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Characterization of the α-mannosidase Gene Family in Filamentous Fungi: N-glycan Remodelling for the Development of Eukaryotic Expression Systems

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Abstract Although filamentous fungi are used extensively for protein expression, their use for the production of heterologous glycoproteins is constrained by the types of N-glycan structures produced by filamentous fungi as compared to those naturally found on the glycoproteins. Attempts are underway to engineer the N-glycan synthetic pathways in filamentous fungi in order to produce fungal expression strains which can produce heterologous glycoproteins carrying specific N-glycan structures. To fully realize this goal, a detailed understanding of the genetic components of this pathway in filamentous fungi is required. In this review, we discuss the characterization of the α-mannosidase gene family in filamentous fungi and its implications for the elucidation of the N-glycan synthetic pathway.

Keywords: glycosylation, filamentous fungi, N-glycans, genetic manipulations

INTRODUCTION

Filamentous fungi are widely used for the heterologous expression of proteins as they are capable of producing up to 40 grams of protein per litre of culture [1-3]. The composition of N-glycans added to expressed glycoproteins can be an important consideration for the production of recombinant human therapeutic products such as epidermal growth factor (EGF), interleukin-6 (IL-6) and corticosteroid binding globulin (CBG) [4]. The post-translational addition of aberrant N-glycans to such proteins can result in reduced activity and/or stability, increased serum clearance, and can sometimes result in an adverse immune response [5,6]. To utilize filamentous fungi for the production of such specialized glycoproteins, it is preferable to produce glycoproteins which carry carbohydrate structures as similar as possible to the natural product. This relies upon detailed knowledge of protein glycosylation in filamentous fungi. Understanding this process may allow manipulation of the N-glycosylation pathway to produce glycoproteins with 'correct' N-glycan structures. While the pathways of mammalian and yeast systems have been well characterized [7,8], less is known about these pathways in filamentous fungi. To fully realize the potential of filamentous fungi as highly flexible expression hosts it is necessary to examine the genetic components of their protein N-glycosylation pathways.

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Protein glycosylation is a post-translational modification of proteins which involves the attachment of sugar residues to newly synthesized polypeptides. Whereas mammalian glycoproteins generally contain complex type N-glycans, which may be necessary for the correct function of the glycoprotein, recombinant production of these same glycoproteins in lower eukaryotic expression systems leads to the addition of oligomannosidic N-glycans (reviewed in [1]). The initial focus of current research on the genetics of the N-glycosylation pathway concerns the first stages of the processing pathway, namely the role of the α-mannosidases in N-glycan processing. In mammalian systems, the α-mannosidases play a key role in modification of N-glycan chains prior to further elongation and production of complex N-glycans. Attempts to produce complex N-glycans in filamentous fungi would thus require elucidation of this part of the pathway. We will review the evolution of multigene families in the N-glycosylation pathway and focus on the characterization of the α-mannosidase gene family of the filamentous fungi.

GENE FAMILIES INVOLVED IN PROTEIN GLYCOSYLATION

Protein N-glycosylation occurs primarily in the endoplasmic reticulum (ER) and Golgi apparatus, and involves a series of discrete catalytic steps. A diverse series of enzymes have evolved to carry out the complex steps of this pathway. It is becoming clear that for many of these catalytic steps, gene families have evolved to generate a number of similar genes to perform a diversity of specialized yet related functions.

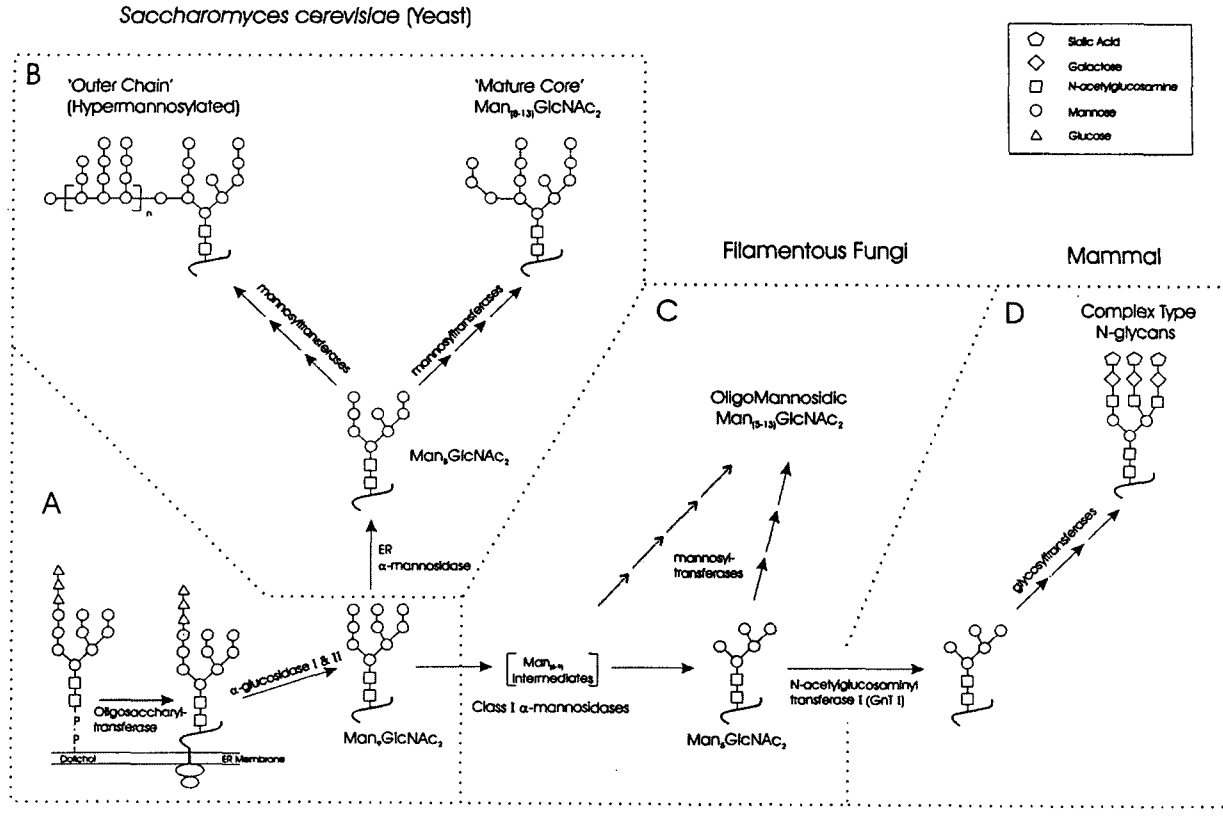

Fig. 1. N-glycosylation synthesis pathway. The initial stages of the pathway are common to all systems [A]. Modification of $Man_9GlcNAc_2$ produces different final N-glycan structures in yeast [B], filamentous fungi [C], and mammalian systems [D]. The final structures shown represent the most common structures found in these species. Variations on these structures are found in some species.

Indeed, virtually every enzyme involved in the protein glycosylation pathway is a member of a multigene family. An extensive classification system has been developed to catalogue the related glycosidases and glycosyltransferases involved in carbohydrate processing [9,10].

The N-glycosylation synthesis pathways have been fairly well characterized in mammalian systems (reviewed in [7]) and yeast expression systems (reviewed in [8,11]), but are not as well characterized in filamentous fungi. Protein N-glycosylation occurs when an oligosaccharide precursor ($Glc_3Man_9GlcNAc_2$) is transferred to newly synthesized proteins in the endoplasmic reticulum (ER) and Golgi apparatus [7]. As the glycoprotein moves through the ER and Golgi to its final destination within or outside the cell, the N-glycan is modified by a series of glycosidases and glycosyltransferases to produce a large diversity of structures. The types of modifications and final glycan structures formed are quite different between more complex higher eukaryotes and simpler lower eukaryotes.

In all eukaryotes the initial stage of N-glycan processing is the removal of three glucose molecules from the $Glc_3Man_9GlcNAc_2$ by α-glucosidase I. In higher eukaryotes, up to four mannose residues are then removed by Class I α-1,2-mannosidases to produce $Man_5GlcNAc_2$, which is the precursor for complex N-glycan formation (Fig. 1). Complex N-glycans are formed in the Golgi by the addition of GlcNAc by N-acetylglucosaminyl transferase I (Gnt I), removal of two mannose residues by α-mannosidase II, followed by the addition of various sugars, such as galactose and sialic acid [7]. In the lower eukaryote *Saccharomyces cerevisiae*, the removal of glucose is followed by the removal of one mannose residue to produce $Man_8GlcNAc_2$. Glycans with fewer than 8 mannose residues are not found in yeast. Mannosyltransferases then sequentially add a number of mannose residues to produce oligomannosidic N-glycans which typically contain core structures of up to 13 mannose but can also lead to the production of very large mannans containing up to 200 mannose residues [8,11,12]. This latter process is known as hyperglycosidic mannosylation.

The potential diversity of N-glycans and modification steps in filamentous fungi are less clear than yeast or mammalian systems (Fig. 1). Filamentous fungi produce N-glycan structures which share properties of both yeast and mammalian systems. N-glycans containing as few as 5 mannose units ($Man_5GlcNAc_2$) have been found in filamentous fungi [13,14], suggesting processing of the $Man_9GlcNAc_2$ in a manner similar to

higher eukaryotes. Some filamentous fungal species, such as *Aspergillus*, also produce hyperglycosidic oligomannans, similar to those found in yeast [1], however, this is not a common feature of filamentous fungi. The filamentous fungi thus appear to have an N-glycosylation system which is intermediate to yeast and mammalian systems. Like yeast systems, filamentous fungi only produce oligomannosidic N-glycans. Like mammalian systems, however, filamentous fungi produce N-glycans which can be fully trimmed by α-1,2-mannosidases to $Man_5GlcNAc_2$ [1]. This has important consequences for the remodelling of N-glycans in these expression systems, as the $Man_5GlcNAc_2$ serves as the substrate for all subsequent modifications leading to complex N-glycans in mammalian systems. An investigation of the α-mannosidase gene family is the first step in fully differentiating this pathway in filamentous fungi.

EVOLUTION OF GENE FAMILIES

The principles of gene evolution can be invoked to explain how the various gene families involved in protein glycosylation arose. Duplication of genes and even whole genomes can be responsible for the generation of multiple copies of genes having the same function and for the creation of novel proteins with diverse functions [15-17]. Duplication events can involve anywhere from a few nucleotides to entire genomes. Equally important to the original duplication event is the maintenance and divergence of the duplicated DNA in the genome [18-23].

Two genes which have descended from a common ancestral gene are known as 'orthologous' genes. Generally, orthologous genes follow from speciation events. Gene duplication, however, will lead to multiple copies of the gene in a single species. Upon divergence, these duplicated genes are then called 'paralagous' genes [16]. These duplications have historically led to the evolution of large multigene families which are characteristic of eukaryotic genomes. The size and abundance of multigene families (and superfamilies) increase with the evolutionary complexity of organisms, thus their formation is an extremely important evolutionary process [24].

Assuming that DNA duplications were selectively neutral, their invasion into a population would be influenced mainly by random genetic drift. If the duplication itself conferred some selective advantage to an organism [19], either through a direct advantage conferred by the duplicated gene product, or by the relaxation of selective constraints on the duplicated DNA, then duplications would have a greater probability of fixation. It has been hypothesized that duplications may confer a selective advantage since the duplicated locus would be more tolerant to deleterious mutations due the functional redundancy created by the extra gene copy [19,23].

Large scale duplication events, such as whole or partial genome duplications can be as important for the expansion of gene families as single gene duplications. Most genes, especially in eukaryotic genomes, are involved in complex gene networks, such as regulatory networks and physiological pathways. Duplication events have the least disruptive effect on gene networks if they involve either a small number of genes in the network or all of the genes in the network [25]. Duplications involving ~40% of the genes in the network would be the most disruptive and likely would not be tolerated. Duplication of entire networks would occur with whole genome duplications, or with chromosomal duplications involving tightly clustered gene networks. Chromosomal duplications may arise from nondisjunction during meiosis, in which homologous chromosomes or sister chromatids fail to segregate, leading to aneuploidy and polysomy in the daughter cells. Whole genome duplications can arise by genome hybridization and polyploidy which may be followed by a period of chromosome loss [16]. Both of these scenarios could be tolerated by filamentous fungi and are likely factors in the evolution of gene families in these organisms.

The generation of duplicated gene networks may result in novel physiological or regulatory pathways, which may confer a selective advantage. It is hypothesized that *S. cerevisiae* may have utilized this process in the acquisiton of anaerobic growth [26]. Several large duplicated blocks with very similar gene arrangements have been found throughout the *S. cerevisiae* genome. It is thought that genome duplication occurred after the yeasts *Saccharomyces* and *Kluyveromyces* diverged and was followed by a period of degeneration, in which approximately 85% of the duplicated genes were lost and the remaining blocks were shuffled somewhat by reciprocal translocation events. Several pairs of duplicated genes are differentially regulated during aerobic and anaerobic growth, suggesting that genome duplication may have allowed *Saccharomyces* to adapt to anaerobic growth, a feature that is lacking in *Kluyveromyces*.

It is expected that the duplication of an entire genome would result in a large amount of redundant genetic material. In the absence of selection, genetic redundancies created by gene duplications are expected to be lost, either through acquisition of deleterious mutations leading to formation of pseudogenes, or through genetic divergence leading to genes with novel function. Such redundancies have been shown, however, to be able to persist in populations for a long time [27]. It is possible that genetic redundancies are maintained to increase the efficiency and fidelity of physiological pathways in the cell and to safeguard against any loss of information in such pathways [28]. This situation appears to have occurred in the N-glycan synthetic pathway. For example, many species, including filamentous fungi, carry multiple Class I α-mannosidases with overlapping functions in the N-glycan synthesis pathway [29,30]. Genetically redundant material produced by large scale duplications may thus be maintained in populations, increasing the fidelity and flow of information in the cell, and providing genetic material for the evolution of proteins with novel functions. The diversification of physiological and developmental path-

ways will be more likely to occur if all genes involved in the pathway are duplicated simultaneously [31]. Localized tandem duplications, however, occur more frequently and are readily tolerated. In fungi, functional gene clustering is quite common, which illustrates the potential role of localized tandem duplications in gene family evolution [32]. Both mechanisms, whole genome and tandem duplications, appear to have had a role in the diversification of gene families of fungi.

EVOLUTION OF THE α-MANNOSIDASE GENE FAMILY

Research on the gene families of the N-glycan processing pathway illustrates the similarities and differences of the glycosylation pathways in various lineages. The α-mannosidase gene family is a diverse groups of genes conserved throughout eukaryotic evolutionary history. Members of this family have been found in mammals, insects, filamentous fungi and yeasts. Duplication events which led to the diversification of this family from an ancestral gene appear to have occurred quite early in the eukaryotic evolutionary history. The α-mannosidases have a variety of cellular functions and localizations, and are involved in N-glycan processing in the endoplasmic reticulum and Golgi apparatus, as well as N-glycan degradation in the lysosome, vacuole, and cytoplasm [30,33]. The α-mannosidases can be classified into two independently derived lineages, termed Class I and Class II, based on protein sequence alignments [30,33,34]. There is significant correlation of biochemical and physiological roles of the various member genes for each of these two classes.

The Class I α-mannosidases are involved in the early stages of N-glycan processing in the ER and Golgi by catalyzing the removal of terminal α-1,2-linked mannose residues from N-glycan chains. Several genetic pathways seem to exist for the removal of the α-1,2-linked mannose residues from N-glycans and there is significant genetic redundancy in this gene family [33]. In most cases, there appear to be multiple paralagous genes which have evolved somewhat specialized, yet overlapping functions in each species.

Single members of the Class I α-mannosidase gene family have been identified in *Aspergillus satoi* [35], *Penicillium citrinum* [36], and *Trichoderma reesei* [37]. More recently, three different Class I α-mannosidase genes have recently been identified in *Aspergillus nidulans* [29]. We have also found two Class I α-mannosidases in another filamentous fungus, *Ophiostoma novoulmi* and it is likely that other filamentous fungi also contain multiple members of this gene family (Fig. 2). The presence of multiple Class I α-mannosidases in filamentous fungi is a reflection of the similarities which exist between the glycosylation pathways of these organisms with the pathways of higher eukaryotes. Mannose removal during N-glycan processing in filamentous fungi is achieved by multiple Class I α-mannosidases which may have partially overlapping or

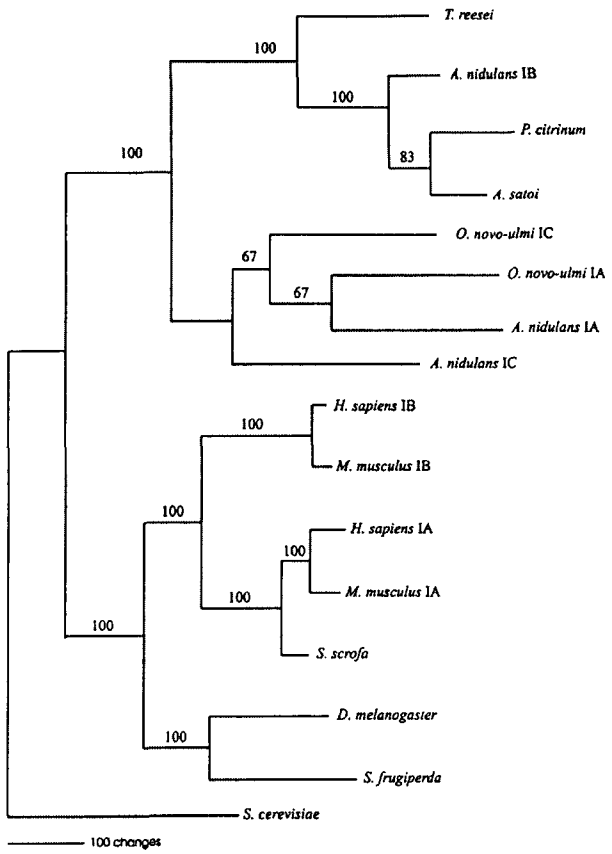

Fig. 2. Phylogenetic analysis of the Class I and Class II α-mannosidases. Multiple alignment of protein sequences was performed using the Multiple Alignment Program [48], and the most parsimonious tree was generated using PAUP 4.0b software [49]. Confidence values were generated by bootstrap analysis and are shown as percentage values above each node. Sequences used in the alignment: *Asperillus nidulans* IA, IB, IC [29], *Ophiostoma novo-ulmi* IA (Accession#: AF12945), *Ophiostoma novo-ulmi* IC (Eades and Hintz, *unpublished results*), *Penicillium citrinum* [36], *Aspergillus satoi* [35], *Homo sapiens* IA [50], *Homo sapiens* IB [51], *Mus musculus* IA [52], *Mus musculus* IB [53], *Sus scrofa* [54], *Drosophila melanogaster* [55], *Spodoptera frugiperda* [56] and *Saccharomyces cerevisiae* [57].

redundant functions, and disruption of specific members may have little physiological effect. Such redundancies are found in other lineages, as well. Roberts *et al.* [38] analyzed N-glycans produced by *mas-1* null mutants of *Drosophila melanogaster* and found that the N-glycan structures were not significantly changed, but that the production of these N-glycans was not as efficient as in the wild-type. A reduction in efficiency of protein glycosylation may reduce the overall fitness of the organism and may favor the maintenance of redundancy in this pathway.

Phylogenetic analyses of the Class I α-mannosidases have produced rather complicated results [29,30,34], but a pattern is beginning to emerge to explain the mo-

lecular evolution of this gene family (Fig. 2). The mouse and human species each contain two Class I α-mannosidases which are highly related and arose from a recent duplication event, which occurred after the divergence of this lineage from insect and fungal lineages. The multiple Class I α-mannosidases found in *A. nidulans* and *O. novo-ulmi* also appear to have arisen from recent duplication events, in that these proteins are more similar to each other than to either of the mammalian Class I α-mannosidases. This is significant, because it suggests that the Class I α-mannosidase gene families in both lineages have independently undergone expansion and evolution through gene duplication and divergence. Characterization of the biochemical properties of the various α-mannosidases in *A. nidulans* and *O. novo-ulmi* will provide further insight into the purpose of such duplication and divergence.

The second family of mannosidases, the Class II α-mannosidases are more diverse in their biochemical properties and physiological functions [33]. This group of enzymes consists of three subfamilies of genes (Classes IIA, IIB and IIC) with distinct cellular functions. The Class IIA subfamily is involved in N-glycan synthesis in the Golgi, while the Class IIB and Class IIC are involved in N-glycan breakdown, removal and recycling in the cytoplasm, lysosome and vacuole. The first subfamily of Class II genes (Class IIA) are responsible for removal of α-1,3- and α-1,6-linked mannose residues from N-glycans during their synthesis, a process which occurs in the higher eukaryotes, but does not occur in lower eukaryotes, such as fungi. The second subfamily (Class IIB) are also found only in higher eukaryotes. These enzymes are involved in N-glycan degradation in the lysosome. The third subfamily (Class IIC) contains a more heterogeneous set of enzymes, with a diversity of functions and cellular localizations. Members of this family are found in higher and lower eukaryotes and are likely involved in many aspects of N-glycan degradation and recycling. Sequence analysis clearly resolves the various inter-relationships of these proteins [34]. The Class IIC subfamily has very low sequence similarity to the other two subfamilies. Phylogenetic analysis of the sequences shows that the Class IIA and Class IIB subfamilies have diverged more recently than the Class IIC subfamily (Fig. 2). A likely scenario is that a single common ancestor was duplicated after the divergence of lower eukaryotes, such as fungi, from the higher eukaryotes. The lower eukaryotes thus only contain the orthologue of the common ancestor. Subsequent duplication in the higher eukaryotes led to the formation of the three subfamilies of Class II genes found in higher eukaryotes. These gene sequences diverged and evolved more specialized functions, such as the more complex N-glycan pathways (Class IIA), and more efficient degradation pathways (Class IIB).

It is intriguing that the two classes of α-mannosidases have such similar and overlapping functions. The Class I genes and the Class IIA genes have complementary functions in the N-glycans synthesis pathway of higher eukaryotes. The other Class II genes have broad substrate specificities and are able to cleave α-1,2 (as well as α-1,3, and α-1,6) mannose linkages, a property it shares with the Class I genes. The Class I and Class II genes show no sequence similarity and appear to have originated independently and represent a classic case of convergent evolution.

TOWARDS N-GLYCAN ENGINEERING

Remodelling of the glycosylation pathway by the transfer of specific pathway components may allow the development of expression systems which produce complex N-glycans typical of mammalian cells in an easily manipulated lower eukaryote. The first steps in this ambitious goal are being taken in two fungal expression systems, *Aspergillus nidulans* and *Trichoderma reesei*. The production of complex type N-glycans relies upon the addition of GlcNAc to $Man_5GlcNAc_2$ by the enzyme Gnt I (Fig. 1), the first committed step in the production of mammalian-type N-glycans. This enzyme is not found in filamentous fungi, hence Kalsner *et al.* [39] inserted the mammalian Gnt I gene into the genome of *A. nidulans*. Expression of the Gnt I alone did not result in the production of N-glycans containing an additional GlcNAc ($GlcNAcMan_5GlcNAc_2$). This is likely because the substrate $Man_5GlcNAc_2$ was in limiting amounts. There may thus be a 'bottleneck' preventing the production of significant amounts of $GlcNAcMan_5GlcNAc_2$. Efficient removal of mannose in the ER and Golgi could provide the necessary precursors for the production of complex N-glycans. The identification of three Class I α-mannosidase genes in *A. nidulans* may help resolve the process of mannose removal during N-glycan processing in filamentous fungi. Controlled overexpression of specific Class I α-mannosidases may clear the 'bottleneck' and permit production of complex N-glycans in *A. nidulans*.

Manipulation of the N-glycan processing pathway has progressed a little further in *T. reesei*. Maras *et al.* [13] were able to convert oligomannose glycans from cellobiohydrolase I (CBHI) to $GlcNAcMan_5GlcNAc_2$ by *in vitro* treatment with Gnt I. Only a small proportion of the N-glycans were converted, however, due to the fact that only a small fraction of the N-glycans provided a suitable substrate ($Man_5GlcNAc_2$) for Gnt I. Pretreatment with α-1,2-mannosidase significantly increased the yield of complex N-glycans, illustrating the need for efficient production of suitable substrate. Maras *et al.* [1] also reported the *in vivo* conversion of oligomannose N-glycans to complex N-glycans by heterologously expressed Gnt I, although the efficiency of conversion was low. Again, the conversion process may be blocked by a 'bottleneck' preventing production of significant amounts of substrate for Gnt I. These strains may need to be further manipulated to clear this bottleneck, either by overexpression of α-1,2-mannosidases to produce $Man_5GlcNAc_2$, or by elimination of mannosyltransferase activity which may be converting $Man_5GlcNAc_2$ (or intermediates) into glycans which are

unsuitable for conversion to complex N-glycans.

Characterization of N-glycan processing gene families has other potential biotechnological uses. The substrate specificity of the various glycosidases purified from filamentous fungi are currently being used to sequence glycans and to aid in monosaccharide composition determination. The identification of novel glycosidases with differing substrate specificities will increase the number of tools available for this type of research. Protein glycosylation is known to be important in many aspects of fungal growth and/or pathogenicity [40-44]. Many phytopathogenic fungi, for example, secrete cell wall degrading enzymes such as endopolygalacturonase and pectate lyase to aid in invasion of host plant tissues. Inhibition of N-glycosylation has been shown to reduce the secretion level and activity of these enzymes in certain fungal species [45-47]. The diversity that exist in the glycosylation machinery of fungi, plants, and animals may provide ideal targets for the development of anti-fungal targets and effect control of such pathogens.

CONCLUSION

Gene families are common in eukaryotes and have allowed for the evolution of increasing complexity in eukaryotes. Expansion and evolution of gene families through such processes as gene duplication, genetic drift, and positive selection have been very important in the development of complex pathways such as protein N-glycosylation. The study of gene families such as the α-mannosidases allows us to better understand the possible functional relationships that exist between the genetic components of these types of pathways [9]. Clarification of the inter-relationships and functions of these enzymes across various species will add to our understanding of the evolution of N-glycosylation. This will in turn enable the precise manipulation of the glycosylation machinery in fungal expression systems to allow production of glycoproteins carrying complex N-glycans.

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