010; Wang et al., 2008) to express in *N. benthamiana* plants. SP-D, CV-N and hMBL are classified as high-mannose-binding lectins, whereas Gal-9 is a galactose-binding lectin. CV-N is isolated from the cyanobacterium *Nostoc ellipsosporum* (Boyd et al., 1997), and the other lectins are derived from humans. We set out to understand why mannose-binding lectins induce necrosis and determine whether there is a common mechanism involved. This would be a logical first step to engineer a more efficient production system for GRFT and other pharmaceutical lectin proteins in plant.

Plant codon-optimized SP-D, CV-N, hMBL and Gal-9 sequences were cloned into the magnICON vector with the apoplast signal peptide. The lectins were expressed in *N. benthamiana* by agroinfiltration. When the lectins were fused with an apoplast-targeting signal, all three mannose-binding lectins induced necrotic symptoms, but plants expressing Gal-9 did not (Figure 56a). In the necrotic plants, cell death, H2O2 generation and increased PR1 expression levels were detected as in GRFT-Apo-induced necrosis (Figure S6a,b). To investigate whether the necrosis by the mannose-binding lectins is dependent on the SA pathway as shown with GRFT, we suppressed the SA signal pathway using NahG. NahG and the mannose-binding lectins were co-inoculated into *N. benthamiana*. The co-expression of NahG and each mannose-binding lectin inhibited cell death and H2O2 generation (Figure 3a). We also detected the amount of the lectin expression in the plants by Western blot. PVX-NahG expressed with magnICON-SP-D and magnICON-hMBL, respectively, inoculated plants expressed more protein than magnICON-SP-D and magnICON-hMBL (Figure 3b). However, the PVX-NahG and magnICON-CV-N inoculated plants expressed the same level of protein. Like GRFT, these secreted mannose-binding lectins induce SA-dependent HR-like cell death, which could be rescued by NahG expression. Moreover, NahG could boost protein expression in some cases.

As we showed, GRFT’s high-mannose binding ability has an important role in the induction of cell death. We investigated whether SP-D, CV-N and hMBL use the same mechanism as GRFT to induce HR-like cell death through their mannose-binding activity. SP-D, CV-N and hMBL are well-studied lectins, and the amino acid substitutions which disrupt their mannose binding activities eliminate their pharmacological effects (Larsen et al., 2004; Liu et al., 2009; Ogasawara and Voelker, 1995). To test whether the mannose binding activities in SP-D, CV-N and hMBL also induce HR-like cell death in *N. benthamiana*, we engineered lectin activity-deficient SP-D, CV-N and hMBL by substituting alanine for the specific amino acid residues involved in glycan binding (see Experimental procedures). The mutated lectins, fused with the apoplast signal, were expressed in *N. benthamiana* using the agrobacterium-mediated magnICON system. The mutated lectin accumulation levels in *N. benthamiana* were confirmed by Western blot (Figure S7). The accumulation levels of lectins and their mutated allele differed in the Western blot analysis. The mutated lectins did not induce necrotic symptoms in *N. benthamiana* plants, and PR1 expression activation and H2O2 generation were not detected (Figure 4a,b). These results indicate that, as for GRFT, the high-mannose N-glycan binding activity in SP-D, CV-N and hMBL is essential for cell death induction in plants.

**GRFT-plant protein interactions detected by in situ PLA system**

To investigate the possibility that GRFT-Apo interacts with a plant protein localized in the apoplast and that this specific interaction leads to HR-like cell death, interaction of GRFT with one or more plant proteins was analysed by in situ proximity ligation assay (PLA), a method to detect proteins using two primary antibodies and quantification of specific protein-protein interactions in situ (Soderberg et al., 2008). We created mouse antibodies against *N. benthamiana* total proteins and secreted proteins. These antibodies were confirmed to recognize *N. benthamiana* proteins by immunostaining (Figure S11). First, we evaluated the GRFT-plant

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**Figure 3** NahG expression suppressed carbohydrate-binding lectins-induced cell death in *N. benthamiana*. (a) Necrosis induction, H2O2 generation and cell death by the carbohydrate binding lectins were inhibited by NahG expression in *N. benthamiana*. Detection of cell death and H2O2 generation in the inoculated plant leaves. Dead cells were stained by Evans blue, and H2O2 was detected using the **H2DCFDA** fluorescent probe. (b–d) Measurement of the lectins’ accumulation levels by Western blotting. Anti-His (rabbit) and anti-rabbit HRP were used for detection. CBB stain indicates loaded protein amounts are equal.
identified an candidate GRFT-interacting host factor. AtXYL1 (At1g68560) was obtained partial XYL1 (two-hybrid assay. We screened independent yeast transformants with GRFT using an Arabidopsis cDNA library (Clontech) in a yeast tion plays an important role in its HR-like cell death induction.

To analyse the mechanism by which secreted GRFT induces HR-like cell death induction and GRFT accumulation level using 35S promoter-driven AXYL1 overexpression. The N. benthamiana plants where AtXYL1 was overexpressed showed delayed necrosis symptom development accompanied with H₂O₂ when secreted GRFT was co-expressed (Figure 5a). In addition, overexpression of AXYL1 strongly enhanced GRFT-Apo accumulation, similar to what was observed in plants where NahG was co-expressed with apoplast-targeted GRFT (Figure 5b). The fact that AXYL1 is a regulated factor for secreted GRFT-induced necrosis is supported by XYL1 knock-down experiments. XYL1 targeted pTRV-based virus-induced gene silencing (VIGS) accelerated the GRFT-induced necrosis symptoms (Figure S9). We then identified the NbXYL1 sequence and created a specific antibody based on this nicotiana homologue of AXYL1 and GRFT through the PLA assay lends support to the hypothesis that the interaction is indeed glycan-mediated.

We investigated the effect of AXYL1 on secreted GRFT-mediated HR-like cell death induction and GRFT accumulation protein interactions using mouse antibody against total proteins and goat antibody against GRFT. Plants expressing GRFT-Apo showed a strong PLA signal, and this signal localized in the plant membrane (Figure S8). On the other hand, there was no signal detected in either the empty vector or GRFTec- infiltrated samples.

Next, we evaluated interactions between secreted GRFT and secreted plant proteins. Again, only the plants expressing GRFT-Apo, and not GRFTec-Apo or empty vector, displayed signal indicating ligation of proximal proteins. This signal localized to the space outside the plant cells. These results indicate that GRFT is interacting with plant proteins via its carbohydrate-binding domain. We speculate this secreted GRFT-host protein interaction plays an important role in its HR-like cell death induction.

To analyse the mechanism by which secreted GRFT induces HR-like cell death, we attempted to isolate host factors that interact with GRFT using an Arabidopsis cDNA library (Clontech) in a yeast two-hybrid assay. We screened independent yeast transformants and obtained partial XYL1 (AXYL1, AT1G68560) fragments as a candidate GRFT-interacting host factor. AXYL1 (At1g68560) was identified an α-xilosidas against xylglucan (XG), which localized in cell wall and apoplast (Sampedro et al., 2010). AXYL1 is a 915-amino acid protein with eight N-glycosylation sites (Sampedro et al., 2001). In the yeast two-hybrid system, AXYL1, a natively secreted protein, is fused with a nuclear localized protein, GAL4 activation domain (AD). In general, the fusion of GAL4 AD transport targets protein to the nucleus, not through the endoplasmic reticulum and secretory pathway, so the N-glycosylation that would occur in the endoplasmic reticulum might be disrupted. However, there are N-linked glycoproteins present in the cytoplasm and nucleus (Hart and West, 2009), with evidence that certain glycoproteins return to the cytoplasm after entry into the ER or after secretion (Chandra et al., 1998; Kung et al., 2009; Pedemonte et al., 1990; Reeves et al., 1981). Nonconventional soluble glycosyltransferases may exist in the cytoplasm or nucleus and directly modify the proteins in these compartments. Another possibility is that soluble N-glycosylated proteins may be flipped across membranes or originated from secretory pathways (Varki et al., 2009). While we cannot rule out that the GRFT-AXYL1 interaction we discovered via the yeast two-hybrid screen is the result of a direct protein-protein interaction, direct evidence of glycans-mediated interaction between the nicotiana homologue of AXYL1 and GRFT through the PLA assay lends support to the hypothesis that the interaction is indeed glycan-mediated.

We investigated the effect of AXYL1 on secreted GRFT-mediated HR-like cell death induction and GRFT accumulation level using 35S promoter-driven AXYL1 overexpression. The N. benthamiana plants where AtXYL1 was overexpressed showed delayed necrosis symptom development accompanied with H₂O₂ when secreted GRFT was co-expressed (Figure 5a). In addition, overexpression of AXYL1 strongly enhanced GRFT-Apo accumulation, similar to what was observed in plants where NahG was co-expressed with apoplast-targeted GRFT (Figure 5b). The fact that AXYL1 is a regulated factor for secreted GRFT-induced necrosis is supported by XYL1 knock-down experiments. XYL1 targeted pTRV-based virus-induced gene silencing (VIGS) accelerated the GRFT-induced necrosis symptoms (Figure S9). We then identified the NbXYL1 sequence and created a specific antibody based on this nicotiana homologue of AXYL1 and GRFT through the PLA assay lends support to the hypothesis that the interaction is indeed glycan-mediated.
when secreted SP-D or hMBL was expressed in *N. benthamiana*. The PLA assay showed that SP-D and hMBL also interacted with NbXYL1. Surprisingly, SP-D and hMBL accumulation levels were drastically suppressed by AtXYL1 overexpression. In contrast to SP-D and hMBL, CV-N-induced HR-like cell death was not altered by AtXYL1 overexpression, and an interaction between CV-N and NbXYL1 could not be detected (Figure S12). We speculate that CV-N interaction with another distinct host apoplast-located protein may cause the SA-mediated cell death phenomenon. Further understanding of this phenomenon could lead to a generalized strategy to enhance expression of lectins of pharmaceutical and industrial interest in plants.

**Discussion**

In this study, we demonstrated that GRFT-Apo induces HR-like cell death accompanied by increased PR1 expression and generation of ROS in *N. benthamiana*. GRFT-Apo-induced cell death was dependent on salicylic acid (SA)-mediated signalling. Our data prove that HR-like cell death induction is regulated by the interaction between GRFT and host proteins, specifically the key host protein, XYL1. GRFT inhibits HIV entry and infection by binding to HIV envelope glycoprotein, gp120 (Mori et al., 2005). Abrogating the activity of GRFT in the lectin activity-deficient mutant GRFT<sup>lec</sup> not only eliminated its biochemical and pharmacological activities, but also eliminated GRFT’s ability to interact with XYL1.

GRFT, SP-D, CV-N and hMBL are all mannose-binding lectins with diverse pharmacological activities: SP-D inhibits cell proliferation, migration and invasion by suppression of epidermal growth factor signalling (Hasegawa et al., 2015); CV-N is known to block entry of the human immunodeficiency virus (HIV) into human cells (Boyd et al., 1997); hMBL binds and neutralizes influenza A virus and reduces the virus’ infection of respiratory epithelial cells (Hartshorn et al., 1993; Reading et al., 1997). hMBL works as a therapeutic microbicide against Ebola virus (Michelow et al., 2011). Like GRFT, their pharmaceutical activities depend on their mannose binding activities (Ferguson et al., 1999; Larsen et al., 2004; Liu et al., 2009). Several lectins that bind high mannose glycans, including GRFT and CV-N, accumulate well in various plants such as tobacco, marshmallow, soya bean and rice, and do not cause necrosis symptoms (Drake et al., 2013; O’Keefe et al., 2009, 2015; Vamvaka et al., 2016). These studies support our data that demonstrate that cytosolic-targeted expression of lectins do not induce necrotic responses in the host plant.

Here, we showed that similar to GRFT, SP-D, CV-N and hMBL induced SA-dependent HR-like cell death when they accumulated in apoplast. We showed the mutations which impair these lectins’ high-mannose N-glycan binding abilities prevented induction of cell death. These results suggested GRFT and other mannose binding lectins induced the same type of SA-dependent cell death and their high-mannose N-glycan binding ability is responsible for the induction. However, GRFT, CV-N and hMBL have shown little...
inflammatory effects or ROS generation induction in mammalian cells (Buffa et al., 2009; Nelson et al., 2014; O’Keefe et al., 2009). Only SP-D induces oxidative burst and leads to apoptosis in human cells (Mahajan et al., 2013). These facts indicate the lectins might induce HR-like cell death by interacting with a specific plant glycoprotein or activation of plant-specific immune responses through their high-mannose binding activities.

Using GRFT as bait in a yeast two-hybrid screening system, AtXYL1 was identified as a key host factor with which GRFT interacts. In the context of the yeast two-hybrid assay, we cannot tell whether the interaction between GRFT ‘bait’ and the AtXYL1 fragment ‘prey’ is mediated through the lectin activity of GRFT binding a AtXYL1 glycan expressed in the yeast system, or whether the interaction is at the amino acid level. There is precedent for N-glycosylation of nuclear proteins (Chandra et al., 1998; Kung et al., 2009; Pedemonte et al., 1990; Reeves et al., 1981; Wariki et al., 2009), and we speculate that the AtXYL1 fragment was directed to the secretory pathway through its own signal peptide in the yeast host and redirected to the nucleus, permitting interaction with GRFT through an N-linked glycan. The PLA demonstrates that the interaction between GRFT and NbXYL1 requires lectin activity in planta.

We showed that secreted GRFT interacted with NbXYL1 in the apoplast in N. benthamiana. Other mannose binding lectins, SP-D and hMBL also interacted with NbXYL1, but CV-N did not. Xyloglucan (XyG) is a ubiquitous plant polysaccharide, a component of hemicellulose in most dicot species, which cross-links cellulose microfibrils in the wall and builds a cellulose-XyG network in the cell wall (Somerville et al., 2004). XyG structure in cell walls is dynamic; it undergoes structural maturation and degradative turnover by apoplastic plant enzymes (Frankova and Fry, 2013). Plant endoglucanases including endo-β-1,4-glucanases reduce the XyG polymer chain length and degrade XyG into oligosaccharides (XGO, Shigeyama et al., 2016). Apoplast localized enzymes including XYL1, a galactosidase (BGAL10), and a fucosidase (AXYB) are involved in XGO degradation into its various monosaccharides (Gunl and Pauly, 2011; Gunl et al., 2011; Sampedro et al., 2001, 2010, 2012). Recently, it was reported that agrobacterium-mediated overexpression of the pepper xylolucan-specific endo-β-1,4-glucanases inhibitor protein (CaXEGIP1) induced cell death in N. benthamiana (Choi et al., 2013). The HR-like cell death was accompanied with salicylic acid-dependent PR gene expression indicating XyG metabolism might be involved in the induction of the process (Choi et al., 2013). Our results showed XYL1 is a negative regulator of secreted GRFT-induced HR-like cell death (Figure 6). It is possible that AtXYL1 protects NbXYL1 by simply sequestering apoplast-targeted GRFT. It is also possible that GRFT-NbXYL1 complexes induce HR, while GRFT-ApXYL1 complexes do not. Interestingly, secreted GRFT accumulation reduced levels NbXYL1, and we also saw this phenomenon in plants overexpressing secreted SP-D and hMBL. We demonstrated that NbXYL1 interacts with SP-D and hMBL in the apoplast of N. benthamiana, and overexpression of AtXYL1 reversed HR-like cell death induced by secreted SP-D and hMBL. Furthermore, a XYL1 knock-down in N. benthamiana or Arabidopsis xyl1 knockout mutant under normal growing condition do not show cell death (Sampedro et al., 2010). We speculate that secreted lectins may impact other apoplast localized XGO degradation enzymes in addition to XYL1 and inhibit XyG metabolism, leading to HR-like cell death as shown in CaXEGIP1 overexpression (Figure 6).

We do not yet fully understand why only GRFT, SP-D and hMBL protein accumulation levels were enhanced by NahG expression while CV-N accumulation levels seemed unaffected. Reports show that recombinant production of CV-N in several systems including bacteria and transgenic plants resulted in relatively low protein yields (Colleluori et al., 2005; Gao et al., 2010; Sexton et al., 2006; Xiong et al., 2010). Recent research showed SA is involved in germination, photosynthesis, respiration, flowering and senescence (Rivas-San Vicente and Plasencia, 2011). In fact, NahG expression enhances plant growth associated with enlarged cell size and extensive endoreduplication (Scott et al., 2004; Xia et al., 2009). Due to the combination of CV-N instability in plant cell and cellular environmental changes brought on by NahG, CV-N protein accumulation level might not be increased in plants where NahG is expressed, even when the plants were rescued from cell death.

In many plant species, lectins are reported to be involved in defence responses to pathogen infection (Peumans and Van Damme, 1995). Plant lectins interact with the glycoproteins on the surfaces of fungi, leading to pathogen growth inhibition (Broekaert et al., 1989; Fliegmann et al., 2004). Moreover, it has been reported that Arabidopsis lectins inhibit the systemic spread of plant viruses (Chisholm et al., 2000; Yamaji et al., 2012). With reports that lectins have potent antiviral activity and may also be useful in cancer diagnostics and therapeutics, there is likely to be interest in developing scalable manufacturing systems (Fu et al., 2011; Liu et al., 2009). We have shown some of the mechanisms that impact accumulation of GRFT and the other two lectins in plants. Clearly suppression of the SA pathway can rescue expression as this is commonly involved in the necrosis response to lectin expression. Similarly, it may be possible to overexpress the host ‘off-target’ binder protein, such as XYL1, to relieve the necrosis and enhance accumulation.

In general, HR is a resistance response induced by interaction with plant resistance gene (R gene) and an avirulence factor of a pathogen (Avr gene) in plant. However, our data showed GRFT and other lectins including SP-D, CV-N and hMBL fused with apoplast signal expression directly induced HR-like cell death in the absence of factors derived from pathogens. Our data are consistent with the research reported by a group who showed that pepper mannose-binding lectin (CaMBL1) causes SA-dependent cell death when CaMBL1 is overexpressed (Hwang and Hwang, 2011). It is possible that exogenous high mannose lectins located to the apoplasm phenoypic a plant pathogen innate immune response induced by a pathogenic lectin.

Engineering plant hosts for biomanufacturing or ‘pharming’ of high value proteins is an emerging strategy, best demonstrated by efforts to ‘humanize’ plant glycosylation machinery (Daskalova et al., 2010; Grill et al., 2005). By characterizing the molecular basis of the necrosis response associated with secretion of mannose targeted lectins, we have demonstrated practically useful strategies to enhance expression of GRFT-Fc lectibodies, BSA fusions or other engineered proteins by suppression of the SA expression pathway, or by augmenting levels of a target protein that is more rapidly metabolized in complex with the lectin.

**Experimental procedures**

**Expression vector construct and plant growth conditions**

The magnICON (Icon Genetics GmbH, Germany) system was used to express wild-type griffithsin (GRFT), surfactant protein D (SP-D),...
cyanovirin-N (CV-N), human mannose binding lectin (hMBL), galectin-9 (Gal-9), GRFT lec-(D30A, D70A and D112A), SP-D lec-(E321Q and N323D), CV-N lec-(K3N, Y7A, E23I and N93A) and hMBL lec-(G51E, R52C, G54D, G57E and G60E), respectively (Chang and Bewley, 2002; Larsen et al., 2004; Ogasawara and Voelker, 1995; Xue et al., 2012). The optimized lectin sequences for
Nicotiana benthamiana
were synthesized at Integrated DNA Technologies (IDT). The synthetic encoding GRFT and GRFTlec-cDNA were cloned into magnICON vector pICH11599 in the NcoI and SacI restriction enzyme sites. NcoI and PstI sites were used to clone SP-D, CV-N, hMBL and Gal-9 and lectin-deficient mutant fragments (each lectin containing C-terminal 6\textsuperscript{His} tag) into pICH11599.

The NahG sequence, which was optimized for
Nicotiana benthamiana
plants, was synthesized at Integrated DNA Technologies (IDT) and cloned into pICH38077 PVX-based magnICON vector using the BamHI and SacI sites.

Nicotiana benthamiana were used in these experiments. Seeds were directly sown in peat pellets and grown at 26°C with a 16-h photoperiod.

Viral vector-based expression of GRFT in
Nicotiana benthamiana
Agrobacterium vacuum infiltration was performed as described in Matoba et al. (Matoba et al., 2010). pICH11599-GRFT, pICH14011 integrase promodule, pICH17388 apoplast module (Marillonnet et al., 2004), pICH38077-NahG (PVX one component expression vector) and pR1101-AN vector (for AXYL1 overexpression, Clontech) were transformed into Agrobacterium tumefaciens GV3101 by electroporation. Each Agrobacterium culture was suspended in infiltration buffer (10 mM MES, 10 mM MgSO\textsubscript{4}, pH 5.5) and mixed at OD\textsubscript{600} 0.1. The Agrobacterium suspension was infiltrated into leaves using vacuum pump. At 5 days postinfiltration (dpi), infected leaves were harvested and analysed.

Detection of cell death and \textsubscript{H\textsubscript{2}}O\textsubscript{2}
The leaves of
Nicotiana benthamiana
were submerged in 0.25% Evans blue (Alfa Aesar) solution for 10 min under vacuum. The leaves were then washed three times with water and destained in 70% ethanol for 2 weeks. \textsubscript{H\textsubscript{2}}O\textsubscript{2} generation in leaf tissues was detected using 2\textsuperscript{0}70\textsuperscript{0}dichlorofluorescein diacetate (H2DCFDA, Sigma-Aldrich, Kim et al., 2008). Briefly, leaves were reacted with loading buffer (10 mM Tris-HCl and 50 mM KCl, pH 7.2) containing 1 mM H2DCFDA under vacuum infiltration for 5 min. The samples were incubated in the dark for 20 min at room temperature, and washed two times in loading buffer. Fluorescence was observed under the UV lamp (365 nm).

Quantitative real-time RT-PCR
Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacture’s protocol. For the real-time RT-PCR, 1 μg of total RNA was used as a template for cDNA synthesis. The first-strand cDNA was synthesized by the Takara RNA PCR Kit with a random primer. Real-time RT-PCR analysis was performed using the iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Real-time RT-PCR was carried out using 1 μL of the RT reaction mixture and SYBR Green mixture (Bio-Rad). For the amplification of the
Nicotiana benthamiana
PR1 genes, the following primer pairs were used (Table S1): NbPR1F (5’-CCCTCCCACA TGTCACTTCT-3’) and NbPR1R (5’-ATTTCGCTCACTGTGGT-3’). For real-time PCR, the actin gene was used as an internal control using primer pair NbActinF (5’-ATC GCACAAAT
TATGCTTCC -3') and NbActinR (S'-TCTCATCGACCCACATCTC -3'). PR1 gene expression was normalized to actin.

**GRFT detection by enzyme-linked immunosorbent assay**

ELISA was performed according to the method of Boyd et al. (Boyd et al., 1997). Briefly, a microtiter plate (NUNC, Nalgene, Thermo Scientific, Waltham, Massachusetts, USA) was coated with HIV gp120 (Protein Sciences Corp., Meriden, CT). Plates were blocked with 5% PBS (phosphate-buffered saline [pH 7.4], 0.05% [vol/vol] Tween-20, 5% nonfat dry milk) for 1 h at room temperature. Total proteins extracted from the plants agro-infiltrated with magniCON were incubated in each well. As a standard, recombinant GRFT purified from *N. benthamiana* was diluted twofold from 200 to 0.78 ng GRFT/well. Positive controls and *N. benthamiana* plants extracted protein were incubated as 50 μL volumes. Rabbit polyclonal anti-GRFT diluted 1:1000 in PBS was added to the wells followed by goat anti-rabbit HRP-conjugated antibody (Sigma-Aldrich) 1:2500 in PBS. Wells were washed (twice after coating and blocking and four times after sample and antibody addition) with PBS supplemented with 0.1% Tween-20 (Merck, Darmstadt, Germany), and incubation periods were 2 h at 37 °C or overnight at 4 °C. TMB substrate (tetramethylbenzidine, Sigma-Aldrich) was added in 50 μL to each well. The reaction was stopped with equal volumes of 1 M H2SO4, and the absorbance was measured at OD450.

**Western blot analysis**

Total proteins from *N. benthamiana* plants, inoculated with wild-type and mutant lectins, were extracted with 30 mM acetate NaCl solution, 2 mM ascorbic acid, and 8 mM sodium metabisulphite as described by O’Keefe et al. (O’Keefe et al., 2009). The samples were boiled for 10 min, separated on a 12% SDS-PAGE gel (Bio-Rad) and transferred to a membrane (Trans-Blot Turbo PVDF Transfer Pack, Bio-Rad, Richmond, CA). The membranes were blocked with 5% PBS (phosphate-buffered saline [pH 7.4], 0.05% [vol/vol] Tween-20, 5% nonfat dry milk) for 1 h at room temperature, incubated with anti-GRFT rabbit antibody overnight at 4 °C, and then rabbit IgG for 1 h at room temperature. For detection of the signal, we used the ECL Western blot system. For detection of SP-D, CV-N, hML and Gal-9, the anti-his antibody (Abcam) and the anti-rabbit HRP were used as primary and secondary antibody respectively.

**Protein infiltration**

GRFT and GRFTlec- were purified as previously described (O’Keefe et al., 2009) and were >99% pure. *N. benthamiana* leaves were infiltrated with 0.9 mg/mL GRFT or GRFTlec- using a vacuum pump. Five days after the infiltration, Evans blue staining and H2O2 detecting were performed.

**In situ PLA assay**

magniCON-GRFT and GRFTlec- agro-infiltrated *N. benthamiana* leaves were harvested 5 days after the infiltration. The leaves were fixed with 4% paraformaldehyde, and tissue embedding was performed according to the protocol (Javelle et al., 2011). The sectioning and fixing onto slide glasses were carried out by Research cores service (University of Louisville). The fixed leaf samples were incubated with GRFT rabbit antibody and *N. benthamiana* total protein mouse antibody or secreted protein mouse antibody, with GRFT goat antibody and NbXYL1 rabbit antibody. PLA was performed according to the manufacturer’s protocol using the Duolink Detection Kit (Sigma-Aldrich). Proximity signal was detected with Axio Observer Z1 microscope (Carl Zeiss, Thornwood, NY).

Total protein extracts were prepared from *N. benthamiana* by grinding the tissue using extraction buffer (20 mM NaAc, 50 mM NaCl, 1% Triton-X 100, pH7.0). Secreted protein extracts were prepared by vacuum infiltrating *N. benthamiana* leaves with Tris- NaCl buffer (20 mM Tris, 100 mM NaCl, 20 mM sodium acetate, 4 mM sodium metabisulphite, pH7.0). Buffer inoculated leaves were spun at 2000 g for 15 min. Centrifuged protein was collected into 1.5-mL tubes. Antibodies against total and secreted proteins from *N. benthamiana* were produced in mice by injection of 50μg/50μL of each of the protein preparations five times. After all subsequent injections, mouse sera were used for detection.

For identifying the N-terminus sequence of NbXYL1, we used *N. sylvestris* XYL1 (XM009800839) sequence which is known. This sequence has 100% homology Fdewith NbXYL1. The cDNA generated using the primers (NbXYLF:5'-GGTTGAGTACCGATGGTGTAGTGGTGTTCAATCATCTCACAATGTATA-3', NbXYLR:5'-AGCCGAATTCTCAGGTGAAATTCCCACTTTCC-3') was cloned using TA cloning system (Promega). After sequencing seven clones, the sequence of NbXYL1 was determined.

**Statistical analyses**

Data are presented as the mean ± SEM; the number of observations (n) refers to technical replicates. Data analysis was carried out by either an unpaired Student’s t-test or one-way analysis of variance with Bonferroni’s multiple comparison test (MCT), as indicated in the text.

**Acknowledgements**

We thank Amanda Lasnik for making the antibodies against total and secreted *N. benthamiana* proteins as well as her critical comments on the manuscript. We would like to thank the Helmsley Charitable Trust and NIH/NIAID grants (AI076169 and AI113182) for financial support. The authors have no conflict of interest to declare.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1 GRFT accumulation in the apoplast induced an HR-like cell death response.

Figure S2 Schematic representation of MagnICON promodule vectors used for GRFT and NahG expression.

Figure S3 GRFT accumulation in the apoplast induced an HR-like cell death response using binary vector.

Figure S4 NahG and GRFTrec did not induce cell death in N. benthamiana.

Figure S5 Vacuum infiltration of purified GRFT protein induces HR-like cell death in N. benthamiana.

Figure S6 Expression of galactoside-binding protein, Galectin-9 (Gal-9) and mannose binding lectins with apoplast signal in N. benthamiana plants.

Figure S7 Expression level of lectin deficiencies of GRFT, SP-D, CV-N, hMBL and Gal-9 inhibits the cell death in N. benthamiana.

Figure S8 In situ association between GRFT and membrane protein in N. benthamiana.

Figure S9 GRFT induced severe necrotic symptom in NbXYL1 silenced N. benthamiana plants using pTRV-based VIGS.

Figure S10 In situ association between GRFT and NbXYL1 in N. benthamiana.

Figure S11 Immunostaining using single antibody.

Figure S12 Interaction of XYL1 and lectins in N. benthamiana plants.

Table S1 Primers and applicant characteristics for RT-qPCR.