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Research article

Inhibitors of protein glycosylation or secretion change the pattern of extracellular proteins in suspension-cultured cells of *Arabidopsis thaliana*

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Abstract

Cell walls are essential for plant development and morphogenesis. The majority of wall proteins are glycosylated, either as N- or O-glycans. Various inhibitors of glycosylation and secretion are used to determine the importance of wall proteins for the functioning of the walls. Tunicamycin is an inhibitor of the first enzyme in the *N*-glycosylation pathway, 3,4-dehydroproline inhibits peptidyl proline hydroxylation, and Brefeldin A is an inhibitor of vesicle trafficking, disrupting the delivery of wall polymers to the apoplast. In inhibitor-treated suspension-cultured *Arabidopsis thaliana* cells, qualitative and quantitative differences in the extracellular proteome were observed for both proteins secreted into medium or ionically-bound in the walls. Lack of *O*-glycosylation resulted in the selective protein loss from the extracellular compartments. Following tunicamycin treatment the secretion of additional proteins as well as ER-resident chaperones from the Hsp70 and Hsp90 families outside the protoplasts was noted. Moreover, changes in the proteolytic degradation pattern of culture filtrate proteins were also observed. Application of Brefeldin A resulted in transient and selective loss of individual proteins from the extracellular compartments of *A. thaliana* cell suspension. We conclude that post-translational modifications are vital for the proper functioning of wall proteins. *N*-glycosylation is crucial for their proper folding and stability. Extracellular compartments could also serve as a sink for improperly folded proteins during the unfolded protein response.

Keywords: Cell wall proteins; Glycosylation; Secretory pathway; Unfolded protein response; Arabidopsis; Proteomics

1. Introduction

Cell walls are essential to plant development and morphogenesis [57]. It is estimated that the genome of *Arabidopsis thaliana* contains more than 1500 genes coding for proteins implicated in cell wall biosynthesis, assembly and functioning [51]. However, the number of true extracellular proteins is still debatable [19]. Cell walls are characterized by a plethora of covalent and non-covalent, inter- and intramolecular interactions, and their destruction is usually required to analyze any wall component. No single method of extraction releases all wall proteins [43] thus indicating the existence of higher-order self-organized macromolecular networks. Despite this, modern approaches are beginning to unravel the true intricacy of the wall proteome [20]. In most cases, cell walls of plants and/or suspension-cultured cells grown under normal conditions were analysed [2,4,7,56], providing a catalogue of proteins without indication how important are individual proteins for the wall functioning. Notable exceptions were studies of apoplastic proteins involved in (1) the regeneration of cell walls by protoplasts [27], (2) the transition from primary to secondary walls [3] and (3) plant responses to pathogenic elicitors or abiotic stresses [8,38,58,59].

Glycosylation is the most common post-translational modification of secretory proteins. It is thought to be important for correct

Abbreviations: BFA, brefeldin A; DHP, 3,4-dehydroproline; ECM, extracellular matrix; ESI, electrospray ionization; LC, liquid chromatography; MALDI TOF, matrix-assisted laser desorption ionization time-of-flight; UPR, unfolded protein response.

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protein folding, conformational stability and biological activity of proteins, and for protection against proteolytic degradation [31,36]. Plant wall proteins can be both *N*- and *O*-glycosylated. Various substances, like tunicamycin and 3,4-dehydroproline, were used to modulate the glycosylation process. Tunicamycin is an inhibitor of UDP-*N*-acetyl-D-glucosamine:dolichol phosphate *N*-acetyl-D-glucosamine-1-phosphate transferase (GPT), the first enzyme in the *N*-glycosylation pathway which transfers the first *N*-acetyl-D-glucosamine onto dolichol phosphate carrier. In effect, tunicamycin prevents the synthesis of glycans for subsequent *N*-glycosylation [25]. However, as this affects also the protein folding, tunicamycin indirectly evokes a cellular reaction known as unfolded protein response (UPR; reviewed in [48,49]). UPR is used to maintain the proper physiological state of ER, especially with respect to its folding and processing capacities. Perturbations in either of these lead to the accumulation of unfolded proteins and protein aggregates in ER and this modulates the expression of various sets of genes and activates ER-associated protein degradation (ERAD) [52]. As a consequence, UPR results in the remodelling of the secretory pathway to minimize the amount and/or lifetime of unfolded proteins in the ER [45].

The still fragmentary knowledge on UPR in plants has been recently reviewed [54]. It seems, however, that in its major aspects, plant UPR is similar to that of mammals and yeast. For example, sensory proteins, such as IRE1 [26], activation of transcription factors from the bZIP family by specific ER-located S1P protease [32,33] or involvement of plant derlins in ERAD processes [23], have already been demonstrated. For the purposes of this paper, we would like to point out that the treatment of plant cells or tissues with tunicamycin induces both up- and down-regulation of gene expression [22,35], and results in, e.g. accumulation of transcripts for ER chaperones, such as luminal binding protein (BiP) [12]. Functional studies showed that overexpression of either BiP [30] or GPT [25] greatly reduces UPR and makes plants resistant to tunicamycin. Interestingly, application of tunicamycin also induces cell death with apoptotic features [11,34], suggesting a link between UPR and programmed plant cell death [18].

3,4-Dehydroproline (DHP) acts at micromolar concentrations to rapidly and irreversibly inhibit peptidyl proline hydroxylation. DHP, as an analogue of proline, is incorporated into the polypeptide chain and blocks the attachment of carbohydrate residues. DHP treatment caused the disappearance of the major proline-rich proteins from the wall of cultured soybean cells [47]. DHP-treated onion root cells showed a 56% decrease in hydroxyproline content [13]. DHP treatment also caused the synthesis and secretion of structurally abnormal proteins, and this indicated that glycoproteins are necessary for cell survival and proliferation [9,10].

The secretion of proteins into the walls occurs *via* the endomembrane system. Perturbing the functioning of this system can provide information on its functioning, and, at the same time, on the role of secreted macromolecules, in the extracellular milieu. Brefeldin A (BFA), a fungal macrocyclic lactone, is a reversible inhibitor of the secretory pathway [14], acting through inhibition of specific guanine nucleotide exchange factors thus affecting vesicle formation [42]. The indirect effects, such as, e.g. BFA action on polar auxin transport [16] or secondary cell wall synthesis [44], have also been shown.

In order to understand the roles of various wall (glyco)proteins we have undertaken a proteomic analysis of changes in wall proteome evoked by various modulators of protein glycosylation and/or secretion. Suspension-cultured cells were treated with tunicamycin, DHP or BFA, and proteins were analysed following electrophoretic separation. Wall (glyco)proteins up- or down-regulated in response to various treatments were identified using mass spectrometry approaches.

2. Results

2.1. Effect of DHP on patterns of extracellular proteins in A. thaliana cell culture

Cells of *A. thaliana* suspension were cultured for 7 days in the presence of an inhibitor of *O*-glycosylation, 3,4-dehydro-D,L-proline (DHP D,L) or its inactive analogue, *cis*-4-hydroxy-L-proline (DHP L), at four different end concentrations. Electrophoretic analysis of proteins released into the medium or bound in the walls revealed that the DHP treatment did not drastically affect the pattern of proteins, although both quantitative and qualitative changes were observed (Fig. 1). Unexpectedly, slight changes were also visible in the cultures treated with DHP L. The protein bands marked on Fig. 1 are those which changed the most upon DHP treatment. Following DHP D,L treatment the disappearance of some wall protein bands could be noted, while cells grown in the presence of DHP L seemed to secrete even more proteins into the walls.

2.2. Patterns of extracellular proteins in Arabidopsis cells treated with tunicamycin

Four-day-old suspension cultures of *A. thaliana* were treated with tunicamycin at a final concentration of 20 μg ml^{-1}. After 24 h and 48 h of inhibitor treatment, ionically-bound wall proteins and proteins released into culture medium were isolated. Electrophoretic separation enabled comparison of the protein patterns. Both qualitative and quantitative differences were detected (Fig. 2). Protein bands with the most pronounced changes in their intensities were cut out and subjected to LC-MS/MS analysis. The results are summarised in Table 1. Two important observations should be noted. Some of the proteins, like subtilisin-like protease, putative FAD-linked oxidoreductase and putative receptor-like kinase were secreted into medium throughout the experiment. They were, however, identified in the protein bands of various molecular masses depending on the time of sampling. As tunicamycin blocks *N*-glycosylation and proteases are also secreted into the culture medium, it was not possible to determine precisely if these changes are due to underglycosylation, susceptibility to proteolytic cleavage or both. Interestingly, following tunicamycin treatment, proteins not normally found in the extracellular compartments, such as elongation factor 2, and molecular chaperones, like BiP and SHEPHERD