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Enhanced accumulation of secreted human growth hormone by transgenic tobacco cells correlates with the introduction of an N-glycosylation site

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ABSTRACT

Extracellular secretion of recombinant proteins from plant cell suspension culture will simplify the protein purification procedure and greatly reduce the production cost. Our early work indicated that presence of hydroxyproline-O-glycosylation at the C- or N-terminus of the target protein boosted the secreted yields in the culture medium. Inspired by early successes, we tested the possibility of introducing an N-glycosylation site to facilitate the secretion of human growth hormone (hGH) from cultured tobacco cells. Three N-glycosylated hGH fusion proteins, designated NAS-EK-hGH, NAS-Kex2-hGH and hGH-NAS, were expressed in tobacco BY-2 cells. Where NAS denotes the "Asn-Ala-Ser" consensus sequence for N-glycosylation; EK denotes an enterokinase cleavage site and Kex2 a sequence to be cleaved by a Golgi-localized Kex2p-like protease. Our results indicated that a single N-glycan attached either at the N-terminus or C-terminus of hGH correlated with enhanced extracellular accumulation of the transgenic proteins; the secreted yield of NAS-EK-hGH and hGH-NAS was 70–90 fold greater than the control targeted, non-glycosylated hGH. NAS-Kex2-hGH was subject to partial cleavage of the N-glycan tag at the Kex2 site in Golgi apparatus, and therefore gave lower yields than the other two constructs.

1. Introduction

Plant cell suspension cultures show promise for production of recombinant proteins having a wide range of applications (Hellwig et al., 2004; Huang and McDonald, 2009). Products isolated from plants are unlikely to be contaminated by the viruses, pathogens and toxins that occur in mammalian or bacterial systems (Desai et al., 2010; Ma et al., 2003). Suspension cells in particular are well-suited for product isolation when the product is secreted into the culture medium as plant cells are grown on inexpensive defined media lacking proteinaceous substances that complicate purification of the secreted products (Lee et al., 2000). Examples of recombinant proteins that have been produced in plant cell suspension cultures range from antibodies such as full size immunoglobulins and Fab fragments (Sharp and Doran, 2001a,b; Yano et al., 2006) to cytokines such as interferon (Xu et al., 2007) and growth regulators like human growth hormone (Xu et al., 2010). However, the bottleneck to exploiting this technology for commercial purposes has been low productivity: protein yields usually fall in the range from 0.1 μg/L to 5.0 mg/L (James and Lee, 2001; Su and Lee, 2007). Exceptions include human glycoproteins such as α1-antitrypsin (Huang et al., 2001; McDonald et al., 2005), interleukin-12 (Shin et al., 2010) and granulocyte–macrophage colony stimulating factor (GM-CSF) (Shin et al., 2003) expressed in rice cells using a sucrose inducible *RAmy3D* promoter, where an extremely high protein yield up to 247 mg/L was achieved (McDonald et al., 2005). However, the growth rates and characteristics of rice cell lines are inferior to those of tobacco BY-2 (*Bright Yellow*-2) and NT-1 (*Nicotiana tabacum*-1) cell lines (Hellwig et al., 2004) and rice cell viability is significantly decreased when cultivated in a sucrose-starvation medium (Huang and McDonald, 2009). Furthermore medium exchange to create a sucrose-starvation environment in the cultures imposes technical difficulty in large-scale cultures (Su and Lee, 2007).

Recently we expressed recombinant green fluorescent protein (GFP), human growth hormone (hGH) and interferon α2b (IFNα2) as fusion glycoproteins in tobacco BY-2 cells and noted that the presence of hydroxyproline (Hyp)-O-glycosylation at the C- or N-terminus of GFP, IFNα2 or hGH boosted the secreted yields (i.e. in the culture medium of suspension cells) of the recombinant glycoproteins 500-fold (up to 120 mg/L) and the glycoproteins retained nearly 100% of their biological activity (Fernandes et al., 2010; Shpak et al., 1999, 2001; Tan et al., 2003; Xu et al., 2005, 2007, 2010). This work was consistent with the earlier suggestion of Eylar that protein glycosylation in general facilitates protein secretion (Eylar, 1966) and raises the possibility that N-glycosylation of the

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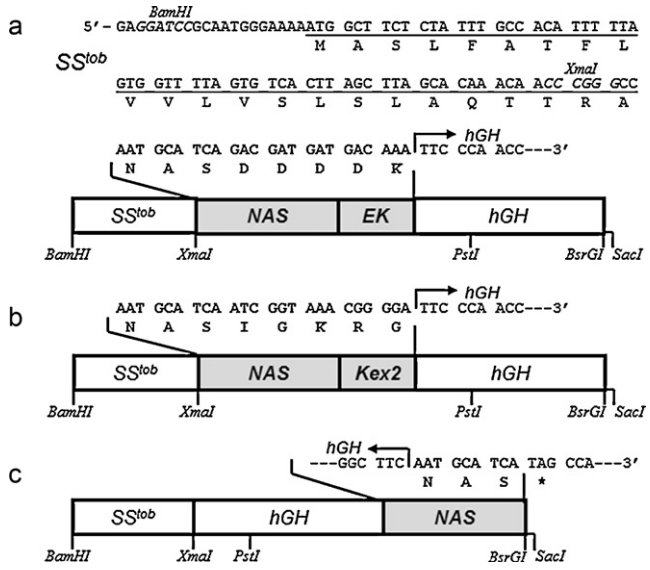

Fig. 1. Schematic representation of the hGH fusion gene constructs and their DNA sequence (a) *SS^tob-NAS-EK-hGH* construct; (b) *SS^tob-NAS-Kex2-hGH* construct; (c) *SS^tob-hGH-NAS* construct. The DNA sequence encoding a signal peptide *SS^tob* is underlined.

same proteins will give yields similar to those in yeast (Sagt et al., 2000).

To test this possibility using an approach analogous to that used earlier for Hyp-O-glycosylation of fusion glycoproteins, we expressed N-glycosylated hGH in tobacco BY-2 cells, the glycans occurring at either the N- or C-terminus. Three fusion constructs were made, designated NAS-EK-hGH, NAS-Kex2-hGH and hGH-NAS, respectively (Fig. 1). NAS denotes the "Asn-Ala-Ser" motif which directs Asn N-glycosylation in eukaryotic cells; EK denotes the "Asp-Asp-Asp-Asp-Lys" motif that is specifically cleaved at Lys by the restriction protease enterokinase; Kex2 denotes the "Ile-Gly-Lys-Arg-Gly" motif that is cleaved by a Golgi-localized Kex2p-like protease in plant cells. Kex2 protease (Kex2p) is the prototype of a Golgi-resident protease responsible for the processing of prohormones in yeast and mammalian cells (Bosshart et al., 1994; Wilcox and Fuller, 1991). The Kex2p-like protease also occurs in the Golgi of tobacco cells (Jiang and Rogers, 1999). Thus the expression of the NAS-Kex2-hGH construct in BY-2 cells will allow us to evaluate the role which the N-glycan tag plays in directing the secretion of the protein from the Golgi apparatus to the extracellular environment.

2. Materials and methods

2.1. Construction of plant expression vector

Plasmids *pUC-SS^tob-hGH* and *pBI121-SS^tob-hGH* for the expression of secreted but non-glycosylated hGH were constructed earlier (Xu et al., 2010); *SS^tob* denotes the tobacco extensin signal peptide. To make the *NAS-EK-hGH* construct, the gene encoding "Asn-Ala-Ser" and an enterokinase restriction site (Asp-Asp-Asp-Asp-Lys) was extended from the 5′-terminus of *hGH* gene in the plasmid *pUC-SS^tob-hGH* by PCR using the following primer set: 5′-ACAA*CCCGGG*CCAATGCATCAGATGACGATGACAAATTCCCAACCAT-TCCCTTATCC-3′ and 5′-TACT*TGTACA*GCTAGAAGCCACAGCTGCCCTC-3′. Similarly, the *NAS-Kex2-hGH* construct was made from the plasmid *pUC-SS^tob-hGH* by PCR using the following primer set: 5′-ACAA*CCCGGG*CCAATGCATCAATCGGTAAACGGGGATTCCCAACCAT-TCCCTTATCC-3′and 5′-TACT*TGTACA*GCTAGAAGCCACAGCTGCCCTC-3′. To add the N-glycosylation signal at the C-terminus of hGH, the gene encoding "Asn-Ala-Ser" was extended from the 3′-terminus of *hGH* gene in the plasmid *pUC-SS^tob-hGH* by PCR using the following primer set: 5′-AATACCCGGGCCTTCCCAACCATTCCCTTATCC-3′and 5′-TACTTGTACAGCTATGATGCATTGAAGCCACAGCTGCCCTC-3′

These PCR amplified gene fragments were sub-cloned into *pUC-SS^tob-EGFP* as *XmaI*/*BsrGI* fragments to generate plasmids *pUC-SS^tob-NAS-EK-hGH*, *pUC-SS^tob-NAS-Kex2-hGH* and *pUC-SS^tob-hGH-NAS*, respectively. The extra nucleotides introduced in these gene cassettes for cloning purposes were then removed via site-directed mutation using a QuickChange Mutagenesis kit (Strategies, CA). The DNA sequencing of the constructed genes was performed in the Department of Environmental and Plant Biology, Ohio University.

The entire *SS^tob-NAS-EK-hGH*, *SS^tob-NAS-Kex2-hGH* and *SS^tob-hGH-NAS* constructs were then cloned into the plant vector *pBI121* (Clontech, CA) as a *BamHI* and *SacI* fragment in place of the *β*-glucuronidase reporter gene. The expression of the fusion gene was under the control of the 35S cauliflower mosaic virus (CaMV35S) promoter.

2.2. Plant cell transformation and selection

The *pBI121* plasmids containing the *SS^tob-NAS-EK-hGH*, *SS^tob-NAS-Kex2-hGH* and *SS^tob-hGH-NAS* gene cassettes were introduced into *Agrobacterium tumefaciens* strain LBA4404 by the freeze–thaw method (Holsters et al., 1978), and then transformed into suspension-cultured tobacco cells (*Nicotiana tabacum*, BY-2) as described earlier (An, 1985). Transformed cell lines were selected on solid Schenk & Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) containing 0.4 mg/L 2, 4-dichlorophenoxyacetic acid (2,4-D), 200 mg/L kanamycin (Sigma) and 400 mg/L timentin (SmithKline Beecham, PA). Positive clones (30–50) selected for kanamycin resistance were grown in liquid SH medium comprised of the same components as above, except it lacked timentin. After 8–10 days of cultures at room temperature on an Innova gyrotary shaker (New Brunswick Scientific, Edison, NJ) rotating at 90 rpm, the culture medium for each cell line was screened for hGH expression and secretion by dot blotting. Five high-yield cell lines for each construct were then chosen for subculture under the same conditions as above.

2.3. Isolation of hGH and its fusion glycoproteins

Recombinant hGH and its fusion glycoproteins were isolated from the culture medium via a hydrophobic-interaction chromatography (HIC) and a Superose-12 gel permeation chromatography (GPC) as before (Xu et al., 2007, 2010). The hGH or its fusion glycoproteins was mainly eluted in the Tris buffer fraction applied to the HIC column. The hGH rich fraction collected from the GPC column was further purified by reversed phase HPLC after injecting into a Vydac® C_4 (214TP) analytical column (4.6 mm × 250 mm, Varian Instruments, Walnut Creek, CA) equilibrated in solution A (0.1% trifluoroacetic acid). Proteins were then eluted with solution B (0.1% trifluoroacetic acid + 80% acetonitrile, v/v) with two step linear gradient of 0–30% B in 15 min, followed by 30–70% B in 90 min at a flow rate of 0.5 ml/min.

2.4. Sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) assay

Samples were mixed with an equal volume of 2× reducing sample buffer, heated at 90 °C for 10 min, cooled and resolved on a 12% SDS-PAGE gel (BioRad, Hercules, CA) as described by Laemmli (1970). The gel was then stained with Coomassie Blue.