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Protein glycosylation during infection by plant pathogenic fungi

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Summary

Glycosylation is a conserved set of post-translational modifications that exists in all eukaryotic cells. During the last decade, the role of glycosylation in plant pathogenic fungi has received significant attention and considerable progress has been made, especially in *Ustilago maydis* and *Magnaporthe oryzae*. Here, we review recent advances in our understanding of the role of N-glycosylation, O-glycosylation and glycosylphosphatidylinositol (GPI) anchors during plant infection by pathogenic fungi. We highlight the roles of these processes in regulatory mechanisms associated with appressorium formation, host penetration, biotrophic growth and immune evasion. We argue that improved knowledge of glycosylation pathways and the impact of these modifications on fungal pathogenesis is overdue and could provide novel strategies for disease control.

Key words: Post-translational modification, glycosylation, GPI anchor, cell wall, fungal infection, pathogenesis, plant-fungus interaction

I. Introduction

Glycosylation is a highly conserved process in eukaryotes that leads to a wide range of post-translational modifications. These involve the synthesis and addition of different polysaccharide cores to specific amino acids within a consensus sequence of the protein. Most glycoproteins acquire glycosyl groups from the secretory system involving endoplasmic reticulum (ER) and Golgi apparatus, and are secreted to plasma membrane-associated cell wall or extracellular spaces (Helenius & Aebi, 2004). Glycosylation can regulate the function of glycoproteins by affecting their folding, sorting, localization, abundance and activity (Helenius & Aebi, 2004).

Protein glycosylation can be divided into three main types– N-glycosylation, O-glycosylation, and Glycosylphosphatidylinositol (GPI) anchoring (Fujita & Kinoshita, 2012; Breitling & Aebi, 2013; Loibl & Strahl, 2013). Detailed glycosylation modification processes have been identified in *Saccharomyces cerevisiae* and comprehensively reviewed (Wildt & Gerngross, 2005; Deshpande *et al.*, 2008). During the past decade, some components of all three main glycosylation pathways have been investigated in plant pathogenic fungi, but our understanding is far from complete. Important roles for glycosylated proteins have, however, been identified in a number of plant pathogenic fungal species, particularly in the corn smut fungus *Ustilago maydis* and the rice blast fungus *Magnaporthe oryzae* (see Table 1), both of which are tractable genetic models for fungal pathogenesis. Emerging evidence suggests that glycosylation contributes to fungal infection mechanisms in multiple ways. In this insight article, we highlight recent advances in our understanding of glycosylation in phytopathogenic fungi, including roles in appressorium-mediated penetration and biotrophic growth. We argue that these processes are, however, often overlooked in studies of fungal effector proteins and cell wall modifications essential for fungal pathogenesis.

II. Glycosylation and fungal virulence

Proteins that serve functions in N-glycosylation, O-glycosylation and GPI anchoring pathways have recently been found to be pathogenicity determinants in plant pathogenic fungi (Table 1). In *M. oryzae*, for instance, the N-glycan synthesis pathway gene *ALG3* is required for infection hypha development and suppression of the plant immune response (Chen *et al.*, 2014). The α-1,2-mannosyltransferase *MgALG2* gene of the Septoria leaf blotch fungus *Mycosphaerella graminicola,*

meanwhile, is important for switching from the yeast-like phase to the hyphal growth form and is essential for virulence (Motteram *et al.*, 2011). Several genes in the N-glycosylation ER quality control (ERQC) system have also been reported to be essential for virulence in *U. maydis* as shown in Table 1 (Schirawski *et al.*, 2005; Fernandez-Alvarez *et al.*, 2009), suggesting a wider role for such post-translations modifications in plant infection. Consistent with this idea, N-glycosylation pathway proteins such as α-1,6-mannosyltransferase Och1, N-acetylglucosaminyl transferase Gnt2 also play an important role in virulence of a range of different fungal pathogens (Table 1) (Li *et al.*, 2014; Zhang *et al.*, 2019; López-Fernández *et al.*, 2013).

Fungal O-glycosylation has also been shown to be significant in fungal pathogenesis. The initial O-mannosyltransferase reaction is mediated by integral ER membrane protein mannose transferases, which can be classified into three subfamilies (PMT1, PMT2, and PMT4) (Lommel & Strahl, 2009). Mutations in *PMT* family genes have been linked to reduced pathogenicity of several plant pathogenic fungi, such as *U. maydis*, *Botrytis cinerea*, *Fusarium oxysporum*, *M. oryzae* and *Penicillium digitatum* (Table 1) (Fernández-Álvarez *et al.*, 2009; Fernández-Álvarez *et al.*, 2012; González *et al.*, 2013; Harries *et al.*, 2015; Guo *et al.*, 2016; Pan *et al.*, 2019; Xu *et al.*, 2020).

Several studies have also addressed the roles of GPI anchoring during infection by plant pathogenic fungi (Rittenour & Harris, 2013; Oliveira-Garcia & Deising, 2016; Liu *et al.*, 2020). To date, only a few GPI anchoring pathway-related genes have been successfully deleted in fungi (Table 1). These include GPI7 orthologs in *M. oryzae* (Liu *et al.*,2020) and in *F. graminearum* (Rittenour & Harris, 2013), as well as GPI12, GAA1 and GPI8 orthologs in *Colletotrichum graminicola* (Oliveira-Garcia and Deising, 2016). GPI anchoring therefore plays diverse and significant roles in vegetative development and pathogenicity in plant pathogenic fungi (Table 1).

III. Regulatory mechanisms of glycosylation in appressorium-mediated penetration

Many plant pathogens have evolved specific mechanisms to penetrate the host cuticle directly, such as the development of specialized infection structures called appressoria (Howard *et al.*, 1991; Talbot, 2019). Recent studies have reported that glycosylation plays vital roles in appressorium-mediated penetration.

O-glycosylation for example, is important in fungal cell wall composition and affects the relative abundance and distribution of β-1,3-glucans, chitin and glycoproteins (Fernandez-Alvarez *et al.*, 2009). In *U. maydis*, deletion of the O-glycosylation pathway gene *PMT4* significantly reduced the frequency of appressorium formation and function (Fernandez-Alvarez *et al.*, 2009). O-glycosylation of the transmembrane mucin protein Msb2 appears to be responsible for the defect of Δ*pmt4* in appressorium differentiation of *U. maydis*, which may be due to an effect on the downstream activation of the MAP kinase Kpp2 (Fernandez-Alvarez *et al.*, 2012). Interestingly, the Msb2 ortholog in *M. oryzae*, MoMsb2, which is also predicted to be heavily glycosylated in the extracellular domain, was also reported to regulate appressorium development via activation of the analogous Pmk1 MAPK signaling pathway (Figure 1) (Liu *et al.*, 2011).

Some proteins related to glycogen utilization, lipid utilization, cell wall biogenesis, glycosylation pathways, and ER quality control are also highly N-glycosylated in *M. oryzae* (Figure 1) (Chen *et al.*, 2020). Mutants of the N-glycosylation pathway gene *ALG3*, for example, are reduced in their frequency of appressorium-mediated penetration (Chen *et al.*, 2014).

Cell wall glycoproteins are also important in determining fungal cell wall composition, and can also be modified by the GPI anchors (Fujita and Kinoshita, 2012). In *M. oryzae*, GPI7-mediated GPI anchoring regulates appressorial cell wall integrity, which is required for turgor generation and penetration (Liu *et al.*, 2020), as shown in Figure 1. The glucan elongation factor (Gel) β-1,3-glucan glucanoyltransferase– members of family 72 of glycoside hydrolases (GH72) –have furthermore been found to play key roles in appressorium cell wall structure (Lesage & Bussey, 2006; Samalova *et al.,* 2017). Interestingly, accumulation of all five Gel proteins are regulated by GPI anchoring in *M. oryzae* (Liu *et al.*, 2020). The vital roles played by the Gel proteins in the polymerisation, distribution and branching of β-1,3-glucans in cell walls may partially explain why GPI anchoring is essential for appressorium-mediated penetration.

IV. Regulatory mechanisms of glycosylation in biotrophic establishment during fungal growth and proliferation

After penetration, fungal pathogens must undergo rapid invasive growth within host tissue. Pathogens must evade or suppress plant immune responses to ensure their survival and intracellular

proliferation. For biotrophic and hemibiotrophic fungi, the host interaction is finely tuned to ensure survival of infected plant cells, as they are invaded and occupied by the pathogen. Glycosylation is clearly involved in the interactions of fungal secreted proteins within the plant host, particularly in the apoplast. Therefore, mutants in glycosylation pathway genes often induce a strong host immune response. For example, mutants affecting N-glycosylation and GPI anchoring pathway genes induce host cell ROS accumulation (Chen *et al.*, 2014; Liu *et al.*, 2020), suggesting that cell wall modifications dependent on glycosylation may be important in preventing PAMP-triggered immunity. It also suggests that secreted effector proteins involved in immunity suppression, particularly in the apoplastic compartment, may require glycosylation for their activity. By contrast, O-glycosylation appears less important in this regard, because deletion mutants lacking the O-glycosylation pathway gene PTM4 do not significantly accumulate reactive oxygen species (ROS) or induce plant cell death (Fernandez-Alvarez *et al.*, 2009).

The ERQC system, based on recognition of specific glycosylation intermediates of N-linked glycans, is used for 'proof-reading' newly synthesized glycoproteins, so that only well-folded glycoproteins can be delivered to the secretory system and reach their final destinations. Misfolded glycoproteins are retained and undergo ER associated degradation (ERAD) and recycling (Ellgaard & Helenius, 2003). Based on studies in both *U. maydis* and *M. oryzae* the ERQC system appears to be fundamental to invasive growth. (Schirawski *et al.*, 2005; Fernandez-Alvarez *et al.*, 2013; Chen *et al.*, 2020). The *U. maydis* ERQC system glucosidase I, Gls1, for instance, is required for the initial stages of infection following appressorium-mediated penetration, whereas glucosidase II β-subunits, Gas1 and Gas2, are required for intracellular expansion in host cells (Schirawski *et al.*, 2005; Fernandez-Alvarez *et al.*, 2013). Therefore, the ERQC system is important for both biotrophic growth and subsequent spread of *U. maydis* dikaryotic hyphae in host tissue. Interestingly, similar functions for ERQC components have been reported in *M. oryzae* (Chen *et al.*, 2020). These ERQC components, including orthologs of Gas1, Gas2 and Gls1, were identified as N-glycosylated proteins via a quantitative N-glycoproteomic analysis of *M. oryzae*. Importantly, as an example, the N-glycosite of Gls1 (N497) is essential for its ER localization and protein stability, and is required for biotrophic growth (Chen *et al.*, 2020). Therefore, the N-glycosylation system helps to maintain protein stability of ERQC components (Figure 1).

The identification of mechanisms of host recognition by immune evasion strategies is a very active area of investigation in fungal-plant interactions (Rovenich *et al.*, 2016). Chitin and β-1,3-glucan are the main structural components of the fungal cell wall, which is vital for fungal growth and development. They also function as PAMPs and can therefore be recognized by host receptors and activate plant immune responses. Fungi have developed different strategies to evade host immunity involving recognition of chitin and β-1,3-glucan. For example, the outer layer of the fungal pathogen cell wall can provide structural protection, which can protect the inner cell wall from recognition by host cells. Accumulation of α-1,3-glucans on the surface of invasive hyphae can, for instance, be used to mask chitin and β-1,3-glucans and thereby block host recognition (Fujikawa *et al.*, 2012). GPI-anchored proteins can furthermore function as a shield in both appressoria and invasive hypha of *M. oryzae*, to protect the fungus from host immunity recognition (Figure 1) (Liu *et al*, 2020). Depletion of GPI-anchored proteins in *M. oryzae*, for example, resulted in chitin and β-1,3-glucans exposure and elicit host defence responses. A similar mechanism was also reported in the human fungal pathogen *Candida albicans* (Shen *et al.*, 2015), suggesting that GPI anchoring-mediated immune evasion may be widespread among pathogens of both plants and animals. Chitin deacetylases, which catalyze the conversion of chitin to chitosan and help shield chitin from host perception, are also predicted to be GPI-anchored proteins (Liu *et al*, 2020), but it is not yet known whether the GPI anchor is important for their function.

During infection, fungi secrete a large repertoire of effector proteins to suppress host immune functions and thereby facilitate invasive growth. Some effector proteins, destined for delivery to the apoplast, contain cysteine-rich regions, which are suitable for disulfide bond formation that is often necessary for their active conformation (Lanver *et al.*, 2017). In *U. maydis*, efficient N-glycosylation of the disulfide isomerase Pdi1, the protein required to catalyze generation of disulfide bonds, is necessary for secretion of virulence factors, including effector proteins (Figure 1). N-glycosylation of Pdi1 affects its electrophoretic mobility, but not cellular location or stability (Marín-Menguiano *et al.*, 2019). GPI anchoring may also be necessary for the function of some effector proteins (Figure 1). Many putative effector proteins have, for example, been predicted to be GPI-anchored proteins in *M. oryzae* (Liu *et al.*, 2020). The GPI anchor in this context may be important for attachment of effector proteins to the cell wall.

Fungi can also evade plant immunity by effector-mediated suppression of immune responses (Valent & Khang, 2015). In *M. oryzae*, an apoplastic effector Slp1 can compete with the rice chitin pattern recognition receptor CEBiP to sequester chitin, and therefore evade host immune response (Mentlak *et al.*, 2012). The stability and chitin-binding activity of Slp1 is tightly regulated by Alg3-mediated N-glycosylation, and this is mediated by N- glycosylation of three sites (Chen *et al.*, 2014). Interestingly, bioinformatic prediction of N-glycosylation suggests that many other effector proteins may also be N-glycosylated, and this may be important in their ability to suppress host immune responses. Recently, for example, it was found that in the oomycete pathogen *Phytophthora sojae*, N-glycosylation also shields an effector PsXEG1 against degradation by host aspartate proteases (Xia *et al.*, 2020).

O-glycosylation may, similarly, regulate the function of a sub-set of effector proteins (Figure 1). For example, Um03749 is a putative secreted effector protein of *U. maydis*, whose function is affected by Pmt4-mediated O-glycosylation (Fernandez-Alvarez *et al.*, 2012). O-glycosylation also regulates the function of the plasma membrane protein Pit1, and subsequently affects the activity of the secreted effector Pit2 for fungal biotrophic growth (Fernandez-Alvarez *et al.*, 2012). Therefore, O-glycosylation may regulate effector function, either directly or indirectly, to facilitate biotrophic growth during fungal infection. It is, however, clear that glycosylation of effectors has not yet been systematically studied. This is important in the context of the analysis of effectors, which are often expressed heterologously in bacteria for structural analysis, or transiently expressed in host plants using *Agrobacterium* to identify interacting plant proteins. Effectors which require N- or O-glycosylation to be active may not adopt their correct structure, if not produced by the fungal pathogen, and this could lead to artifacts.

V. Conclusions and future perspectives

It is becoming clear that all three types of glycosylation play fundamental roles in the infection processes of plant pathogenic fungi. This is likely to be a function of the requirement for fungi to have specialized modifications in their cell walls, associated with invasive growth and formation of the intimate plant-fungal interface in biotrophic interactions, as well as deployment of the battery of secreted effector proteins necessary to suppress host immunity. Many apoplastic effectors, in

particular, are glycosylated and these modifications are likely to be critical for their function. In the past decade, significant new information has been gained regarding the function and molecular mechanisms of glycosylation and these processes are important for appressorium-mediated penetration and biotrophic growth. However, relatively few fungal pathogen systems have been analyzed so far and therefore the findings reported to date cannot yet be generalized. In addition, the patterns and types of glycosylation that occur during plant infection by pathogens and how this is regulated remain to be elucidated.

Our current understanding of glycosylation has largely been established through functional genetic studies of specific glycosylation pathway proteins, while glycosylated target proteins executing biological functions have received far less attention. Future research may need instead to focus on systematic global analysis of glycosylation in virulence through proteomic and glycomic analyses. Systematic study of glycosylation targets, including the profiling of the global repertoire of glycoproteins and their glycosites, will be critical to understand the mechanistic roles of glycosylation during fungal infection process. Although N-glycoproteomes of *M. oryzae* and *F. graminearum* (Yu *et al.*, 2016; Chen *et al.*, 2020), and O-glycoproteomes of *F. oxysporum* and *B. cinerea* have been generated and investigated recently (González *et al.*, 2014; Xu *et al.*, 2020), few O-glycoproteome and GPI anchoring proteomics studies have been performed to date. A comparative study of *Penicillium* species, for example, shows how much diversity in modifications is likely to exist within fungal glycoproteomes (Hykollari *et al.,* 2016). In the future, it will therefore be necessary to systematically screen the full repertoire of glycosylated fungal effectors, for example, through proteomic analysis of invaded host tissue in which pathogen effectors are highly expressed. A range of mass spectrometry and fractionation approaches will also be necessary because glycans of the same mass can have very different structures. LC-MALDI-ToF-mass spectrometry, in combination with chemical and enzymatic treatment, as well as reverse phase HPLC, may collectively be necessary to define the glycosylation patterns associated with the secreted effector population. Functional analysis of genes encoding glycosylated proteins has also been hard to carry out because they often exist as gene families. The development of efficient CRISPR-Cas9 genome editing (Foster *et al.*, 2018), offers the chance to delete entire gene families which, due to redundancy in function, may lead to discernible phenotypes. In this way the full extent of the effect of glycosylation on infection structure

development, invasive growth and effector function can be evaluated. Such studies may also reveal whether interfering with specific types of glycosylation could become a novel disease-control strategy.

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Figure legend

Fig. 1 Potential mechanisms of glycosylation during fungal infection process. (a) Regulatory mechanisms of glycosylation pathways during appressorium formation and penetration. The O-glycosylated mucin Msb2 senses plant surface signals to activate the MAPK signaling pathway required for appressorium formation, and to regulate appressorial penetration. Glycosylphosphatidylinositol (GPI) anchoring contributes to appressorial penetration by affecting cell wall integrity, through accumulation of GPI-anchored proteins, especially Gel family proteins. Many N-glycosylated proteins also affect appressorium-mediated penetration, such as proteins involved in glycogen and lipid utilization, cell wall biogenesis and ER quality control (ERQC) system. (b) Regulatory mechanisms of different glycosylation pathways for biotrophic establishment during fungal invasive growth. The ERQC components are regulated by N-glycosylation, then control protein folding and secretion for fungal biotrophic growth. The N-glycosylation pathway also regulates functions of *Ustilago maydis* Pdi for effector secretion. It can also directly modify effector proteins such as *Magnaporthe oryzae* Slp1, to regulate their function in immune evasion. O-glycosylation modifies *U. maydis* Pit1 protein to affect function of effector Pit2. GPI anchored proteins function as a shield to protect fungal cell wall PAMPs for immune evasion. GPI anchoring also regulates biotrophic growth by affecting effector secretion. EHIM, plant-derived extra invasive hyphal membrane; BIC, biotrophic interfacial complex. Solid arrow indicates the reported regulation with evidence, dashed arrow indicates predicted regulation.

Table 1 Glycosylation pathway genes discussed in this review.

Fungus species	Protein	Glycosylation	Molecular function	Biological function	Reference
Magnaporthe oryzae	Alg3	N-	α-1,3-mannosyltransferase	mycelial growth, conidiation, invasive growth, reactive oxygen spieces (ROS) detoxification, virulence	Chen *et al.*, 2014
	Cnx1	N-	calnexin		
	Gls1	N-	glucosidase I	mycelial growth, conidiation, invasive hyphal growth, virulence	Chen *et al.*, 2020
	Gls2	N-	glucosidase II β-subunit		
	GTB1	N-	glucosidase II β-subunit		
	PMT2	O-	O-mannosyltransferase	fungal adhesion, conidial germination, cell wall integrity, invasive hyphae growth	Guo *et al.*, 2016
	PMT4	O-	O-mannosyltransferase	hyphal growth, conidiation, penetration and biotrophic invasion, virulence	Pan *et al.*, 2018
	GPI7	GPI	phosphoethanolamine transferase	cell wall biogenesis, penetration, invasive growth, immune evasion	Liu *et al.*, 2020
Ustilago maydis	Gls1	N-	glucosidase I	initial stages of biotrophic growth, virulence	Fernandez-Alvarez *et al.*, 2013
	Gas1	N-	glucosidase II α-subunit	intracellular expending, virulence	Schirawski *et al.*, 2005
	Gas2	N-	glucosidase II β-subunit	intracellular expending, virulence	Fernandez-Alvarez *et al.*, 2013
	Pdi1	N-	disulfide isomerase	effector secretion, virulence	Marin-Menguiano *et al.*, 2019
	PMT4	O-	O-mannosyltransferase	appressorium formation, plant cuticle penetration, virulence	Fernandez-Alvarez *et al.*, 2009
Botrytis cinerea	PMT1	O-	O-mannosyltransferase	morphogenesis, fungal adherence, cell wall integrity and virulence	Gonzalez *et al.*, 2014
	PMT2	O-	O-mannosyltransferase		

	PMT4	O-	O-mannosyltransferase		
Fusarium oxysporum	Gnt2	N-	N-acetylglucosaminyl transferase	conidium morphology, hyphal fusion, secretion of trafficking vesicles	Lopez-Fernandez *et al.*, 2013
	Och1	N-	α-1,6-mannosyltransferase	fungal growth, cell wall integrity, hyphal adhesion, virulence	Li *et al.*, 2014
Fusarium graminearum	GPI7	GPI	phosphoethanolamine transferase	growth, macroconidia formation, cell wall integrity, virulence	Rittenour and Harris, 2013
Verticillium dahliae	Och1	N-	α-1,6-mannosyltransferase	growth, conidia production, microsclerotia formation, cell wall integrity, virulence	Zhang *et al.*, 2019
Colletotrichum graminicola	GPI12	GPI	N-acetylglucosaminylphosphatidyl-inositol deacetylase	cell wall integrity, infection hyphae	Oliveira-Garcia & Deising, 2016
	GAA1	GPI	metallo-peptide-synthetase	differentiation, virulence	
	GPI8	GPI	cystein protease		
Mycosphaerella graminicola	Alg2	N-	α-1,2-mannosyltransferase	cell wall integrity, yeast‐like to filamentous growth switch	Motteram *et al.*, 2011
Penicillium digitatum	PMT2	O-	O-mannosyltransferase	cell wall integrity, conidiogenesis, virulence, sensitivity to fungicide	Harris *et al.*, 2015

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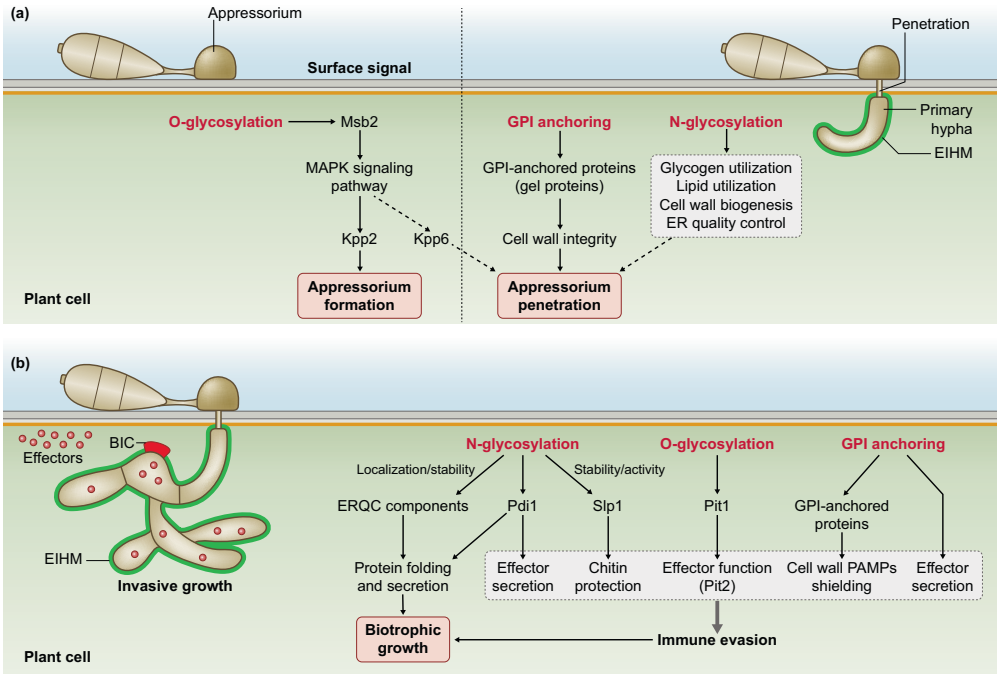

Figure 1

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