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Glycosylation Analysis of the Avr9 Effector of *Cladosporium fulvum*, the Fungal Pathogen of *Solanum lycopersicum*

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ABSTRACT

Tomato (*Solanum lycopersicum*) is one of the most important industrial crops, and is threatened by various fungal pathogens like *Cladosporium fulvum*. This fungus secretes the Avr9 effector into the apolast of tomato leaves and is recognized by the tomato receptor-like protein Cf-9. The Avr9-enoding gene is highly expressed during colonization of the tomato apoplast. Avr9 shares significant structural similarity with carboxypeptidase inhibitors, expecting to target host apoplastic proteases. So far, despite using sophisticated methodologies, no definitive Avr9-interacting proteins have been successfully identified. One hypothesis is that glycosylation of Avr9 might be crucial for interaction with host target(s). In this study, native proteins secreted by *C. fulvum* expressing Avr9 were isolated. Mass spectrometry analysis revealed that Avr9 is N-glycosylated when secreted by *C. fulvum*, containing at least two N-acetylglucosamine (GlcNAc) and six mannose residues. The necrosis-inducing activity of glycosylated and non-glycosylated Avr9 was determined and it was found that both caused a comparable Cf-9-mediated hypersensitive response. This research represents a crucial advancement in the comprehension of the molecular interactions pertaining to the Avr9 effector. Nevertheless, more thorough examinations are required to completely elucidate the biological implications of glycosylation. Refining the experimental parameters and investigating additional potential interactors would presumably augment the reliability of the results.

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1. Introduction

Tomato (*Solanum lycopersicum*) is one of the most commercially important members of Solonaceae family. The total world tomato production is currently about 185 milion metric tonnes for both processing and fresh consumption (FAO, 2022). Tomato production is treated by many biotic and abiotic factors including fungal pathogens. *Cladosporium fulvum* is a non-obligate biotrophic fungus causing leaf mould of tomato (*S. lycopersicum*) (de Wit *et al.*, 2012). During infection, *C. fulvum* secretes many effectors into the apoplast of tomato including Avr2, Avr4, Avr4E, Avr5 and Avr9, facilitating infection and colonization of tomato leaves (Mesarich *et al.*, 2014; Stergiopoulos and de Wit, 2009). Avr2 and Avr4 provide protection against basal defense enzymes deployed by the host. Avr2 is an offensive virulence factor that inhibits apoplastic tomato cysteine proteases (Rooney *et al.*, 2005; van Esse *et al.*, 2008), while Avr4 is a defensive virulence factor, that specifically binds to chitin present in fungal cell walls, thereby providing protection against hydrolytic activity of plant chitinases (van den Burg *et al.*, 2006; van Esse *et al.*, 2007). The functions of Avr4E and Avr5 are still unknown. Although Avr9 is the first effector from *C. fulvum* of which the encoding gene has been cloned (Van Kan *et al.*, 1991), its intrinsic function is not known yet. Expression of the *Avr9* gene is highly induced during plant colonization (Van Kan *et al.*, 1991), but could hardly be detected during mycelial growth *in vitro*. *Avr9* encodes a 63 amino acid pre-pro-protein that is processed by fungal and/or host proteases into a 28

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amino acid mature peptide that accumulates in the apoplast (Van den Ackerveken *et al.*, 1993). Nuclear Magnetic Resonance (NMR) studies revealed that Avr9 consists of three anti-parallel strands forming a rigid region of β-sheet with six cysteine residues that form disulfide bridges, resulting in a cystine knot structure (van den Hooven *et al.*, 2001; Vervoort *et al.*, 1997). Such structure is commonly found in carboxypeptidase inhibitors, ion-channel blockers and growth factors (Pallaghy *et al.*, 1994) and it was therefore suggested that Avr9 might inhibit tomato carboxypeptidases (Vervoort *et al.*, 1997). However, such an activity has not been experimentally demonstrated, but this hypothesis has so far only been tested with commercially available carboxypeptidase (van den Hooven *et al.*, 2001) and not with various classes of tomato proteases. The tomato resistance protein Cf-9 belongs to the Receptor-Like Protein (RLP) family and recognizes Avr9 triggering a hypersensitive response (HR) (van der Hoorn *et al.*, 2005). This interaction likely requires additional interactors because no evidence for direct interaction between Avr9 and Cf-9 could be found when the Cf-9 protein was produced in insect cells, COS cells or in *Arabidopsis thaliana* (Luderer *et al.*, 2001).

Using the I^{125} labelled Avr9 as a ligand, a high affinity binding site (HABS) was detected in plasma membranes of solanaceous and some non-solanaceous plant species, irrespective of the presence or absence of the *Cf-9* gene (Kooman-Gersmann *et al.*, 1996). Introduction of *Cf-9* gene in tomato, potato and tobacco (containing the HABS) triggered an HR upon Avr9 infiltration. In contrast, *Cf-9*-transgenic *A. thaliana*, lacking the HABS, failed to induce HR after Avr9 infiltration (Kooman-Gersmann, 1998), suggesting that the HABS is essential for Avr9-triggered Cf-9-mediated HR. In addition, mutational analysis of the Avr9 peptide showed a positive correlation between binding affinity to the HABS and its ability to trigger a Cf-9-mediated HR (Kooman-Gersmann *et al.*, 1998). However, it is not known whether this HABS is a target of Avr9 or a co-receptor required for its recognition. The HABS may function similarly to the Receptor-Like Kinases (RLK) *Sl*SOBIR1 or BAK1 to facilitate Avr9-triggered Cf-9-mediated defense signaling (Liebrand *et al.*, 2014). These two RLKs were shown to interact with Cf-4, Cf-9 and Ve1 *in planta* and are required for the Cf-4-, Cf-9, and Ve1-mediated HR and immunity to the fungal tomato pathogens *C. fulvum* and *Verticillium dahliae*, respectively (Liebrand *et al.*, 2013). Avr9 contains a potential N-glycosylation site (NSS signature). Most of the Av9 peptides purified from tobacco or tomato plants expressing the *Avr9* gene using the PVX expression system contained one N-acetyl glucosamine (GlcNAc) residue attached to the asparagine residue of the NSS glycosylation signature (Kooman-Gersmann *et al.*, 1998). It is likely that Avr9 is also N-glycosylated when it is secreted by *C. fulvum*. The Avr9 peptide carrying one GlcNAc residue showed a lower affinity for the HABS than non-glycosylated Avr9. However, it is not certain that the Cf-9 protein is correctly folded when expressed in heterologous hosts and these experiments could not exclude that correctly folded Cf-9 still binds to Avr9 (Luderer *et al.*, 2001). In the present study, we analyzed the glycosylation pattern and the HR-inducing activity of Avr9 on Cf-9 expressing tomato. The bottlenecks, challenges and approaches to identifying the intrinsic biological function(s) and the glycosylation of Avr9 are discussed.

2. Materials and methods

2.1. Plant and fungal materials

Tomato cultivars MoneyMaker-Cf-4 (MM-Cf-4), -Cf-9 (MM-Cf-9) and the sequenced cultivar Heinz were used for all *in planta* studies, including assays for Cf-mediated hypersensitive response (HR) and virulence assays. Tomato plants were grown in a greenhouse at 70% relative humidity, 23-25°C during daytime and 19-21°C at night, with a light/dark regime of 16/8 hours and 100 W m^{-2} supplemental light when the sunlight influx intensity was less than 150 W m^{-2} (Mesarich *et al.*, 2014). *C. fulvum* race 0 (the sequenced strain) (de Wit *et al.*, 2012), and strain IPO2559 (this strain lacks the *Avr9* gene (race 2.4.4E.9) and a *C. fulvum* transformant (race 5) that over-expresses the *Avr9* gene under the control of *Aspergillus nidulans gpd* promoter (*GPD*::*Avr9)* (van den Ackerveken *et al.*, 1993) were used in this study.

2.2. Preparation of fungal inoculum

C. fulvum strains were grown on 1% Oxoid™ Potato Dextrose Agar (PDA) plates containing 100 µg/mL of streptomycin sulfate salt (Sigma-Aldrich) for 12 days at 20°C. Spores were harvested from PDA plates by flooding the sporulating plates with sterile water.

Fungal mycelia were removed using Miracloth (Calbiochem) and spores were collected by centrifugation at 3,700xg for 15 min, re-suspended in sterile water and the concentration was adjusted to 10^6 spores/mL (Mesarich *et al.*, 2014).

2.3. Isolation of apoplastic fluid from Cladosporium fulvum*-infected tomato leaves*

Inoculation of tomato plants with *C. fulvum* was performed as previously described (Ökmen *et al.*, 2013). Fungal spore suspensions were prepared from *C. fulvum* strains 0 (sequenced strain carrying all functional Avr genes) and IPO2559 (carrying non-functional *Avr4* and *Avr9* genes), adjusted to 10^6 spores/mL and each strain was sprayed on the abaxial side of leaves from 4-week-old tomato plants (6 plants of cv. Heinz). Leaf samples were collected at 0, 2, 4, 6, 8 and 10 days post inoculation (dpi) for apoplastic fluid (AF) isolation using a previously described method (de Wit and Spikman, 1982). AF samples were cleared by centrifugation at 12,000xg for 20 min at 4°C.

2.4. Isolation and purification of glycosylated Avr9 from culture filtrates of a Cladosporium fulvum *Avr9 overexpressing strain grown in vitro*

To identify the sugar moiety present at the N-glycosylation site of Avr9, spores of transgenic *C. fulvum* overexpressing *Avr9* were inoculated (1×10^5 spores/mL) in 10 sterile baffled flasks, each containing 100 mL of sterile Difco™ Potato Dextrose Broth (PDB). Flasks were incubated in an orbital shaker incubator at 22°C and 200 rpm, and cultures from five flasks were harvested at 6 and 12 dpi, respectively. Mycelia were removed using Miracloth and culture filtrates were cleared by a two-step centrifugation at 3,700xg for 15 min and subsequently at 12,000xg for 20 min at 4°C. To purify secreted Avr9, culture filtrates were passed through Amicon filters to remove proteins above 30 kDa in size. The flow-through was concentrated 100-fold by passing over a 1kDa Amicon filter.

2.5. Native gel electrophoresis and HR-inducing activity

To demonstrate the presence of Avr9, 15 µL of concentrated culture filtrates were loaded on a 15% low pH-PAGE gel as previously described (Van den Ackerveken *et al.*, 1993) and run under non-denaturing conditions using pyronine Y as a front marker. Electrophoresis was carried out at 200 V for 90 min. A part of gel was cut and subsequently stained with Coomassie brilliant blue. The stained part was used as a guide to cut the gel bands and to purify the Avr9 peptide from the non-stained parts of the gel. To test their capacity to induce an HR on Cf-9 tomato plants, bands were cut from low pH-PAGE and then gel pieces were incubated in water to elute proteins from the gel. The concentration of eluted glycosylated and non-glycosylated Avr9 proteins was measured and infiltrated into MM-Cf-9 tomato at 0.3, 1 and 3 µM to determine their ability to induce Cf-9-mediated HR.

2.6. Glycosylation analysis using LC-ESI-MS

To identify sugar residues present at the N-glycosylation site of Avr9, the masses of the eluted Avr9 proteins were analysed by LC-ESI-MS. Spectral peaks in the chromatogram were de-convoluted and de-isotoped. The de-convoluted and de-isotoped masses of selected spectra were used for calculating the number of hexoses (presumably mannose), and GlcNAc residues attached to Avr9.

3. Results and discussion

3.1. Secreted Avr9 by strains of Cladosporium fulvum *specifically induces HR in Cf-9 tomato plants*

The HR-inducing activity of naturally secreted Avr9 by infiltration of apoplastic fluid was harvested from cv. Heinz inoculated with *C. fulvum* strains. Infiltration of AF obtained from Heinz plants inoculated with the Avr9-expressing race showed clear HR-inducing activity on MM-Cf-9 plants, while strain IPO2559 that lacks the *Avr9* gene did not induce any HR on MM-Cf-9 plants (Fig. 1). Infiltrating apoplastic protein fractions into leaves of MM-Cf-4 did not induce HR indicating the specific recognition of the secreted Avr9 only by Cf-9 (Fig. 1).

3.2. Avr9 peptide produced by Cladosporium fulvum *is glycosylated*

To determine whether *C. fulvum*-secreted Avr9 is glycosylated, a *C. fulvum* transformant constitutively expressing *Avr9 in vitro* (*GPD*::*Avr9*) (van den Ackerveken *et al.*, 1993) was grown in liquid culture for either 6 or 12 days. Secreted proteins smaller than 30 kDa and larger than 1kDa were collected using columns with a cut-off of 30 and 1 kDa, respectively.

Collected and concentrated Avr9 proteins were separated on low pH-PAGE gel (Fig. 2A). Staining with coomassie brilliant blue revealed two major bands that might represent differently processed and glycosylated Avr9 peptides. Proteins present in these bands were isolated and analysed by ESI-MS. Based on the calculated masses of amino acids present in the different Avr9 peptides, the masses and numbers of sugar residues could be calculated (Table 1).

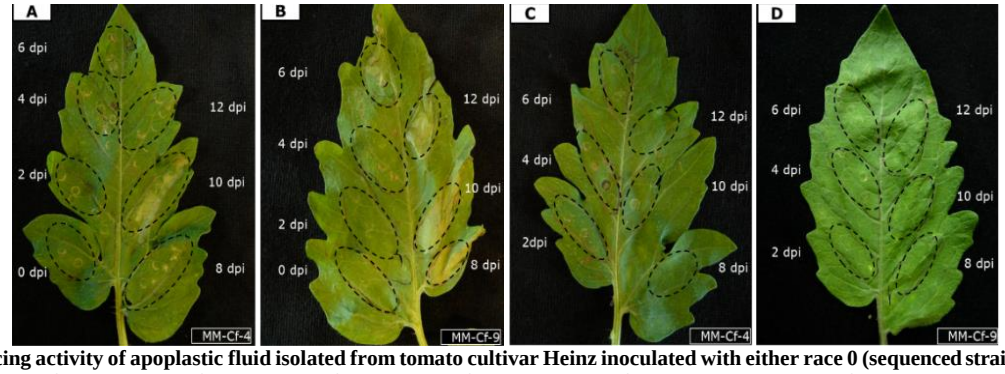

Figure 1. HR-inducing activity of apoplastic fluid isolated from tomato cultivar Heinz inoculated with either race 0 (sequenced strain) or strain IPO2559 (lacking the *Avr9* gene) of *Cladosporium fulvum*. MM-Cf-4 and MM-Cf-9 plants were infiltrated with apoplastic fluid isolated at 0-, 2-, 4-, 6-, 8-, 10- or 12-days post inoculation (dpi) from tomato cultivar Heinz inoculated with race 0 (A) and (B) or strain IPO2559 of *C. fulvum* (C) and (D).

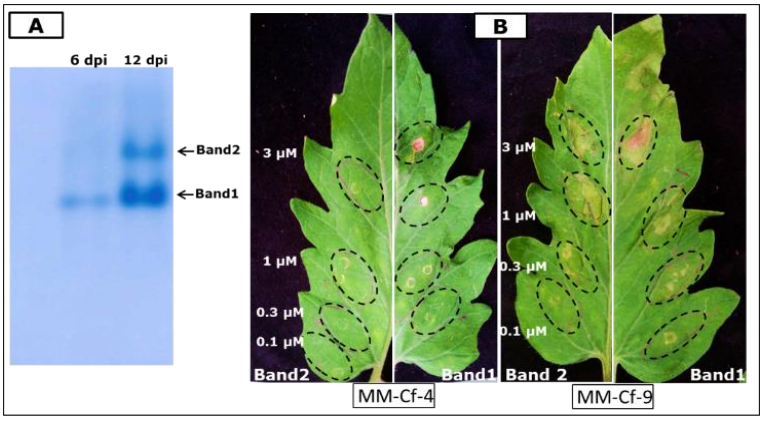

Figure 2. Cf-9-mediated HR activity of (non-) glycosylated Avr9 peptides secreted by transgenic *Cladosporium fulvum* constitutively producing Avr9. Cultures of *C. fulvum* overexpressing Avr9 were harvested after 6- or 12-days post incubation (dpi) on potato dextrose broth and proteins below 30 kDa were collected using an Amicon filter with 1kDa cut-off. (A) 15 µl of 100x concentrated culture filtrate harvested at 6 or 12 dpi were loaded on a 15% low pH-PAGE gel. Bands indicated by arrows were excised for mass spectrometry analysis. (B) glycosylated- (band 2) and less/non-glycosylated Avr9 (band 1) belonging to 12 dpi were eluted from band of non-stained low pH gel and their HR-inducing activity was tested on MM-Cf-4 and MM-Cf-9 at four different concentrations.

Table 1. Theoritical masses of Avr9 containing different numbers of amino acid and sugar residues.

Avr9 # amino acids	MW (Da) of Avr9	MW (Da) of Avr9 + 1GlcNAc	MW (Da) of Avr9 + 2GlcNAc	MW (Da) of Avr9 + 2GlcNAc plus 6 mannoses
28	3415.39	3618.47	3821.55	4793.87
29	3475.41	3678.49	3881.57	4853.89
30	3571.48	3774.56	3977.64	4949.96
31	3628.50	3831.58	4034.66	5006.98
32	3741.58	3944.66	4147.74	5120.06
33	3798.60	4001.68	4204.76	5177.08
34	3897.67	4100.75	4303.83	5276.15

Band 1 contained Avr9 molecules consisting of 31 or 32 amino acids. These Avr9 carried no sugar residue at 6 days post incubation, but they contained one GlcNAc residue at 12 days post-incubation. Band 2 also contained Avr9 consisting of 31 and 32 amino acids at both 6- and 12-days post-incubation, but Avr9 consisting of 34 amino acids was only found at 12 days post-incubation. All Avr9 peptides present in band 2 carried two GlcNAc and six mannose residues at 6- and 12-days post incubation (Table 2). These results indicate that the majority of Avr9 produced *in vitro* by *C. fulvum* is glycosylated and partially processed (Fig. 3). To test their Cf-9-mediated HR-inducing activity, Avr9 peptides extracted from gel pieces corresponding to these two bands were infiltrated into MM-Cf-9 as well as MM-Cf-4 plants at three different concentrations (Fig. 2B). No clear difference in HR inducing activity on Cf-9 plants between Avr9 peptides extracted from band 1 and 2 was observed, suggesting no clear effect of N-glycosylation of Avr9 on its HR-inducing activity (Fig 2B).

Table 2. Measured masses of the Avr9 peptides and their sugar composition secreted in culture filtrate obtained from *in vitro*- grown Avr9-transgenic *Cladosporium fulvum*.

Origin of sample [a]	peak retention time (min) [b]	Highest monoisotopic m/z, z[c]	Determined monoisotopic (Da)[d]	# of amino acids in peptide[e]	Glycosylation pattern[f]
Band1-6dpi	20.89	726.71, 5	3628.50	31	-
Band1-6dpi	23.0	749.32, 5	3741.58	32	-
Band2-6dpi	19.5	1002.40, 5	5006.97	31	2GlcNAc6Man
Band2-6dpi	22.0	1025.02, 5	5120.06	32	2GlcNAc6Man
Band1-12dpi	22.0	767.32, 5	3831.58	31	GlcNAc
Band1-12dpi	24.5	789.94, 5	3944.66	32	GlcNAc
Band1-12dpi	26.0	749.32, 5	3741.58	32	-
Band2-12dpi	18.5	1002.40, 5	5006.98	31	2GlcNAc6Man
Band2-12dpi	22.0	1025.02, 5	5120.07	32	2GlcNAc6Man
Band2-12dpi	24.1	1056.24, 5	5276.16	34	2GlcNAc6Man

[a] indicates the origin of samples for mass spectrometry excised form gel (Fig. 2A). [b] indicates the number of minutes that a solute spends in a column (time elapsed from injection to elution). [c] indicates the mass (m) and charge(z) of Avr9 peptide. m/z is the ratio of mass to charge. [d] indicates the whole size of monoisotopic Avr9 peptide carrying N-glycan in Dalton. [e] indicates the number of amino acid residues found in the Avr9 peptide. [f] indicates the number of N-acetyl-glucosamine (GLCNAc) and Mannose (Man) residues present on the asparagine residue of the Avr9 peptide.

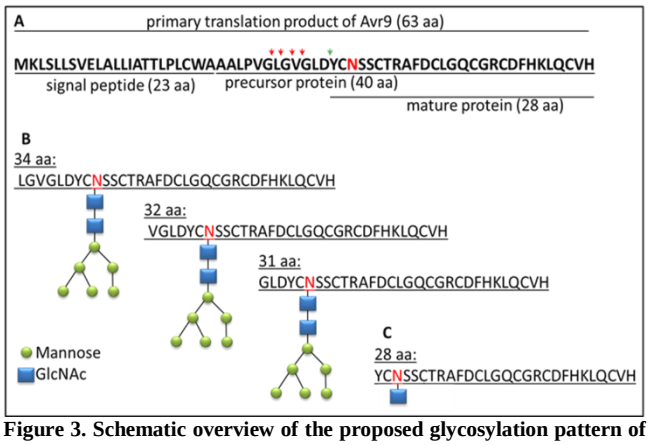

Figure 3. Schematic overview of the proposed glycosylation pattern of Avr9 isolated from culture filtrates of transgenic *Cladosporium fulvum* constitutively producing Avr9 as determined by ESI-MS. (A) The complete Avr9 peptide (63 amino acids) is encoded by the *Avr9* gene. The glycosylation site is highlighted in red. The precursor Avr9 secreted by a *C. fulvum* strain that overexpresses Avr9 is processed by fungal proteases from 40 amino acids to 34, 32 or 31 amino acids by fungal proteases (red arrows) and by plant proteases (green arrow) as shown previously (Van den Ackerveken *et al.*, 1993). (B) The glycosylation site is predicted to carry two GlcNAc and six Mannose residues as measured by ESI-MS from Avr9 secreted by *C. fulvum* during *in vitro* and one HexNAc residue during *in planta* (C). The schematic representation of the proximal N-linked glycan structure is based on observations in yeast and filamentous fungi (Maras *et al.*, 1999).

Avr9 is an effector of *C. fulvum* that in its mature form contains 28 amino acids and three disulphide bridges. Although it was the first cloned fungal effector (van Kan *et al.*, 1991), its intrinsic function is still unknown. Avr9 was discovered in AF of *C. fulvum*-infected tomato plants by its strong HR-inducing activity on tomato plants carrying the extracellular leucine-rich repeat (RLP)-containing Cf-9 receptor-like protein (van der Hoorn *et al.*, 2005). In previous attempts, no direct interaction between Avr9 and Cf-9 was observed (Luderer *et al.*, 2001). However, it was suggested that Cf-9 likely requires an additional membrane protein, HABS, which functions as a co-receptor and consequently activates downstream defense signaling (Kooman-Gersmann *et al.*, 1996).

Based on structural homology of Avr9 with carboxypeptidase inhibitors, it was proposed that its intrinsic biological function might be to target host serine carboxypeptidases (SCPs) or SCP-like (SCPL) enzymes (Pallaghy *et al.*, 1994; van den Hooven *et al.*, 2001). Plants have undergone a significant expansion of SCPs (van der Hoorn, 2008) and SCPLs (Fraser *et al.*, 2005; Milkowski and Strack, 2004) that display antimicrobial activity against pathogens (Mugford *et al.*, 2009; van der Hoorn and Jones, 2004; Zhou and Li, 2005). For successful colonization, pathogens need to overcome these antimicrobial activities by secreting corresponding inhibitors (Misas-Villamil and van der Hoorn, 2008; Mueller *et al.*, 2013; Shabab *et al.*, 2008; Karimi-Jashni *et al.*, 2015). Such an inhibitory action was shown for Avr2 against the apoplastic plant cysteine protease Rcr3 (Rooney *et al.*, 2005) and additional cysteine proteases (Shabab *et al.*, 2008; van Esse *et al.*, 2008). So far, all attempts failed to identify potential Avr9 target(s) in tomato. The first possibility assumes that the interaction of Avr9 with its targets requires a specific condition that differs from those that have been employed in pull-down and farswetern blotting assays as well as other methodologies (Luderer *et al.*, 2001; van Esse *et al.*, 2006; Karimi-Jashni, 2015). The second possibility assumes that Avr9 acts

as ion-channel blocker (Pallaghy *et al.*, 1994) or growth factor as previously proposed (van den Hooven *et al.*, 2001). In this case, processed Avr9 would block anion channels as well as Ca^{2+} and K^{+} channels that along with intracellular signaling proteins and second messengers (Ligterink *et al.*, 1997) are critical components of signal transduction in higher plants (Aducci *et al.*, 1997). The third possibility assumes that glycosylated forms of Avr9 are required to interact with its target(s), whereas the mature 28 amino acid peptide observed *in planta* might be the result of processing after its initial interaction with a host target. Mass spectrometry analysis of isolated Avr9 from culture filtrates of *C. fulvum* overexpressing Avr9 showed that *C. fulvum*-secreted Avr9 is glycosylated (Cummings, 2019). The role of glycan complexes in signalling and biological functions of proteins has been previously described (Arnold *et al.*, 2007; Paulson, 1989).

Fungi have a conserved N-glycosylation pathway (Deshpande *et al.*, 2008), that generates glycoproteins with two GlcNAc and a low or high mannose content as observed in filamentous fungi, (Maras *et al.*, 1999) or yeasts (Herscovics, 1999). In filamentous fungi, the type of N-glycan structures varies between different glycoproteins and glycoprotein enzymes (Anyaogu *et al.*, 2021). For example, a glucose oxidase from *Aspergillus niger* contains maximally seven mannose residues, while an acid carboxypeptidase from *A. saitoi* contains 11 mannose residues attached to the GlcNAc residue (Maras *et al.*, 1999). N-Glycosylation of effectors was reported to be important for their function (Cummings, 2019). The rice blast fungus *Magnaporthe oryzae*, secretes the effector Slp1 (Secreted LysM Protein1) that binds to chitin and competes with the plant CEBiP (Mentlak *et al.*, 2012). Slp1 has three glycosylation sites and is N-glycosylated by an α-1, 3-mannosyltransferase encoded by *Alg3* (Chen *et al.*, 2014). Pathogenicity assay with the mutants of *Alg3* showed that glycosylation of this effector is required for its stability and chitin-binding activity and allows the pathogen to evade the plant immune response (Mach, 2014). We found that *C. fulvum*-secreted Avr9 contains maximally 2 GlcNAc and 6 mannose residues (Fig. 3). It is likely that the structure of the sugar moiety of Avr9 shares homology with that of N-linked oligosaccharides present in glucose oxidase from *A. niger* (Fig. 4). However, it does not exclude that *C. fulvum* produces glycosylated Avr9 with a higher number of mannose residues similar in structure to the N-linked oligosaccharides present on acid carboxypeptidase from *Aspergillus saitoi* (Fig. 4).

Mass spectrometry data of isolated Avr9 from culture filtrates of *C. fulvum* revealed Avr9 peptides with no or only one GlcNAc residue (Fig. 3). This pattern is similar to the plant-produced Avr9 that contains no or only one GlcNAc residue (van den Ackerveken *et al.*, 1993). These Avr9 molecules with fewer sugar residues have likely undergone processing by different types of secreted fungal glycosidases (Naik and Waghmare, 2020). In plants, glycoproteins contain different types of N-glycan structures compared to glycoproteins in fungi. Plant glycoproteins can be decorated by xylose, galactose and fucose residues (Bosch *et al.*, 2013). Glycosylation of Avr9 is not required for HR induction in Cf-9 plants, but it might be required for binding to its host target. Production of sufficient amounts of glycosylated Avr9 for biochemical studies is a challenge. A genetic approach might be more efficient to determine whether glycosylation of Avr9 might be important for interacting with its virulence target or the RLP Cf9 (Cummings, 2019). In the future *C. fulvum* mutants that are unable to produce glycosylated Avr9 should be generated to study the requirement of N-glycosylation of Avr9 to function as a virulence or avirulence factor (Marín-Menguiano *et al.*, 2019).

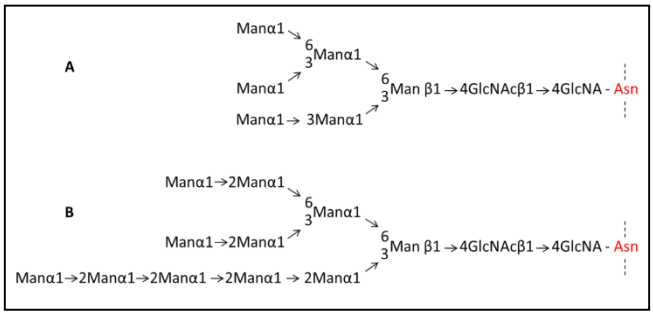

Figure 4. N-glycan linkages as observed in glucose oxidase of *Aspergillus niger* (A) and in acid carboxypeptidase of *Aspergillus saitoi* (B). Figure is depicted and modified from Maras et al. (1999).

4. Conclusion

This study is a significant step toward understanding the molecular interactions of the Avr9 effector. However, more comprehensive investigations are needed to fully elucidate its function and the role of glycosylation. Optimizing the experimental conditions and exploring other potential interactors would likely enhance the robustness of the findings.

Abbreviation

Cladosporium fulvum: Cf, N-acetylglucosamine: GlcNAc, High-Affinity Binding Site: HABS, Receptor-Like Kinases: RLK, Receptor-Like Kinases: RLK, Leucine-Rich Repeat Protein: RLP, MoneyMaker-Cf-4: MM-Cf-4, Hypersensitive Response: HR, Potato Dextrose Agar: PDA, SDS polyacrylamide gel: SDS-PAG, Streptavidin conjugated to Horse Radish Peroxidase: streptavidin-HRP.

Conflict of interests

There is no conflict of interest.

Ethics approval and consent to participate

No humans or animals were used in the present research. The authors have adhered to ethical standards, including avoiding plagiarism, data fabrication, and double publication.

Consent for publications

The final manuscript is approved for publication.

Availability of data and material

All data are embedded in the manuscript.

Informed consent

The author declares not to use any patients in this research.

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