Hippophae rhamnoides N-glycoproteome analysis: a small step towards sea buckthorn proteome mining

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Abstract *Hippophae rhamnoides* is a hardy shrub capable of growing under extreme environmental conditions namely, high salt, drought and cold. Its ability to grow under extreme conditions and its wide application in pharmaceutical and nutraceutical industry calls for its in-depth analysis. N-glycoproteome mining by con A affinity chromatography from seedling was attempted. The glycoproteome was resolved on first and second dimension gel electrophoresis. A total of 48 spots were detected and 10 non-redundant proteins were identified by MALDI-TOF/TOF. *Arabidopsis thaliana* protein disulfide isomerase-like 1-4 (ATPDIL1-4) electron transporter, protein disulphide isomerase, calreticulin 1 (CRT1), glycosyl hydrolase family 38 (GH 38) protein, phantastica, maturase k, *Arabidopsis throrax* related protein 6 (ATXR 6), cysteine protease inhibitor were identified out of which ATXR 6, phantastica and putative ATPDIL1-4 electron transporter are novel glycoproteins. Calcium binding protein CRT1 was validated for its calcium binding by stains all staining. GO analysis showed involvement of GH 38 and ATXR 6 in glycan and lysine degradation pathways. This is to our knowledge the first report of glycoproteome analysis for any Elaeagnaceae member.

Keywords N-glycoproteins · Affinity chromatography · Stains all · Secretory

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Introduction

Sea buckthorn, (*Hippophae* sp.) is a winter hardy deciduous shrub native to Asia and Europe. It is also known as seaberry and sandthorn. *Hippophae rhamnoides* belongs to the family Elaeagnaceae with six species and twelve subspecies, majority of which are found in Asia (Bal et al. 2011). Sea buckthorn is known for its ability to grow in cold, drought and saline conditions. It can survive at $-43\text{ }^{\circ}\text{C}$ – $40\text{ }^{\circ}\text{C}$. It has an extensive and strong root system and forms symbiotic association with nitrogen fixing, nodule forming actinobacteria, *Frankia* (Suryakumar and Gupta 2011). Seed, leaf and berry of sea buckthorn have high antioxidant content that provides high immunomodulatory, anti-bacterial and anti-aging property. It has been used in treatment of gastric ulcers, skin disorders, cardio-vascular disease, wound healing, protection from cold-hypoxia stress, radiation damage, anti-tumor, anti-carcinogenic, memory and stress disorders. Due to its association with nodule forming bacteria it has been used in reclamation of land (Basu et al. 2007; Suryakumar and Gupta 2011). Most of the studies on sea buckthorn have focused on exploring the pharmaceutical properties and its environmental benefits. Unfortunately, the molecular mechanism of its stress tolerance, nutraceutical and medicinal properties are poorly understood.

Therefore, in the last 4–5 years efforts to understand stress tolerance mechanism have been undertaken. Differential expression of genes in seedlings under cold ($4\text{ }^{\circ}\text{C}$) and freeze ($-10\text{ }^{\circ}\text{C}$) stress showed significantly enhanced expression of genes encoding enzymes involved in biosynthesis and metabolism of compounds regulating cold and freeze tolerance (Chaudhary and Sharma 2015). Ghargal et al. (2012) observed increase in expression of tonoplast monosaccharide transporter (TMT), cation exchanger (CAX) and aquaporin genes responsible for

regulation of water and ion uptake. Chitinase and pathogenesis related thaumatin (PRT) protein showed increase in expression by 8 and 12fold respectively under freeze stress. A number of stress related genes were identified from mature seeds of sea buckthorn which are important in seed development and maturation (Fatima et al. 2012).

Very few reports on proteome analysis of sea buckthorn are available. Xu et al. (2008) reported drought stress responsive proteome in the seedling and leaf of sea buckthorn. A total of 55 proteins showed change in abundance in drought. Some novel proteins NitrX, J-type co-chaperone Hsc20, HslU, and NikE-OppD ABC transporter were identified. Secretome analysis from seedlings showed 61 low temperature responsive targets. Polygalacturonase Inhibitor Protein (PGIP) an antifreeze protein was isolated from the apoplast (Gupta and Deswal 2012). This antifreeze protein has the ability to inhibit growth of ice crystal at a temperature below freezing point and presence of these proteins allows the survival of seedlings at -5°C .

Contributing to the effort to understand the tolerance mechanism, N-glycoproteome of sea buckthorn seedling was analyzed for the very first time. Infact this is the first report of glycoproteome analysis in any Elaeagnaceae member. Glycoproteins have glycan (sugar) molecules attached to specific amino acid residues. These are important for localization, enhancing stability and solubility of proteins. These are involved in cell signaling, signal recognition and intermolecular interactions of proteins (Thannhauser et al. 2013). There are very few reports on glycoproteome analysis in plants. Glycoproteome has been analyzed from *Arabidopsis* (Lastovickova et al. 2011; Zhang et al. 2011; Minic et al. 2007), barley grain (Lastovickova et al. 2011), tomato (Catala et al. 2011; Thannhauser et al. 2013; Ruiz-May et al. 2013), maize (Silva-Sanchez et al. 2014), tobacco (Hrubá and Tupy 1999), rice (Komatsu et al. 2009), *Brachypodium* (Zhang et al. 2014) and cotton (Kumar et al. 2013). As glycosylation plays an important role in various biological processes including growth and development (Palmisano et al. 2010). This study could provide a glimpse of the functionally significant proteins at seedling stage. Being a hardy plant this would be an important system that could provide valuable information on its ability to survive in severe stress conditions which might help in developing stress tolerant plants. This study is the first report on the N-glycoproteome analysis of sea buckthorn seedlings.

Methods

Plant material and growth conditions

Seeds of sea buckthorn (*Hippophae rhamnoides* subsp. *turkestanica*) were grown according to the procedure

followed by Gupta and Deswal (2012) where seeds were soaked in teepol for 5 min and then washed with tap water followed by sterilization with 70 % ethanol and washing with double distilled water. The seeds were soaked for 5 days in double distilled water followed by incubation in dark for one day and plated on pre-soaked germination paper rolls and kept under white light ($270\ \mu\text{mol}/\text{m}^2/\text{s}$, 16 h light/8 h dark) at $24 \pm 2^{\circ}\text{C}$ and were watered regularly. Seedlings were harvested after 20 days following Gupta and Deswal 2012.

Protein extraction

Seedlings were ground using liquid nitrogen in pre-chilled mortar and pestle and homogenized in the extraction buffer (50 mM Tris-Cl pH 7.4, 30 % glycerol and 2 % PVPP) in 1: 2 (wt/vol) ratio. The homogenate was centrifuged at 12,500 rpm for 25 min at 4°C . The clear supernatant was used for purification of glycoproteins. Protein concentration was determined using bovine serum albumin (BSA) as standard protein (Bradford 1976).

Lectin affinity chromatography for N-glycoprotein purification

Affinity purification of N-glycoproteins was performed using Con A agarose (0.5 ml) (Sigma–Aldrich). Column was washed with 10 column volume (cv) of wash buffer (1 M NaCl, 5 mM MgCl_2 , 5 mM MnCl_2 , and 5 mM CaCl_2) followed by equilibration with 10 cv of the equilibration buffer (20 mM Tris-Cl pH 7.4, 0.5 M NaCl, 1 mM MgCl_2 , 1 mM MnCl_2 and 1 mM CaCl_2). Protein sample (10 mg) was incubated for 1 h at 4°C . Column was washed with equilibration buffer to remove the unbound proteins. Elution was done with the equilibration buffer containing 0.5 M methyl- α -D-mannopyranoside. The purified protein was concentrated using 10 kDa cutoff centrifugal concentrator (Amicon Ultra-15, Milipore) buffer was exchanged to 20 mM Tris-Cl pH 7.4 followed by methanol/chloroform precipitation.

Resolution of proteins on SDS–PAGE and 2D-GE

One dimensional SDS–PAGE was performed following Laemmli 1970. The crude (20 μg) and purified (10 μg) proteins were resolved by 12 % SDS–PAGE. For second-dimensional gel, 400 μg protein was dissolved in 100 μl lysis buffer (8 M urea, 2 M thiourea, 2 % CHAPS, 2 % Triton-X 100, 2 % ampholyte and 50 mM DTT) mixed with the rehydration buffer (8 M urea, 0.25 % CHAPS, 0.001 mg BPB, 0.28 % DTT and 0.005 % ampholyte). The protein was rehydrated on a 13 cm IPG strip (pH 3–10, non-linear GE Healthcare) overnight at 20°C . Isoelectric

focusing (IEF) was performed using Protean IEF Cell (Biorad) at 500 V-2:30 h, 1000 V-1:30 h, 8000 V-2 h and 8000 V-1 h. The IPG strips were incubated in the equilibration buffer I (1.5 mM Tris, pH 8.8, 50 mM Urea, 6 M Glycerol, 30 % SDS, 2 % BPB, 0.002 %) containing 1 % (w/v) DTT followed by incubation with equilibration buffer II containing 2.5 % (w/v) iodoacetamide. The strip was placed horizontally on a 12 % polyacrylamide gel and the second dimensional gel was performed using Hoefer SE 600 Ruby (GE Healthcare). Separation was carried out at 4 mA for 30 min, 10 mA for 30 min and 19 mA. The experiment was performed with three biological replicates.

Gel staining and image analysis

After SDS–PAGE the gels were stained with colloidal CBB stain (Neuhoff et al. 1988). Gels were washed in double distilled water and stained overnight in CBB G-250 (0.08 % CBB G-250, 1.6 % o-phosphoric acid, 8 % ammonium sulfate and 20 % methanol) at RT on rocker. Gels were de-stained with double distilled water till the background became clear. Gel-image analysis was performed using ImageMaster2D Platinum software (ver 6.0; GE Healthcare, Sweden). Spot detection was performed using a saliency-2, minimum area-4, and smoothness-10 and spots with significant intensity were counted. Molecular weight and pI was calculated on the basis of the molecular marker and the arrangement of pH on the IPG strip.

Western blotting and con A affino-blotting

The crude and purified protein was separated on 12 % SDS–PAGE and was electroblotted onto a nitrocellulose membrane according to Towbin et al. (1979) using transfer buffer (39 mM glycine, 48 mM Tris and 20 % (w/v) methanol). The proteins were blocked with 3 % bovine serum albumin (BSA) in phosphate-buffered saline (PBS), overnight at 4 °C. The membrane was incubated in con A solution (con A (10 µg/µl), 50 mM MnCl₂, 100 mM CaCl₂, 100 mM MgCl₂ and 0.5 % Triton X in PBST) for 6 h at RT. The membrane was washed for 30 min in PBST (PBS with Triton-X 100) and incubated with a solution of HRPO (40 µg/µl) for 1 h. After washing the blot with 1X PBS for 5 min the blot was incubated in substrate of 4-chloro-1-naphthol (3.4 mM) in 50 mM Tris-Cl (pH 7.5) in the presence of H₂O₂ (0.3 %) in dark. The reaction was stopped with double distilled water.

Stains-all staining

Crude and purified samples were separated on one dimensional SDS–PAGE. The gel was fixed overnight in

10 % glacial acetic acid and 25 % isopropanol followed by washing with 25 % isopropanol four times with 30 min each. The gel was incubated in the staining solution (45 mM Tris-Cl pH 9.2, 25 % isopropanol, 7.5 % formamide and 0.01 % Stains-all) for 48 h with constant shaking at room temperature (Myers et al. 1996). The background stain was removed by washing the gel in 25 % isopropanol with gentle shaking in dark. The gel was scanned and destained completely by keeping the gel in white light. Position of the polypeptide was determined by staining the gel with colloidal CBB stain.

In-gel trypsin digestion

Spots were excised and de-stained using 40 mM ammonium bicarbonate and 40 % acetonitrile (de-staining solution). Reduction was carried out with 5 mM DTT in 40 mM ammonium bicarbonate at 60 °C for 20 min followed by alkylation using 20 mM iodoacetamide in 40 mM ammonium bicarbonate for 15 min in dark at room temperature. The gel pieces were washed with de-staining solution and dehydrated using 100 % acetonitrile for 15 min. Trypsin digestion was carried out using 50 µl trypsin (10 ng/µl, Promega) in 25 mM ammonium bicarbonate overnight at 37 °C. The peptides were extracted using 80 % acetonitrile and 0.5 % acetic acid and lyophilized.

Protein identification and bioinformatic analysis

The trypsin digested samples were identified by MALDI–TOF–TOF using AB SCIEX TOF/TOFTM 5800. The samples were mixed with equal volume of MALDI matrix (5 mg/ml CHCA in 50 % ACN and 0.1 % TFA) and subjected to MALDI analysis. Database search was performed using MASCOT (version 2.5, Matrix Science). The search parameters were: NCBI nr sequence database, taxonomy-Viridiplantae, sequence query search, trypsin digestion with one missed cleavage, fixed carbamidomethyl modification, oxidation and deamidated variable modification, monoisotopic mass value, unrestricted protein mass, peptide mass tolerance of 150 ppm and fragment mass tolerance of 0.3 Da. MASCOT protein ion with $p < 0.05$ was considered significant. UniProt database was used to search for the function and localization of the identified proteins. SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) was used to predict the presence of N-terminal SPs for targeting to the secretory pathway and NetNGlyc (<http://www.cbs.dtu.dk/services/NetNGlyc/>) was used to predict N-glycosylation consensus sequences. Sub-cellular localization was predicted using Plant-mPLOC tool (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>; Chou and Shen 2010) and N-glycosylation sites were predicted using

N-GLYCOSITE tool (<http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html>; Zhang et al. 2004). GO (Gene Ontology) annotation was performed using BLAST2GO. Presence of transmembrane helix was predicted by TMHMM tool (www.cbs.dtu.dk/services/TMHMM/). Protein interaction analysis was performed by STRING (STRING version 10.0).

Result and discussion

Work flow

Glycoproteins are important for cellular localization, protein stability, intermolecular interactions and development of complex multicellular organisms. These are also involved in protein secretory pathway via endoplasmic reticulum, Golgi apparatus, vacuole, plasma membrane and extracellular compartment (Zhang et al. 2014). Analyzing the glycoproteome might help us to know which proteins are involved in regulating growth during early developmental stage. Therefore, the main objective of the study was to purify the seedling N-glycoproteome. The analytical workflow (Fig. 1) shows extraction of proteins followed by purification of N-glycoproteome by con A affinity chromatography. The N-glycoproteome was confirmed by affinity blotting using con A-HRPO. The purified N-glycoproteome was resolved on a 2-D gel and 10 most abundant spots with varied pI and molecular weight were in-gel digested using trypsin followed by analysis of the peptides by MALDI TOF/TOF. *In silico* analysis for the prediction of N-glycosylation sites, localization and functions of the identified proteins was performed.

Resolution of crude and the con A elute on the denaturing gel showed difference in protein profile. Compared with crude very few polypeptides were observed in the con A elute. Prominent polypeptide at 30 kDa and 14 polypeptides from 45 to 97 kDa with very faint polypeptides at 17, 20, 40 and 43 kDa were observed in the con A elute. The sizes of the glycopeptides were comparable to that of the crude profile showing that glycopeptides of sea buckthorn occupy a wide range of size. To further confirm the N-glycoproteins con A affinity blotting was performed.

Confirmation of purified proteins by con A affinity blotting

Purification of N-glycosylated proteins was achieved by con A affinity chromatography which was further confirmed by con A affinity blotting. Con A is bivalent and binds specifically to mannose and glucose residues on one side and also to horseradish peroxidase (which is a mannose containing glycoprotein) on the other side. This property

Fig. 1 Experimental workflow for the analysis of N-glycoproteome from seabuckthorn. N-glycoproteome purified from 20 day old seedling by con A affinity chromatography resolved on SDS-PAGE and 2-D gel. Con A affinity blotting confirm the purification of N-glycoproteome. Spots were in-gel digested and analyzed by MALDI-TOF/TOF

has been used to detect the sugar moieties on the protein. When the substrate of HRPO was provided in the presence of H_2O_2 , a dark purple product is formed.

Even with higher crude protein (20 μ g) in the gel the intensity of positive signal were weaker in comparison with the eluted protein (10 μ g) confirming the selective enrichment of N-glycoproteins by con A affinity chromatography (Fig. 2). Signals observed in crude were almost similar with that of elute excepting a polypeptide at ~50 kDa observed in elute were absent in the crude. Affinoblot blot showed positive signals with almost all the polypeptides from 28 kDa and above while no signals were observed below 28 kDa. The absence of signals on blot may be due to the presence of non-glycosylated protein or co-purification of other protein during affinity chromatography. The glycosylated polypeptides showing positive signal on con A affinity blot ranged from 28 to >97 kDa as

Fig. 2 Analysis of N-glycoproteome by electrophoresis and affinity blotting. Crude (20 μ g), con A elute (10 μ g) and BSA (5 μ g) were resolved on 12 % gel **a** stained with colloidal CBB. **b** Con A affinity blot of the same sample using con A-HRPO, BSA (66 kDa) used as negative control and ovalbumin (45 kDa) in marker as positive control. Polypeptides marked with (arrow) shows positive signals on the con A affinity blot

observed for barley (Lastovickova et al. 2011), maize (Silva-Sanchez et al. 2014) and *Arabidopsis* (Zhang et al. 2011). Although very faint polypeptides were observed at 28 and 32 kDa however strong signals were observed on the blot which suggests that these polypeptides were probably heavily glycosylated. RuBisCO, the most abundant protein does not show any signal neither on the gel nor on the affinity blot showing the specificity of the affinity procedure. Only ovalbumin, which is an N-glycosylated protein showed positive signal among the markers confirming the reliability of the affinity blot.

Mass spectrometric (MS) identification of N-glycoproteins resolved on 2-D PAGE

Two-dimensional gel of the N-glycoproteome showed 48 spots. As glycoproteins have bulky glycan molecules protruding on the protein surface which are weakly stained by CBB. The purified protein spots were spread over a pI range of 4–8 and molecular weight of 97–10 kDa (Fig. 4). Out of the 48 spots trypsin digestion was performed for spot 1, 10, 11, 43, 46 and 47 and also one spot each from the trail of three, four, seven and four spots at 38, 31, 30 and 28 kDa (spot 26, 28, 36 and 39) as these spots may be isoforms of the same protein. The spots were picked taking into consideration that these are of different molecular weight, pI and abundance.

These proteins were identified by MALDI-TOF/TOF as putative ATPDIL1-4 (*Arabidopsis thaliana* protein disulfide isomerase-like 1-4) electron transporter (spot 1), protein disulfide isomerase (PDI) (spot 10), calreticulin 1 (CRT1) (spot 11), glycosyl hydrolase family 38 protein (GH 38) (spot 26), phantastica (spot 28), maturase k (spot 36), ATXR6 (*Arabidopsis thaliana* related protein 6) (spot 39), cysteine protease inhibitor (spot 43), con A precursor (spot 46) and an unnamed protein product (spot 47) (Table 1). Out of these four proteins, GH 38 family protein (Catala et al. 2011), PDI (Catala et al. 2011), CRT1 (Komatsu et al. 2009) and con A precursor (Sheldon and Bowles 1992) are reported to be glycosylated earlier. The identified proteins belong to vital physiological processes like protein targeting, redox homeostasis, organ identity, glycan metabolism and stress.

Protein involved in endoplasmic reticulum quality control (ERQC) and redox homeostasis

Putative ATPDIL1-4 electron transporter (spot 1) is a type of protein disulfide isomerase transporter (Onda 2013). It may be involved in redox homeostasis. Protein disulfide isomerase (PDI), (spot 10) a multifunctional enzyme belongs to the superfamily of protein-thiol oxidoreductases and is localized mainly in the lumen of endoplasmic reticulum with a transmembrane domain at its N-terminus. It contains thioredoxin (TRX) domain and acts as catalyst of disulfide bond formation in the oxidizing environment of endoplasmic reticulum stabilizing the tertiary and quaternary structure of protein (Gupta and Tuteja 2011). The presence of N-glycosylated form is reported in ripe tomato fruit by Catala et al. (2011). PDI is involved in oxidation or reduction of disulfide bridges, cell adhesion, have chaperone and anti-chaperone activity and also act as a primary acceptor of peptides translocated into the ER (Noiva 1999; Onda 2013). It is also involved in signal transduction pathway and associates with transcriptional complexes regulating gene expression under various stimuli (Ray et al. 2003). Upregulation of PDI is observed under ER stress (Gupta and Tuteja 2011).

Calreticulin (spot 11), a multifunctional, ubiquitous, calcium binding protein localized mainly in the endoplasmic reticulum (Opas et al. 1996) is involved in various intra- and extracellular processes (Lenartowska et al. 2009). It has been reported to be present in other cellular compartments like Golgi complex, nucleus, nuclear membrane, cytoplasm, vesicles and spindle apparatus of the dividing cells, plasma membrane and plasmodesmata (Bush 1995; Denecke et al. 1995; Nelson et al. 1997). It was reported to be present abundantly in most of the plant parts such as roots, young leaves and different flowering organs and also in germinating seeds (Borisjuk et al. 1998).

Table 1 N-glycosylated proteins identified from *Hippophae rhamnoides* seedlings

Sl. No.	Accession number	Protein name	Spot number	Mowse score (M. S.)	Mass/pI		Sequence coverage (%)	N-glycosylation motif	N-glycosylation site	Signal peptide	Localization	Function
					Theoretical	Observed						
1	gil148807199	Putative ATPDIL1-4 electron transporter [Prunus dulcis]	1	222	27825/4.96	91000/4.39	22	Yes	1 (61)	No	Nucleus and cytoplasm	Cell redox homeostasis
2	gil11133775	Protein disulfide-isomerase	10	177	55811/4.95	62000/4.64	14	Yes	1 (41)	Yes	Endoplasmic reticulum	Oxidation/reduction of disulfide bridges, cell signaling, cell adhesion, chaperone, anti-chaperone
3	gil79320099	CRT1 (CALRETICULIN 1) [Arabidopsis thaliana]	11	90	49283/5.58	58000/4.02	15	Yes	3 (59, 154, 387)	Yes	Endoplasmic reticulum	Regulation of Ca ²⁺ homeostasis, chaperone, signaling, pathogenesis
4	gil15231611	Glycosyl hydrolase family 38 protein [Arabidopsis thaliana]	26	75	115547/6.27	38000/5.38	9	Yes	7 (64, 278, 336, 470, 638, 730, 420)	Yes	Cell wall, Chloroplast, cytoplasm, extracellular, nucleus and vacuole	Synthesis of N-glycoproteins
5	gil5230656	Phantastica [Solanum lycopersicum]	28	64	41399/9.25	35000/3.98	29	Yes	1 (175)	No	Nucleus	Determine the identity of dorsal leaf initial cells, lateral organs, dorsiventrality of bracts and petal lobes
6	gil22128197	Maturase K [Baeckea tetragona]	36	48	60896/9.54	31000/6.01	28	Yes	5 (38, 50, 225, 338, 409)	No	Chloroplast	Post-transcriptional processing, cell cycle regulation
7	gil15237940	ATXR6; DNA binding / protein binding [Arabidopsis thaliana]	39	64	40299/8.81	30000/5.06	19	Yes	1 (294)	No	Nucleus	Monomethylation of H3K27
8	gil290889339	Cysteine protease inhibitor [Polygonum sibiricum]	43	71	12265/5.08	26000/7.9	23	No	0	No	Chloroplast, cytoplasm and nucleus	Inhibit Cysteine protease, involve in abiotic stress tolerance and resistance to biotic stress
9	gil17979	ConA precursor [Canavalia ensiformis]	46	135	31502/5.85	17000/7.44	10	Yes	2 (73, 152)	Yes	Cytoplasm	Modified to form con A
10	gil215767432	Unnamed protein product [Oryza sativa Japonica Group]	47	42	21098/4.93	11000/5.36	51	Yes	1 (53)	No	Chloroplast	Unknown

Calreticulin performs two very important functions, chaperoning and regulation of Ca^{2+} homeostasis (Opas et al. 1996; Michalak et al. 1999). It is involved in various other functions such as Ca^{2+} dependent signaling pathways, control of cell adhesion, immune system and apoptosis, wound healing, pathogenesis and also in gene expression (Crofts et al. 1998). Calreticulin along with calnexin promotes proper folding, oligomeric assembly and quality control of newly synthesized glycoproteins (Jia et al. 2009; Michalak et al. 1999). It was found to be developmentally regulated in the rice suspension culture cells and also acts as molecular chaperone under stress conditions. Cold stress affects calreticulin expression and distribution at a very early stage (Li et al. 2003).

Interaction of ATPDIL1-4, PDI and CRT proteins with each other was showed by STRING database (Supplementary Fig. 1). These proteins form an important component of the Endoplasmic Reticulum quality control (ERQC) system which is responsible for proper protein folding, assembly and transport. These proteins were identified from spots 1, 10 and 11 which were the most abundant spots (Fig. 4). Abundance of these proteins during the early stage of the plant might be important in the actively dividing and differentiating cells and also in maintaining environmental homeostasis during the early vegetative growth of the plant. Apart of its interaction these proteins also interacts with proteins involved in glutamate metabolism, other chaperone proteins, stress responsive, other oxidoreductases and proteins involved in glycan metabolism (Supplementary Fig. 2, 3 and 4).

Protein involved in nucleotide modification and metabolism

Maturase K (spot 36) is involved in post-transcriptional processing and is found to be localized in the chloroplast. It catalyzes the binding of the intron RNA during reverse transcription and splicing (Zorb et al. 2010). It was found in the elute fraction of *Nicotiana tabacum* lectin which binds to high mannose and complex type N-glycans (Schouppe et al. 2011). It interacts with various proteins which are involved in the light reaction of photosynthesis. Together with these proteins mat K might be regulating transfer of electrons and proper organization of the photosynthetic pathway to maintain the basic photosynthetic requirement for growth. Mat K interacts with proteins involved in degradation of misfolded proteins and photosynthetic complex I associated proteins (Supplementary Fig. 5). Interaction of these proteins might be regulating the quality control of proteins and photosynthetic machinery of the actively growing seedling.

ATXR (Arabidopsis trithorax related protein) 6 (spot 39), belongs to a class IV H3K27 (Histone 3, lysine 27)

methyltransferases that carries out monomethylation of H3K27 at the heterochromatic chromocenters along with ATXR5. ATXR5 and ATXR6 are the only enzymes that bring about methylation at H3K27 in Arabidopsis. These two enzymes always act together and have an important role in chromatin condensation, gene silencing, genome stability and cell cycle regulation in *Arabidopsis* (Jacob et al. 2009; Pontvianne et al. 2010; Stroud et al. 2012). It interacts with various other methyl transferases involved in DNA replication, DNA repair and cell cycle regulation. These proteins are involved in important biological processes such as flowering, transition from vegetative to reproductive phase and DNA repair. Pathway mapping by KEGG showed it to be involved in catabolism of lysine which might be important for maintaining lysine homeostasis by converting lysine to other metabolites in response to stress or developmental cues (Galili 2002). Proteins involved in DNA repair, cell cycle regulation, replication, other histone methyltransferases and variant of histone 3 showed interaction with ATXR6 (Supplementary Fig. 6).

Protein involved in organ identity determination

Phantastica (PHAN) (spot 28) is a MYB-related transcription factor required for the identity of dorsal leaf initial cells, dorsoventrality of bracts and petal lobes, determination of lateral organ identity (Waites and Hudson 1995). PHAN has an important role in stabilizing and maintaining the leaf adaxial-abaxial polarity, proximodistal axis development in simple and compound leaves and also leaf margin development (Zoulias et al. 2012; Kim et al. 2003; Ge et al. 2014). It was also found to be responsible for drought tolerance in tobacco through an ABA dependent mechanism (Huang et al. 2013). Proteins interacting with phantastica involved in histone modification and transcriptional regulation and together they might be influencing cell differentiation and cell fate determination (Supplementary, Fig. 7).

Protein involved in glycan metabolism

Glycosyl hydrolase family 38 protein (spot 26) which is a class II α -D-mannosidase involved in the synthesis of glycoproteins has been reported in the N-glycoproteome of xylem sap in *B. oleracea* (Ligat et al. 2011), tomato fruit (Catala et al. 2011) and mature stem of *Arabidopsis* (Minic et al. 2007). These are localized in the Golgi apparatus, cytoplasm and vacuole (Kumar et al. 2013). KEGG pathway mapping of GH 38 family protein showed its involvement in glycan degradation pathway. GH 38 family protein hydrolyzes removal of mannose residues for further modification of the glycan residues or for quality control of

Fig. 3 Analysis of Ca^{2+} binding protein in N-glycoproteome using stains all staining. Crude (20 μg) and con A elute (10 μg) resolved on 12 % SDS–PAGE stained with **a** colloidal CBB and **b** stains all. Polypeptides with (asterisk) showed blue stain with stains all

the glycosylated proteins in the ER. Degradation of glycans may either lead to misfolding of protein or further glycan modification and targeting to other components of the secretory pathway (Ma et al. 2016).

Protein involved in abiotic and biotic stress tolerance

Cysteine protease inhibitor (spot 43) also known as plant cystatins or phytocystatins have been identified and characterized in several plants. These are small proteins usually present in high amount in the storage tissues and control the accumulation and degradation of storage proteins (Abe et al. 1992; Koiwa et al. 1997). They have antifungal as well as insecticidal activities which are induced during biotic stress such as herbivory (Haq et al. 2004). A stable complex with their target proteases is formed thereby blocking, altering or preventing access of the substrate to the enzymes active site (Leo et al. 2002). They are also involved in developmental cues, as modulators of programmed cell death and as storage protein. They have an important role in regulating protein turnover during seed development and germination. High salt, drought and cold stress increase its expression. Over-expression of cysteine protease inhibitors prevents cell death, improve tolerance to abiotic (salt, drought and oxidative) stress and resistance to biotic stress during seed germination and early seedling development (Zhang et al. 2008).

Fig. 4 Analysis of N-glycoproteome of sea buckthorn by 2D-electrophoresis. N-glycoproteome (400 μg) purified by con A affinity chromatography was resolved on pH 3–10, non-linear IPG strip for first dimension. The proteins were resolved on a 12 % gel for second dimension and stained with colloidal CBB stain. Total of 48 spots marked with arrow were resolved on the gel. Spots in boxes were in-gel digested using trypsin and analysed by MALDI–TOF/TOF

Concanavalin A precursor (spot 46), a precursor of non-enzymatic carbohydrate binding protein (lectin) present in the endoplasmic reticulum (Herman et al. 1985) is a high-mannose oligosaccharide which is processed to form mature con A which is non-glycosylated (Sheldon and Bowles 1992). Con A precursor in elute may be from the column itself as mature non-glycosylated con A is derived from its glycosylated precursor which upon modification forms the lectin (Chrispeels et al. 1986). The presence of con A in the elute has been reported earlier (Ligat et al. 2011; Zhang et al. 2011; Lastovickova et al. 2011).

As reported earlier by Komatsu et al. (2009), this study also showed the presence of the N-glycosylation of a multifunctional, Ca^{2+} binding protein, calreticulin (Fig. 4; spot 11). To confirm its Ca^{2+} binding 1D-SDS–PAGE gel of the crude and the purified sample was stained with Stains-All (Fig. 3). Stains-all is a cationic carbocyanine dye that stains blue to sialoglycoproteins and phosphoproteins while imparting all other proteins red. The absorption spectrum of the dye changes with interaction of the dye with different molecules. Blue color stain of Ca^{2+} binding protein with Stains-all is due to the interaction of the dye with the anionic sites within the Ca^{2+} binding proteins (Campbell et al. 1983). Two polypeptides at 56 and 54 kDa showed blue stain with stains all similar to 56 and 54 kDa calreticulin isoforms in spinach leaves (Navazio et al. 1995). The molecular weight of the polypeptides were similar to the spot (spot 11) from which calreticulin was identified on 2-D gel (Fig. 4). The

Fig. 5 **a** Prediction of sub-cellular locations of the glycoproteins of sea buckthorn seedlings by Plant-mPLOC tool. GO annotation of identified N-glycoprotein of sea buckthorn seedling **b** molecular function, **c** biological processes and **d** cellular component

presence of calreticulin isoforms has also been reported in barley (Chen et al. 1994). The 54 kDa isoform was thought to be unglycosylated form of 56 kDa, due to similarity in pI and antigenicity. The isoforms showed difference in the N-terminal sequence and in Cleveland peptide maps after partial digestion with *Staphylococcus aureus* (Navazio et al. 1995). N-glycosylation of 54 kDa isoform has not been reported earlier, however in this study both the isoforms were enriched by con A chromatography and also showed positive signals on con A affinoblot.

Bioinformatic analysis of identified proteins

N-glycosylation site prediction and sub-cellular localization

N-glycosylation site of all the identified proteins were predicted based on N-X-S, N-X-T or NX[ST]Z motifs, where X can be any amino acid except proline using

N-Glycosite (Zhang et al. 2004). A total of 22 N-glycosylation sites were predicted with at least one site in each protein (Table 1). However, N-glycosylation site was absent in cysteine protease inhibitor and its presence in the glycoproteome purified by con A affinity chromatography may be because of its association with a glycosylated protein. Glycosyl hydrolase family 38 protein has the maximum number of seven glycosylation sites followed by maturase k with five sites. Of the 22 N-glycosylation sites 14 and 8 were N-X-S and N-X-T sites respectively which shows preferential N-glycosylation of N-X-S over N-X-T.

Sub-cellular localization of the identified proteins showed that most of the proteins were localized in the nucleus (27 %) followed by chloroplast (26 %), cytoplasm (21 %), endoplasmic reticulum (11 %), vacuole (5 %), cell wall (5 %) and extracellular (5 %) (Fig. 5a). Localization of glycoproteins were found to be similar with maize endosperm (Silva-Sanchez et al. 2014).

Gene ontology (GO) annotation

Gene Ontology (GO) annotation categorized the identified proteins in three classes of molecular functions, (a) catalytic activity, (b) binding and (c) molecular function regulator. At least four proteins belong to two different classes and showed multiple functions (Fig. 5b). The proteins were also grouped into twelve classes of biological processes, (a) cellular process, (b) metabolic process, (c) single cell organism process, (d) biological regulation, (e) regulation of biological process, (f) response to stimulus, (g) developmental process, (h) multicellular organism process, (i) cellular component organization or biogenesis, (j) negative regulation of biological process, (k) reproductive process and (l) reproduction (Fig. 5c). Categorization of proteins in twelve biological processes shows that proteins were likely to be involved in multiple functions and process. The proteins were found in ten different cellular components, (a) cell part, (b) organelle, (c) cell, (d) membrane, (e) organelle part, (f) membrane part, (g) extracellular region, (h) membrane enclosed lumen, (i) cell junction and (j) symplast (Fig. 5d). Association of the proteins with different cellular components corresponds with their sub-cellular localization mentioned earlier.

During seedling growth cells are actively dividing and undergoing differentiation which is brought about by cumulative effect of various proteins. The roles of these proteins are determined by various modifications of the proteins. N-glycosylation is one such modification responsible for regulating protein function. In this study N-glycoproteins were found to be involved in diverse forms of molecular functions and biological processes in regulating cell cycle, homeostasis, cell identity determination and also basic photosynthetic machinery. In-depth study of the proteins would help in understanding how its function is being regulated by N-glycosylation.

Conclusions

N-glycoproteome in sea buckthorn seedling was analyzed in this study by con A affinity chromatography. A total of 48 spots were detected in the con A purified N-glycoproteome. Among the proteins identified glycosylation of calreticulin, GH38, maturase k and protein disulfide isomerase are reported earlier. However, glycosylation of ATXR 6, phantastica and putative ATPDIL1-4 electron transporter is not reported earlier and are novel. Categorization of the proteins in a common biological process, functions and cellular component shows that these proteins may be having similar function and might be interacting directly or indirectly in a biological process. In this study we have reported partial glycoproteome of

seabuckthorn. A complete N-glycoproteome of sea buckthorn could be analyzed using a combination of other lectins namely lentil lectin, pea lectin, peanut agglutinin, etc., along with con A. The role of the glycoproteins in providing stress tolerance in sea buckthorn would be analyzed which in future can be translated to improve stress tolerance in crop plants.

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