The *Rhizoctonia solani* LysM and RlpA-like effector proteins contribute to virulence suppressing chitin-triggered immunity and hypersensitive response

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Abstract

Rhizoctonia (in Greek “root-killer”) species and particularly *R. solani* attacks a broad range of plant species and crops. It belongs to Basidiomycota and is a soil-borne pathogen causing mainly damping-off in seedlings, although it can infect plants in any stage. It does not form any kind of asexual spores while the sexual form (*Thanatephorus cucumeris*) is extremely rare. However, it forms microsclerotia, able to survive in soil and plant debris for a long period and under harsh environmental conditions. Despite the severity of this disease, many aspects in *R. solani* infection biology still remain to be elucidated. In this study, we investigated the role of two effector candidates, derived from a *R. solani* AG2-2IIIB strain isolated from infected sugar beet plants. Gene expression analysis showed that genes encoding for a LysM effector and a RlpA-like protein were induced upon early infection stages, while their heterologous expression in *Cercospora beticola* contributed to virulence. Furthermore, the RsLysM effector showed chitin binding affinity, suppressing chitin-triggered immunity but could not protect hyphae from hydrolysis. The RlpA-like effector is homologous to a papain-like inhibitor and to a barwin-like endoglucanase, while our data showed that it was able to suppress hypersensitive response in *Nicotiana bethamiana* leaves. Overall, this study provides us with valuable information on *R. solani* infection biology towards understanding the molecular mechanisms in this pathosystem.

Keywords *Cercospora beticola,* effectors, heterologous expression, LysM, RlpA-like protein, papain-like inhibitor, sugar beet

Introduction

Rhizoctonia solani Kühn is an important soil-borne pathogen causing substantial yield losses in a wide range of crops including cereals, soybean, potato and sugar beet (Adams, 1988). It is responsible for damping-off disease in many hosts (Anderson *et al*., 1982) as well as crown and root rot in sugar beet, leaf spot and root rot in tobacco, sheath blight in rice and black scurf in potato (Bernades *et al*., 2009; Buhre *et al*., 2009; Ceresini *et al*., 2002; Gonzalez *et al*., 2011). The fungus produces persistent sclerotia, which are the major infestation source together with infested debris in the soil. *Rhizoctonia solani* commonly infects roots and hypocotyls but all types of plant organs can be colonized by the mycelia. It lacks conidia formation,

while the sexual stage (*Thanatephorus cuccumeris*) is extremely rare (Adams and Butler, 1983). This pathogen is classified into different anastomosis groups (AGs) based on hyphal cell wall fusion between different isolates. Some AGs are further divided into subgroups based on host range, colony morphology, pathogenicity, zymogram patterns and other characteristics (Ogoshi *et al*., 1987).

Despite the economic impact of *R. solani*, relatively little is known about how it causes disease. A general view is that *R. solani* hyphae adhere to the host plant surface and enter the outer cell layers by the formation of penetration cushions (Gonzalez *et al*., 2011), a process followed by the growth of invasive hyphae that ramify through the host tissue. Mechanisms behind the host plant entry of *R. solani* are scare and hypothesis on gene function difficult to test since *R. solani* is not amenable to molecular manipulations.

Secreted proteins promoting disease were first observed from human pathogenic bacteria (Preston, 2007). Plant pathogens secrete effector proteins, to manipulate plant defenses and to establish a successful infection (Vleeshouwers and Oliver, 2014; Lo Pesti *et al*., 2015,). Fungal effectors suppress and block early plant defence responses such as hypersensitive response (HR) (Hemetsberger *et al*., 2012), stealth hyphae from recognition or protect them from plant chitinases (de Jonge and Thomma, 2009, van den Burg *et al*., 2006), promote necrosis (Qutob *et al*., 2006) etc. However, many aspects in effectors role during plant-microbe interactions remain unclear.

Effectors have been well-characterized in oomycetes (Morgan and Kamoun, 2007), but in filamentous fungal pathogens are less studied. Recent advancements in sequencing technologies, led to the identification of candidate effectors with higher confidence, developing also computational methods for their prediction (Sperschneider *et al*., 2017). More detailed genomic data are presently available from five different *R. solani* strains from monocot and dicot hosts, resulted in prediction of putative effector proteins (Cubeta *et al*., 2014; Hane *et al*., 2014; Wibberg *et al*., 2013, 2016a; Zheng *et al*., 2013). By now, few effectors in *R. solani* have been characterized. In an AG8 strain, a xylanase and a protease inhibitor I9 induce cell death when expressed in *Nicotiana benthamiana* (Andersson *et al*., 2017). Similarly, cell death induction was also observed, when three candidate effectors, a peptidase inhibitor I9, a glycoside transferase family 5 and a cytochrome C oxidase, derived from an AG1-IA strain, were inoculated on rice, maize and

soybean leaves (Zheng *et al*., 2012).

Sugar beet is mainly attacked by the AG2-2IIIB strains. The disease can appear in different forms and with different symptoms; root and crown rot, damping off or as foliar blight (Bolton *et al*., 2010). The demand for sugar beet cultivars with high resistance to *R. solani* is increasing. Effects of a milder and more humid climate are seen in temperate regions influencing the length of crop seasons and simultaneously allow pathogens to multiply for longer periods. Moreover, the extent of *R. solani* AG2-2IIIB soil inoculum is expected to increase in regions particularly where sugar beet and maize are overlapping in the crop rotation schemes since maize can act as a host and thus propagate the pathogen (Buddemeyer *et al*., 2004; Schulze *et al*., 2016).

The *R. solani* AG2-2IIIB strain has a predicted genome size of 56.02 Mb and 11,897 protein-encoding genes whereof 1,142 were secreted and represent potential effectors (Wibberg *et al*., 2016a). Here we refined the dataset and identified 11 AG2-2IIIB specific effector candidates. A rare lipoprotein-A-like (RlpA-like) protein and the chitin-binding lysin motif (LysM) effector were highly induced in diseased sugar beet seedlings. In planta assays using *Cercospora beticola* as a heterologous expression system transfected with RlpA-like and LysM, showed increased virulence. The LysM effector was able to suppress chitin-triggered immunity in plants, while the RlpA-like protein suppressed HR in *N. bethamiana* leaves. These data give us valuable insights towards understanding the mechanisms that this pathogen deploys upon infection.

Materials and Methods

Fungal isolates and sequence analysis

Rhizoctonia solani AG2-2IIIB isolate BBA 69670 (DSM 101808) was used in this study. *Rhizoctonia solani* inoculum for soil infestation was prepared on media containing perlite, corn flour, and water. *Cercospora beticola* isolate Ty1 (MariboHilleshög Research, Landskrona) was kept on PDA at 22°C in darkness and sporulation was induced on tomato growth extract medium. Small cysteine-rich and secreted proteins were retrieved from *R. solani* AG2-2IIIB genome (Wibberg *et al.,* 2016a). Presence of conserved domains was tested using the SMART 6.0 protein analysis tool (Letunic *et al*., 2009) followed by SignalP 4.1 for signal peptide

prediction (Petersen *et al*., 2011). The Phyre[2] server was used for protein structure prediction (Kelley *et al*., 2015)

Quantitative reverse transcriptase - PCR (qRT-PCR)

Three-week old sugar beet plants were planted in soil infested with *R. solani* mycelia (10:1 ratio of fresh soil: inoculum). Total RNA was extracted from the plants 4,5,6 and 7 days post inoculation using the RNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions, while *R. solani* mycelia grown in PDB medium were used as fungal control. Expression was normalized by *G3PDH* expression (Chamoun *et al*., 2015) and relative expression values were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Phylogenetic analyses

Phylogenetic analyses were on RsRlpA and RsLysM homologs, from different fungal species, based on amino acid sequences using all sites, and were carried out using the Neighbor-Joining (NJ) method implemented in the MEGA v.7 software (Kumar *et al*., 2016), using the JTT substitution model (Jones *et al*., 1992). Bootstrap analyses were performed on 1,000 replicates. Alignments were conducted using the CLUSTAL W algorithm.

Construction of *Cercospora beticola* overexpression strains and virulence assays

Overexpression vectors were designed using the In-Fusion HD cloning kit (Takara Bio). The LysM and RlpA sequences were PCR amplified from *R. solani* cDNA using high fidelity Phusion *Taq* polymerase (Thermo Scientific). The destination vector was the pRFHUE-eGFP, conferring resistance to hygromycin (Crespo-Sempere *et al*., 2011). The *PgdpA* promoter drives the gene insert with GFP in C-terminus and the vectors were transformed in *C. beticola* using an *Agrobacterium*-mediated protocol (Utemark and Karlovsky, 2008). Three independent colonies expressing the correct effector genes were selected for further analysis.

For virulence assays, leaves of 3-week-old sugar beet plants were inoculated with 10^5 /ml. *Cercospora beticola* conidia, from overexpression, empty vector and wild-type strains were used. Lesion area was calculated 7 dpi, using the ImageJ software version 1.51n (National Institute of Health, USA). Total genomic DNA was

extracted from the diseased plant material using a CTAB-mediated protocol. Fungal DNA was quantified using the *C. beticola* actin (*act*) reference gene and normalized with *B. vulgaris* elongation factor (*elf-1*) gene.

Confocal microscopy and suppression of hypersensitive response

The RlpA-like effector sequence was cloned into pENTR/D-TOPO Gateway vector (Thermo Fisher Scientific), and entered to pGWB605 tagged with C-terminus GFP fluorescence protein driven by the CaMV:35S promoter and transformed into *Agrobacterium tumefaciencs* C58C1. Overnight cultures were used for agro-infiltration on 4-week old leaves *N. bethamiana*. Protein cellular localization was monitored by a Zeiss LSM 800 confocal microscope. For suppression of hypersensitive response assay, the *RsRlpA* gene entered to the pGWB602 binary vector, driven by the CaMV:35S promoter as well. This candidate effector was agro-infiltrated as described above in transgenic *N. bethamiana* plants, expressing the tomato Cf-4 receptor protein (kindly provided by Wageningen University), and the HR was triggered 24 hours after agro-infiltration with the *Cladosporium fulvum* Avr4 effector. The HR was evaluated using a scale from 0-3 with ‘’0’’ indicating no symptoms and ‘’3’’ indicating severe symptoms.

Heterologous expression of RsLysM in *Pichia pastoris*

The RsLysM protein was cloned in the pPic9 expression vector with N-terminal His and FLAG tags and transformed into the *P. pastoris* strain GS113. A positive clone was cultured in a fermentor (Bioflo 300) as previously described by Rooney *et al*., (2005). His-tagged protein was purified using a Ni-NTA column (Qiagen) and final protein concentration was determined spectrophotometrically at 280 nm, and analyzed by a Pierce BCA Protein assay kit (Thermo Fisher Scientific).

Affinity precipitation assay, measurement of reactive oxygen species and hyphal protection assay of RsLysM effector

A chitin-binding assay of the RsLysM effector protein was performed as described by van de Burg *et al*. (2006). Briefly, the pure protein was incubated with insoluble polysaccharides (crab shell chitin, chitosan, xylan or cellulose) and analyzed on SDS polyacrylamide gel. For suppression of chitin-induced oxidative burst of reactive oxygen species (ROS) assay, a protocol by de Jonge *et al*. (2010) was used. Briefly,

leave discs were inoculated with assay buffer with/without chitin oligomers $(GlcNAc)_6$ and the suppression of ROS was investigated using 10 µM RsLySM protein. For hyphal protection assay a protocol described previously by Kohler *et al*., (2016) was used. Briefly, the protein was incubated with germinated conidia from *T. virens*. Then bacterial chitinase (Sigma) and 10U zymolyase (Zymo Research) were added to the final volume. The Avr4 effector or BSA were used as positive and negative controls respectively and images were taken 6hpi.

Results

***RsLysM* and *RsRlpA* are two effector candidates induced upon early-stage infection**

Previous genome analysis of a *R. solani* AG2-2IIIB isolate showed that it contained almost 100 putative secreted proteins identified as candidate effectors (< 400 aa and small cysteine-rich motifs) (Wibberg *et al*., 2016b). Furthermore, 11 of them appeared to be unique in this isolate (Wibberg *et al*., 2016b). Transcript analysis on sugar beet plants grown in *R. solani* (*Rs*) infested soil and analyzed 4-7 days post-infection revealed elevated levels of *RsLysM* (*RSOLAG22IIIB_4067*) and *RsRlpA* (*RSLAG22IIIB_8473*) genes already at 4 dpi suggesting an important role during *R. solani* host colonization (**Figure 1**). *RsRlpA* was predicted to encode a rare lipoprotein A (RlpA)-like domain protein, which has been previously described in bacteria (Gerding *et al*., 2009). LysM encoding genes harbor no protein domains other than lysin motifs (LysMs). Fungal LysM effectors are versatile proteins that occur in a plethora of species with divergent lifestyles. The general function of LysM is to evade plant recognition but may also have other roles (Takahara *et al*., 2016).

To investigate the genetic relations among *R. solani* candidate effectors, phylogenetic analyses were conducted on homologs derived from a variety of fungal species, including ascomycetes and basidiomycetes, using amino acid sequences and Neighbor-Joining analysis. RsRlpA homologs are mainly clustered in two groups, one includes proteins from the basidiomycetes and the other from ascomycetes (**Figure 2A**). RsLysM effector homologs were categorized mainly in three distinct groups; one group contained LysM effectors from Basidiomycota, a second group contained proteins from mycoparasites and saprophytes (Ascomycota), while a third group contained LysM effectors from plant pathogenic Ascomycota (**Figure 2B**).

These data indicate that these effectors have been differentially evolved in Basidiomycota and Ascomycota fungi.

RsLysM* and *RsRlpA* promote virulence upon heterologous expression in *Cercospora beticola

The amino acid sequences of *RsLysM* and *RsRlpA* were analyzed for conserved domains, using bioinformatic tools. SMART analysis showed that the RsLysM effector has two CBM50 modules (IPR002482; LysM peptidoglycan binding), while the RsRlpA has a conserved double-psi beta-barrel (IPR036908; DPBB) fold (**Figure 3A**). Structural prediction of RsRlpA using the Phyle[2] showed 39% homology to a bacterial cellulose binding protein, 34% to papain inhibitor, 27% to expansin, 26% to kiwellin protein and 26% to barwin-like endoglucanase. Both RsLysM and RsRlpA proteins contain an ER signal peptide at the N-terminus according to SingalP, indicating putative secreted proteins.

To test if these two effector candidates are involved in virulence we heterologously expressed them to the hemibiotroph sugar beet fungal pathogen *Cercospora beticola* (leaf spot disease), which is amenable to genetic modifications. The overexpression *C. beticola* strains (*RsRlpA*+ and *RsLySM*+) displayed no difference in morphology, growth rate or conidiation in comparison to wild-type, while qRT-PCR confirmed the expression of these genes. The *RsLysM*+ *C. beticola* strains, displayed significantly larger necrotic lesions on sugar beet leaves, compared to wild-type and strains where only the empty vector was inserted **(Figure 3B, C)**. In contrast, no clear phenotypic difference was observed in *RsRlpA*+ strains. Similarly, our results showed that RsRlpA did not induce necrosis upon transient expression in *N. bethamiana* leaves. However, the fungal biomass was significantly increased in comparison with wild-type and empty vector for both *RsLysM*+ and *RsRlpA*+ strains, further supporting the role of these candidate effectors in host colonization **(Figure 3D)**.

The localization of RsLysM and RsRlpA was also investigated in *C. beticola* fungal hyphae. Proteins were tagged with GFP at the C terminus driven by the PgdpA promoter. Both candidate effectors were localized to the cell periphery, while the RsLysM was mainly observed in hyphal tips (**Figure 4**).

The RsRlpA effector suppresses hypersensitive response and is mainly localized to the plant plasma membrane

Many plant pathogens rely on an initial short biotrophic stage to establish a successful infection and therefore secrete effectors to work as suppressors of HR (Stergiopoulos and de Wit, 2009). In order to investigate whether RsRlpA was able to suppress HR, this candidate effector was transiently expressed in transgenic Cf-4 *N. bethamiana* leaves and HR was induced by transient expression of the Avr4 effector. Our preliminary data showed that in areas where RsRlpA had been previously agro-infiltrated, reduced HR was observed as compared to the area that only *Avr4* was expressed (**Figure 5**), indicating that this effector potentially suppresses HR.

Despite GFP tagging in *RsRlpA*+ strains, we repeatedly failed to clearly follow RsRlpA events during the infection process in sugar beet possibly due to the special structure of its leaves. Thus, to determine where this effector is localized, we transiently expressed this candidate effector under the constitutive CaMV:35S promoter on *N. bethamiana* leaves, tagged with GFP at C-terminus. RsRlpA mainly localized to the plasma membrane, while localization of this protein was also observed at nucleoplasm and ER. Furthermore, plasmolysis of the *N. bethamiana* leaves, using mannitol, supported the plasma membrane localization (**Figure 6**).

The RsLysM effector binds to chitin

Previous analyses of LysM effector proteins from plant pathogenic ascomycetes have demonstrated their ability to bind chitin (de Jonge *et al*., 2010; Marshall *et al*., 2011; Kombrink *et al*., 2017). Whether LysM effectors from basidiomycetes have a similar chitin-binding affinity is unknown. We expressed in yeast (*Pichia pastoris*) and we purified the protein, which was used for the chitin-binding assay. The *R. solani* LysM effector was able to interact with all tested forms of chitin, such as chitin beads, crab shell chitin and chitosan (**Figure 7A**), while it did not precipitate with the rest polysaccharides such as xylan and cellulose (**Figure 7A**), suggesting that RsLysM is an active chitin-binding effector protein, similar to other characterized LysM effectors.

The RsLysM effector suppresses chitin-triggered immunity but does not protect hyphae from degradation

LysM effectors from ascomycetes suppress chitin-triggered immunity (de Jonge *et al*., 2010; Marshall *et al*., 2011; Mentlak *et al*., 2012; Kombrink *et al*., 2017). To

investigate whether the RsLysM effector also prevent plant chitin-triggered immunity, leaves were treated with chitin oligomers $(GlcNAc)_6$, which led to reactive oxygen species (ROS) burst, while treatment with bacterial flagellin (flg22) was used as a positive control. In leaves, where 10 µM LysM effector had been added, a significant reduction of ROS was observed (**Figure 7B)**, suggesting that RsLysM displays similar non-recognition function as seen in other pathosystems.

Certain LysM effectors are able to protect fungal hyphae from chitinolytic activity (Marshall *et al*., 2011; Kombrink *et al*., 2017) similar to Avr4 effector from *C. fulvum* (van den Burg *et al*., 2006). To investigate whether the RsLysM displays similar function, germinated conidia from *Trichoderma viride* were mixed with 10-20 µM of pure RsLysM, while Avr4 and BSA were used as positive and negative controls respectively. RsLysM was unable to protect fungal hyphae against degradation from bacterial chitinases and zymolyases (**Figure 7C**), in contrast to Avr4 (**Figure 7C).**

Discussion

Rhizoctonia solani is a soil-borne basidiomycete pathogen with a broad host range, infecting more than 200 plants (Anderson, 1982), while it does not fit into either biotrophic or necrotrophic categories. This plant pathogen is commonly described as a saprophyte that is able to switch to pathogenic endotrophic growth thriving on dead or dying plant debris/cells. Spore dispersal is the most common process to initiate new infections. The absence of any asexual reproduction in combination with the complex anastomosis system and the lack of methods to genetically manipulate it, has resulted in little to be known about hampered the elucidation of its infection biology and interaction with hosts.

In this study, we investigated the role of two candidate effectors in *R. solani* AG2-2IIIB, which is aggressive to sugar beets. Sugar beets contribute to 30% of global sugar production and since they have a relatively long growing season they are vulnerable to such pathogens. The availability of *R. solani* AG2-2IIIB genome (Wibberg *et al*., 2016a, b) gave us the advantage to select the most promising candidate effectors. The AG2-2IIIB isolate secretes an elevated number of proteins as compared to other sequenced AG isolates with known genomes (Anderson *et al*., 2017; Wibberg *et al*., 2016b), possibly because of adaptation to hosts. Among the putative secreted ones, more than 100 have been annotated as candidate effectors, (Wibberg *et al*., 2016b).

Since our previous genome analysis showed that *R. solani* AG2-2IIIB isolate contains only one gene coding for LysM effector and a unique gene encoding a putative RlpA-like protein, we selected these two candidate effectors as the most promising ones. A number of LysM effectors have been identified in a plethora of fungal species with variable lifestyles but studies have focused mainly in pathogenic fungal species, while limited studies have also been conducted in mycoparasites and in mammalian fungal pathogens (de Jonge and Thomma 2009; Seidl-Seiboth *et al*., 2013). So far, no LysM effector has been characterized in any plant pathogenic species that belongs to Basidiomycota (Wibberg *et al*., 2016b).

The LysM effector gene in *R. solani* AG2-2IIIB showed induction upon early infection stage, which in agreement with the previously published data of foliar pathogens such as *C. fulvum* and *Mycosphaerella graminicola* (de Jonge *et al*., 2010; Marshall *et al*., 2011), indicating an involvement in plant colonization. However, the situation looks different in *Verticillium dahliae*, where induction of the lineage-specific *Vd2LysM* gene was observed one week post inoculation when fungal accumulation was observed in xylem (Fradin and Thomma, 2006). In contrast, expression studies from three LysM effectors in the *V. dahliae* JR2 strain showed no induction upon any stage of infection (Kombrink *et al*., 2017). Moreover, *C. beticola* strains overexpressing the RsLysM displayed increased fungal biomass and necrotic lesions in infected plants, strongly supporting its role in host colonization. Similarly, the *Vd2LysM* and Ecp6 effectors are required for full virulence of *V. dahliae* and *C. fulvum* respectively (Bolton *et al*., 2008; de Jonge *et al*., 2013). In contrast, deletion of three core LysM effectors from the *V. dahliae* JR2 strain, showed no changes in pathogen virulence (Kombrink *et al*., 2017), indicating functional differentiation among them.

We showed that RsLysM effector was able to suppress ROS burst, induced by chitin, strongly indicating perturbation chitin-triggered immunity, similar to the already characterized ones (de Jonge *et al*., 2010; Marshall *et al*., 2011; Kombrink *et al*., 2017). Our data further support the already formulated concept regarding the universal role of LysM effectors in the suppression of chitin-triggered immunity (Kombrik and Thomma, 2013). However, our results showed that RsLysM did not protect fungal hyphae from degradation, similar to Ecp6 (de Jonge *et a*l., 2010). This result indicates that this protein is not involved in protecting the fungal cell wall from external hydrolytic enzymes. In contrast, the Vd2LySM and Mg3LysM effectors from

V. dahliae and *Z. tritici* showed clear hyphal protection ability (Marshal *et al*., 2011; Kombrink *et al*., 2017), indicating that hyphal protection is not a universal phenomenon for LysM effectors.

The second candidate effector was an RlpA-like protein, containing a DPBB domain. This domain has possibly an enzymatic function and it has been identified in various proteins, such as expansins, dehydrogenases, endoglucanases and proteinases (Castillo *et al*., 1999), while manual inspection showed that it is also present in some papain-like cysteine protease inhibitors, derived from fungal species. The function of this domain in filamentous fungi remains unknown, but previous data showed that proteins containing this domain were identified in the secretome analysis of virulent *Pyrenophora tere*s f. *teres* isolates, an ascomycete causing net blotch disease in barley (Ismail and Able, 2016) and in Basidiomycota species causing brown and white rot (Pellegrin *et al*., 2015). We observed that the gene, encoding for RlpA-like protein in *R. solani*, was induced at an early stage of infection. Similarly, induction of RlpA-like gene was also found in *Magnaporthe oryzae* invasive hyphae in first invaded cells, indicating a role during the biotrophic stage of this pathogen (Mosquera *et al*., 2009). However, there is not any functional study showing the direct involvement of the RlpA-like protein in fungal pathogenicity. Our results showed that *C. beticola* strains, overexpressing the RsRlpA-like protein, displayed increased fungal biomass in infected plants, and to our best of knowledge, this is the first report showing the contribution of an RlpA-like protein to fungal virulence. The protein 3D structural analysis also showed that RlpA-like is homologous to a papain-like protease inhibitor. Previous studies revealed involvement of these inhibitors in the manipulation of plant immunity and contribution to defense (Ronney *et al*., 2005, Ilyas *et al*., 2015). Moreover, our preliminary data revealed that RsRlpA was able to suppress HR on *N. bethamiana* plants. Although more studies are needed in order to characterize the enzymatic function of this protein and to evaluate the HR suppression, we could speculate that *R. solani* AG2-2IIIB possibly relies on a short initial biotrophic stage crucial for a successful establishment of infection.

Acknowledgments

Authors are grateful to Prof. Bart Thomma, Peter Herfs, Nick Snelders and Hui Tian at Wageningen University, Phytopathology Lab, who contributed to RsLysM protein production and purification, to Wageningen University for providing us with *N.*

bethamiana Cf-4 seeds and to Dr. Ioannis Stergiopoulos and Dr. Li Hung-Chen at UC Davis, Phytopathology Lab for helping us with the hyphal protection assay. This work was supported by grants from: the Swedish Research Council VR and Formas, MariboHilleshög Research and the Swedish University of Agricultural Sciences (CD, JF).

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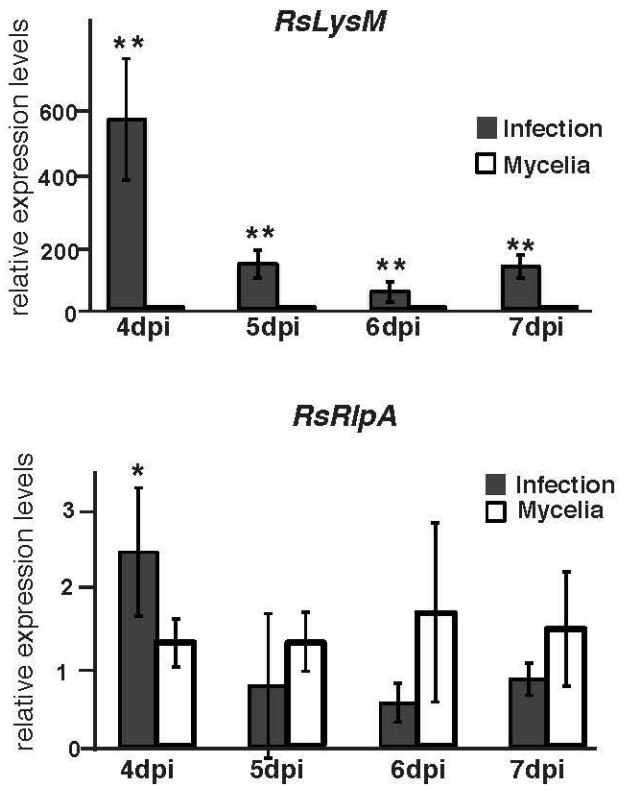

Figure 1. Relative transcript levels of the *Rhizoctonia solani* AG2-2IIIB genes *RsLysM* and *RsRlpA*. Sugar beet plants were grown in infested soil for 4,5,6, and 7 days before harvest for real-time qRT-PCR. *Rhizoctonia solani* mycelia was grown in PDB medium and used as comparison. The *G3PDH* gene was used as internal standard. Error bars represent SD based on at least three biological replicates. Asterisks (* $p < 0.05$, ** $p < 0.01$) indicate statistically significant differences between columns at the same time point according to Student's T test.

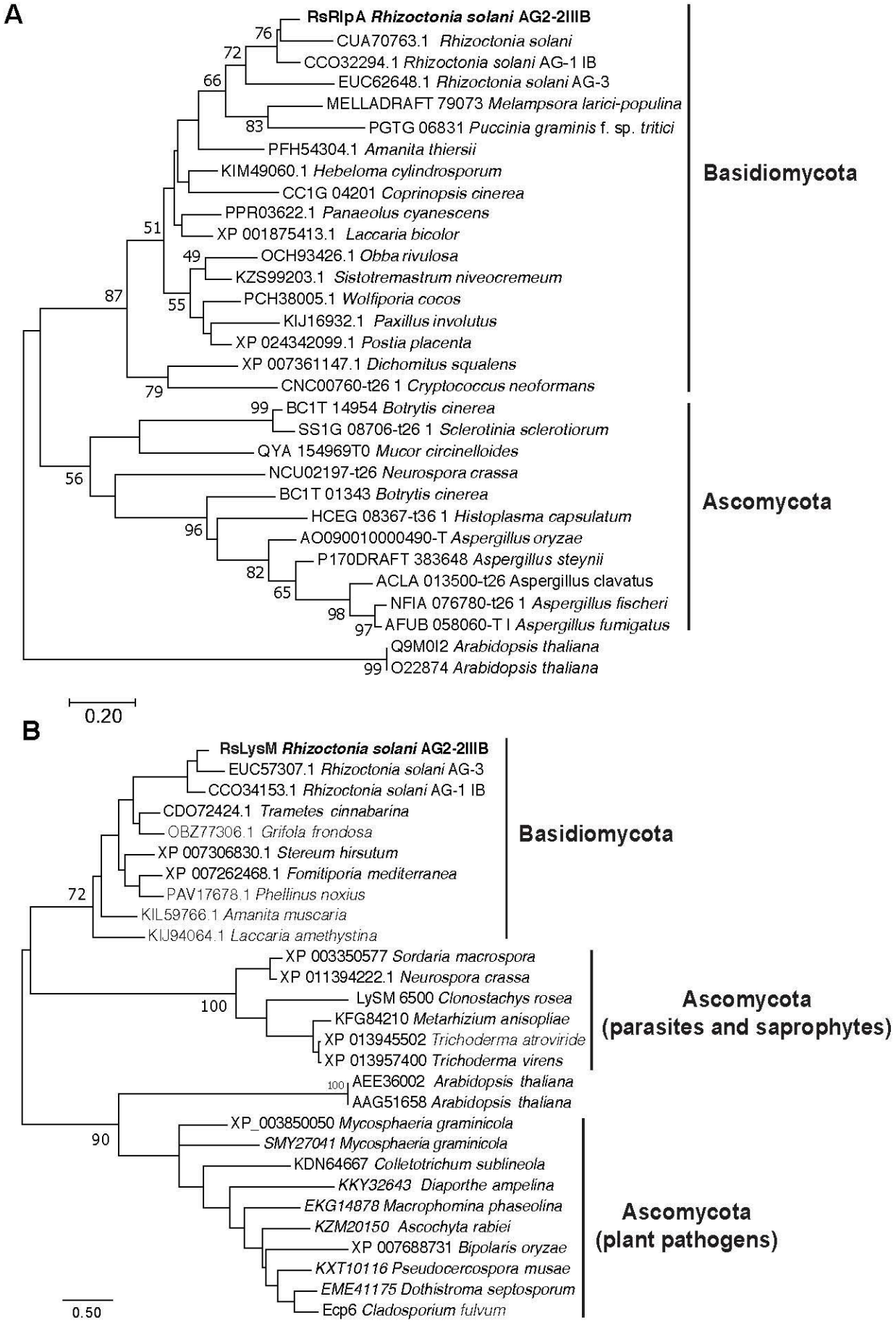

Figure 2. Phylogeny of *R. solani* candidate effector proteins and selected homologs, (**A**) RsRlpA and (**B**) RsLysM. Analyses were based on Neighbor-Joining analysis with the JTT substitution model and complete deletion of missing data. Numbers at nodes indicate the bootstrap value. Bar, indicates the number of amino acid substitutions.

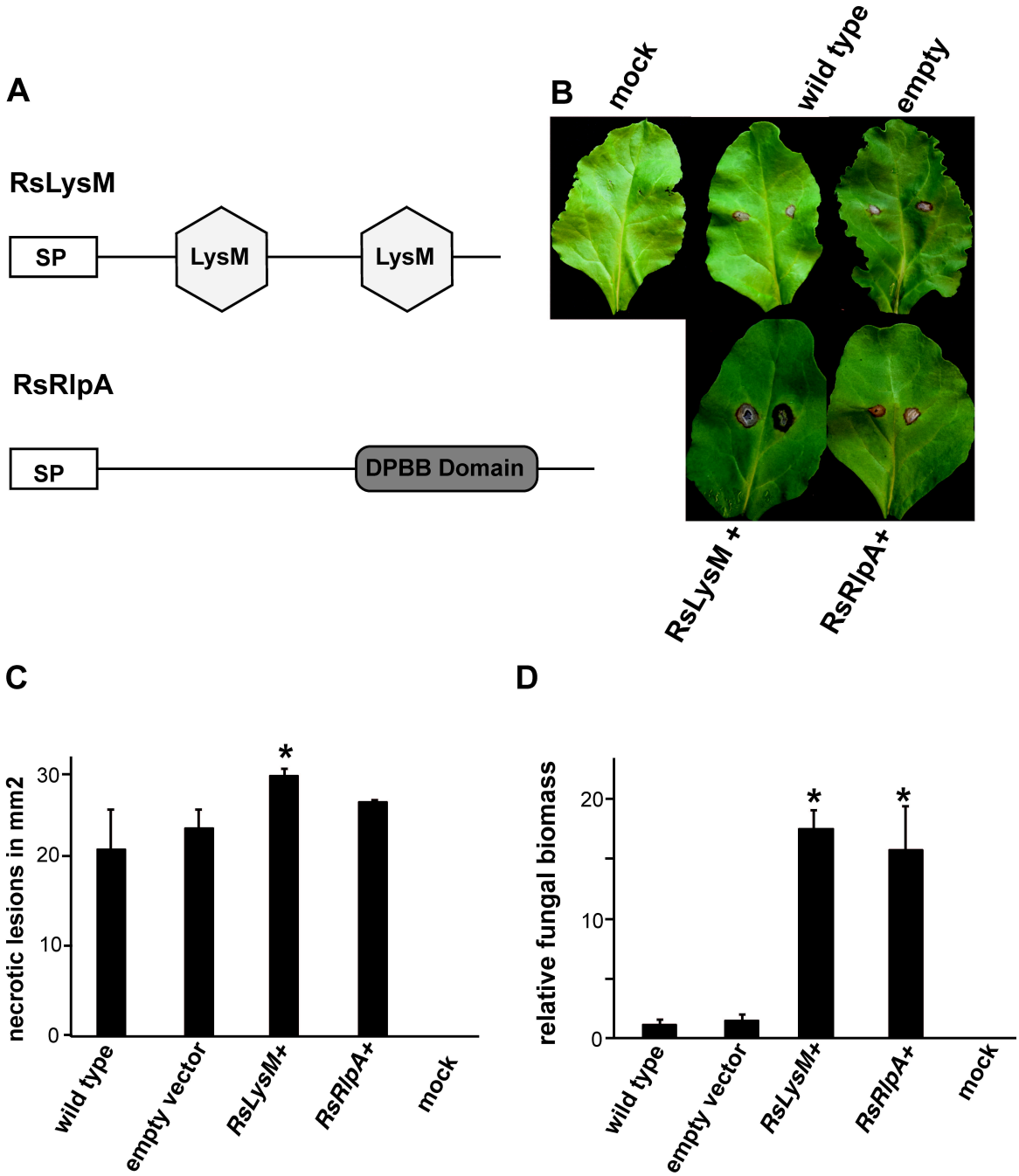

Figure 3. Assays of RsLySM and RsRlpA candidate effectors. (A) Domain structure of RsLySM and RsRlpA proteins. (B) Phenotypes on sugar beet leaves in response to *C. beticola* strains harboring *RsLySM* and *RsRlpA* genes driven by the gdpA promoter, 7dpi. (C) Area of necrotic lesions on sugar beet leaves. (D) Fungal biomass quantification upon infection of sugar beet leaves. For quantitative PCR (qPCR), the *C. beticola* actin (*act*) gene was used. Data were normalized with the elongation factor gene (*elf-1*) from *Beta vulgaris*. Data show the average of three independent overexpression strains each includes three biological replicates. Asterisks indicate statistic significant differences between the wildtype, empty vector and overexpression *C. beticola* strains according to Fisher's test (p value < 0.05). Error bars represent SD based on three independent strains

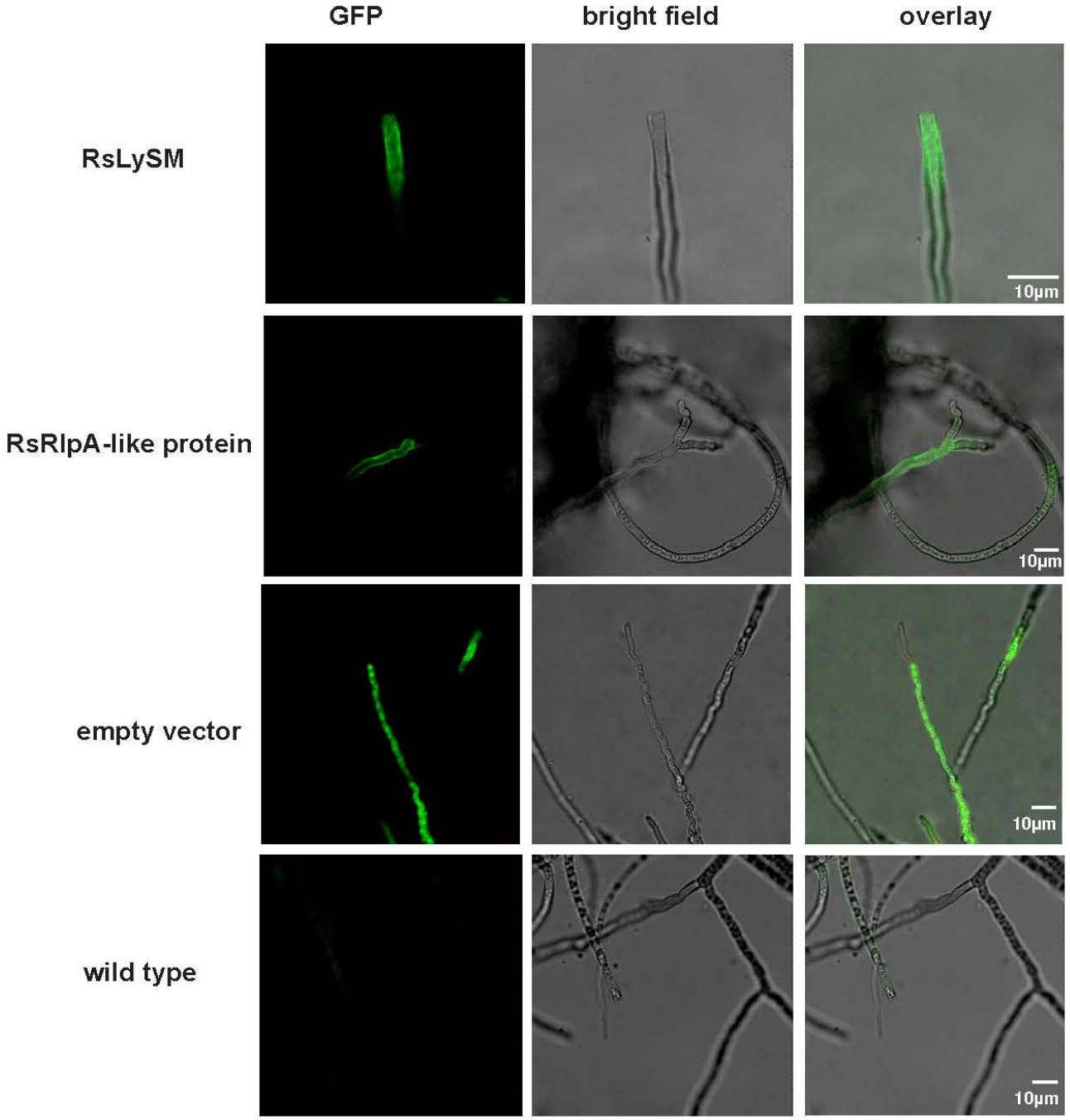

Figure 4. Live-cell imaging of GFP-tagged *R. solani* RsLySM and RsRlpA effector candidates in *C. beticola* hyphae. Live-cell imaging was performed with a laser-scanning confocal microscope with a sequential scanning mode, 7 days after inoculation on potato dextrose agar. GFP was excited at 488 nm and collected at 505–525 nm.

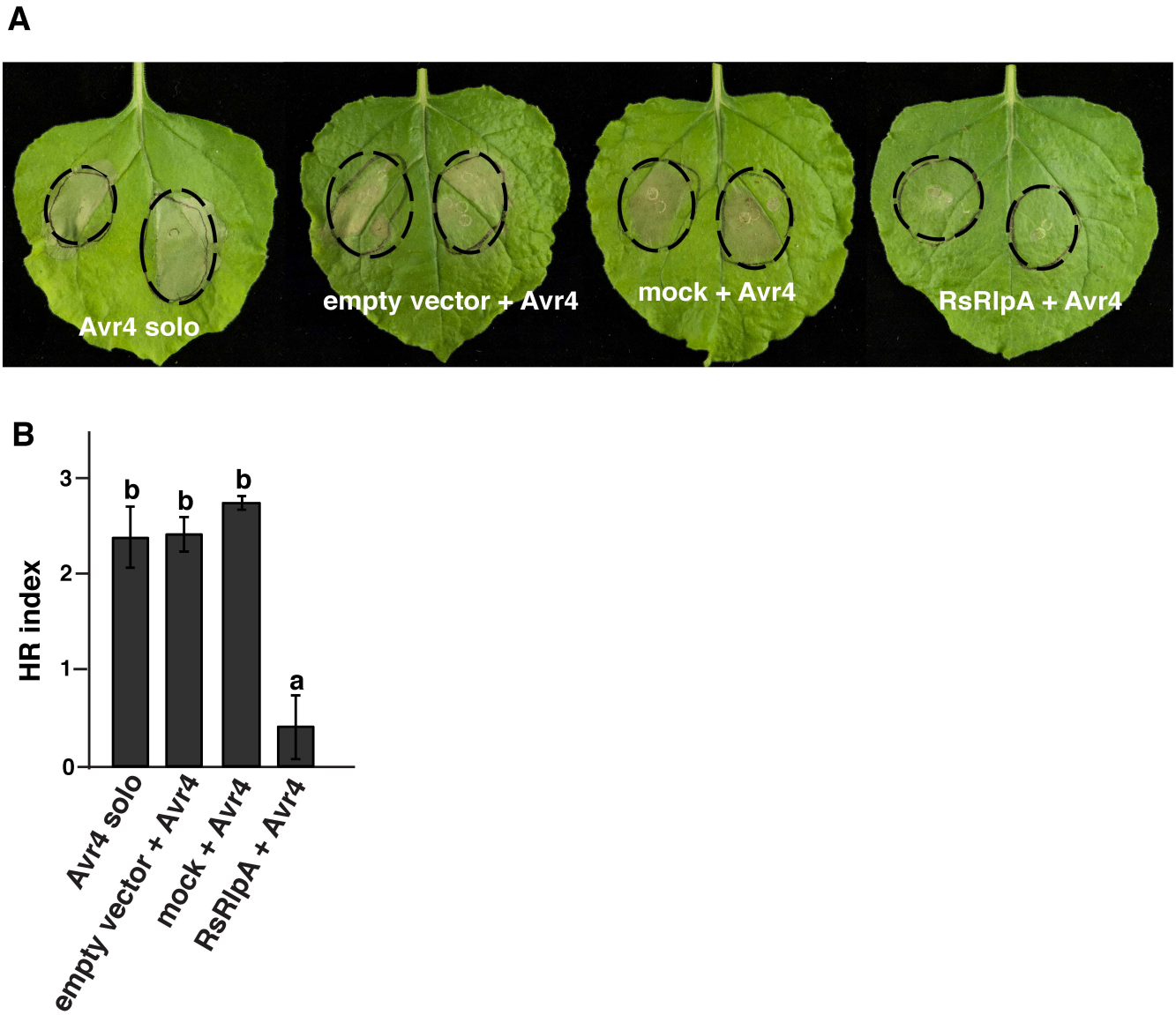

Figure 5. Assay for suppression of Avr4-mediated hypersensitive response (HR). (**A**) Symptoms on transgenic *Nicotiana bethamiana* leaves expressing the tomato Cf-4 receptor. (**B**) HR scale from 0-3 with ''0'' indicates no symptoms and ''3'' severe symptoms on *N. bethamiana*. Leaves were agro-infiltrated first with the RsRlpA effector candidate driven by the CaMV:35S promoter and HR challenged 24hpi with the Avr4 effector. Empty vector and mock inoculation were used as controls. Cycles with dashed lines show the agro-infiltration sites of both effectors. Images taken 3dpi. Different letters (a,b) indicate statistical significant differences according to Tukey test ($p < 0.05$). Error bars represent SD of six plants, each contained two agro-infitrated leaves.

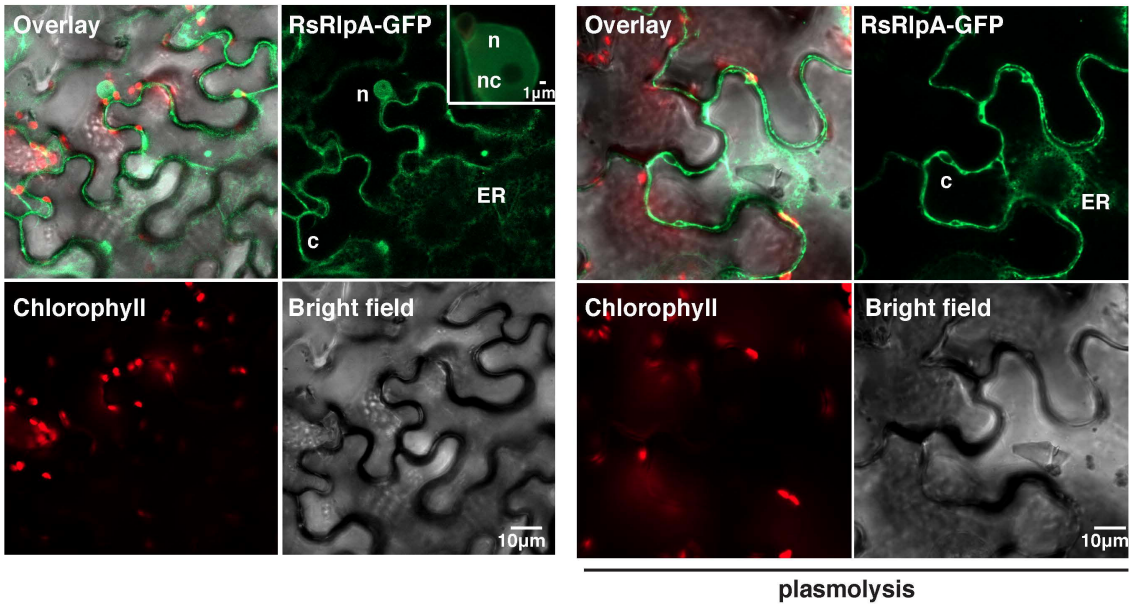

Figure 6. Live-cell imaging of C-terminal GFP-tagged *R. solani* RsRlpA effector candidate in agro-infiltrated *Nicotiana benthamiana* leaves. The localizations were monitored with a laser-scanning confocal microscope with a sequential scanning mode 48 hrs post infiltration. The GFP and the chlorophyll were excited at 488 nm. GFP (green) and chlorophyll (red) fluorescent signals were collected at 505–525 and 680–700 nm, respectively. Plasmolysis was incited by using 1M mannitol for 30 min. (**c**): cytoplasm, (**ER**): endoplasmic reticulum, (**n**): nucleoplasm, (**nu**): nucleolus.

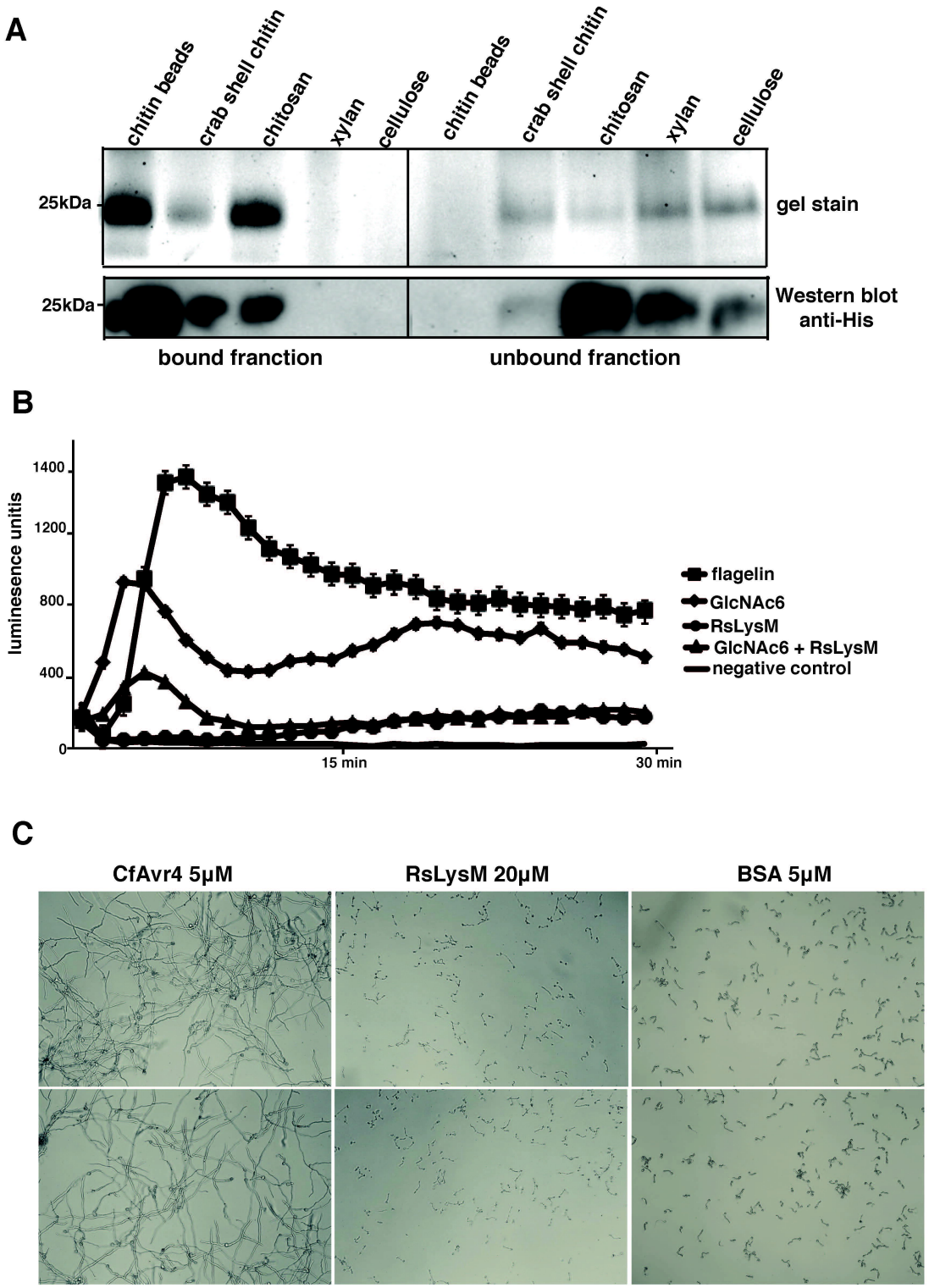

Figure 7. Functional analysis of the RsLysM protein. (**A**) Binding affinity assay on different insoluble polysaccharides. 20 μg/ml of LysM protein was mixed with 5 mg of chitin beads, crab shell chitin, chitosan, xylan or cellulose. Presence of protein in bound (pellet) and unbound fraction (supernatant) separated using SDS-polyacrylamide gel electrophoresis followed by Coomassie staining. (**B**) Chitin-induced oxidative (ROS) burst assay in leafs. Production of ROS was determined using luminol-dependent chemiluminescence. Leaf discs were treated with 10nM flagellin (positive control), 10μM GlcNAc6, 10μM LySM protein, 10μM GlcNAc6 + 10μM LySM protein. The luminol reagent was used as a negative control. At least eight biological replicates were run, and experiment repeated twice. (**C**) Hyphal protection assay. 20μM of LysM protein was incubated with $3x10^5$ *T. virens* conidia for 30min and 0.1U bacterial chitinase and 10U zymolyase. Images were taken 6hpi. 5μM Avr4 or BSA were used as positive and negative controls respectively. Assay was repeated twice with same results.