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ORIGINAL ARTICLE

# Dramatic secretion of recombinant protein expressed in tobacco cells with a designer glycopeptide tag is highly impacted by medium composition

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**Abstract**

*Key message* **Cell growth medium composition has profound impacts on the *O*-glycosylation of a "designer" arabinogalactan protein-based module; full glycosylation is essential in directing efficient extracellular secretion of the tagged recombinant protein.**

*Abstract* Expression of recombinant proteins in plant cells as fusion with a de novo designed hydroxyproline (Hyp)-*O*-glycosylated peptide (HypGP) tag, termed HypGP engineering technology, resulted in dramatically increased secreted protein yields. This is due to the function of the HypGP tag as a molecular carrier in promoting efficient transport of conjoined proteins into culture media. To optimize the cell culture to achieve the best secreted protein yields, the medium effects on the cell growth and protein secretion were investigated using as a model system the tobacco BY-2 cell expressing enhanced green fluorescence protein (EGFP) fused with a $(SP)_{32}$ tag (32 tandem repeats of "Ser-Pro" motif). The $(SP)_{32}$ tag was found to undergo two-stage Hyp-*O*-glycosylation in plant cells with the dramatic secretion of the conjoined EGFP correlating with the *triggering* of the second-stage glycosylation. The BY-2 cell culture in SH medium generated a high secreted protein yield (125 mg/L) with a low cell biomass accumulation (~7.5 gDW/L). In contrast, very low secreted protein yields (~1.5 mg/L) with a high cell biomass accumulation (13.5 gDW/L) were obtained in MS medium. The macronutrients, specifically, the nitrogen supply greatly impacted the glycosylation of the $(SP)_{32}$ tag and subsequent protein secretion. Modified MS medium with reduced nitrogen levels boosted the secreted EGFP yields to 168 mg/L. This study demonstrates the profound impacts of medium composition on the secreted yields of a HypGP-tagged protein, and provides a basis for medium design to achieve the highest productivity of the HypGP engineering technology.

**Keywords** Plant cell culture · Recombinant proteins · Hydroxyproline-*O*-glycosylation · Protein secretion · Medium manipulation · Nitrogen supply

## Introduction

Plant cell culture is emerging as an alternative bioproduction system for recombinant pharmaceuticals as it offers advantages in *safety* and *cost-effectiveness* over other eukaryotic systems (Hellwig et al. 2004; Santos et al. 2016; Xu et al. 2011). Plant cells do not harbor any known human pathogens and bacterial endotoxins, so providing major advantages in product safety, especially for therapeutics. Plant cells can grow in simple synthetic media using conventional bioreactors, thus becoming a cost-effective bioproduction platform. With the recent FDA (Food and Drugs Administration) approval and commercialization of the

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world's first plant cell-based recombinant pharmaceutical, Elelyso® (β-glucocerebrosidase) for human use as an orphan drug for the treatment of Gaucher's disease (Tekoah et al. 2015), plant cell culture is now reaching the stage at which it may challenge those established bioproduction systems that use bacterial, yeast and mammalian cells. However, the major bottleneck to full adoption of this technology for commercial purposes has been generally low productivity with protein yields usually below 10 mg/L (Su and Lee 2007; Xu and Zhang 2014). In addition, recovered product yield is significantly diminished when the expressed protein is retained within the production cells as this dramatically increases the cost of protein purification.

These technical challenges was addressed by a proprietary technology, termed *HypGP engineering technology*, with which increase in secreted yields of recombinant protein as high as 1500-fold compared to control systems was achieved (Kieliszewski et al. 2015; Xu et al. 2007). The HypGP engineering technology exploits the glycosylation "code" of plant cell wall hydroxyproline (Hyp)-rich glycoproteins (HRGPs) for de novo design of proline-rich peptides, such as tandem repeats of a "Ser-Pro" motif, which undergo intensive post-translational modifications upon engineered in plant cells, including hydroxylation (converting Pro to Hyp) and subsequent *O*-glycosylation (adding an arabinogalactan polysaccharide onto each Hyp) (Kieliszewski and Shpak 2001; Xu et al. 2008). These Hyp-*O*-glycosylated peptides (HypGP) appear to function as a "molecular carrier" in promoting efficient transport of the conjoined recombinant proteins into the culture media and protecting the proteins from proteolytic degradation (Xu et al. 2007, 2010), which boosts the secreted protein yields from production cells. From process development point of view, dramatic secretion of proteins from cultured plant cells not only simplifies the protein isolation and purification process, but also allows continuous perfusion culture to be implemented, thereby increasing volumetric productivity overall (Hakkinen et al. 2014; Huang and McDonald 2009). Therefore, the HypGP engineering technology has significant potential to establish plant cell culture-based molecular farming as an economically feasible platform, which is capable of producing a broader range of recombinant therapeutic proteins.

On the way towards commercialization of the HypGP engineering technology, it is necessary to optimize the cell culture system to achieve the best secreted protein yield. In plant cell culture processes, medium or nutrient design is regarded as important as expression vector and cell line development for increasing the protein productivity (Fischer et al. 2015; Xu et al. 2011). The medium type and composition can influence cell growth and protein production, and modify glycosylation as well (Schillberg et al. 2013; Tekoah et al. 2015; Tsoi and Doran 2002). Protein productivity could be substantially improved through manipulating the medium composition. Holland et al. (2010) reports a 10- to 20-fold increase in the production of the full-size human anti-HIV antibody in tobacco BY-2 cell cultures through an extensive medium analysis and optimization, particularly the nitrogen source. Medium manipulation was also reported to result in a fivefold increase in the $IgG_1$ production in tobacco cell culture (Doran 2000).

In this study, transgenic tobacco cell expressing enhanced green fluorescence protein (EGFP) as fusion with a designer HypGP tag comprised of 32 repeats of "Ser-Pro" motif, or $(SP)_{32}$-EGFP (Fig. 1), was used as a model

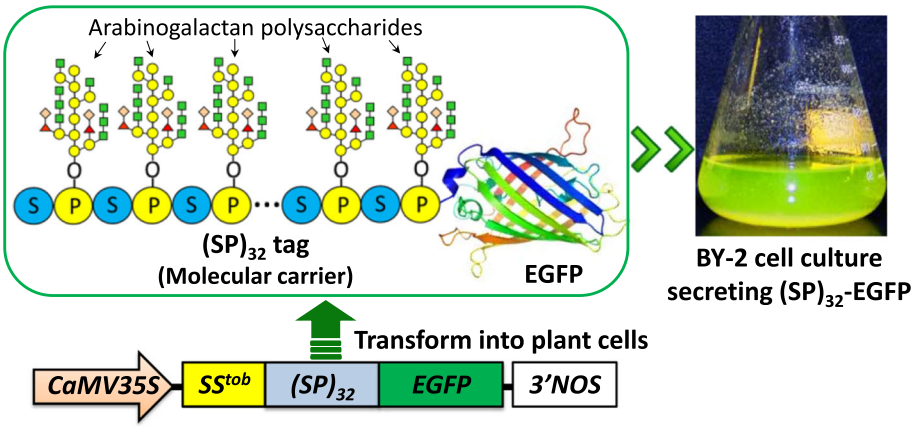


**Fig. 1** Schematic of $(SP)_{32}$-tagged EGFP expressed in plant cells. After transformation of the fusion gene *$(SP)_{32}$-EGFP* into plant cells, the clustered non-contiguous Pro (P) residues in the $(SP)_{32}$ tag are hydroxylated to be Hyp, and subsequently glycosylated with arabinogalactan polysaccharides comprised of a β-1,3-linked galactose backbone with sidechains containing arabinose, rhamnose and glucuronic acids, as identified early (Shpak et al. 1999; Xu et al. 2008). Dramatic secretion of $(SP)_{32}$-EGFP turned the cell culture medium into bright green. *$(SP)_{32}$* 32 tandem repeats of "Ser-Pro" motif, *CaMV35S* 35S cauliflower mosaic virus promoter, *$SS^{tob}$* tobacco extensin signal sequence, *3'NOS* nopaline synthase terminator

system to study the medium composition effects on the *O*-glycosylation of the HypGP tag and the secreted protein yields. The $(SP)_{32}$ tag represents a typical glycosylation module of arabinogalactan glycoproteins (AGPs), one of the major types of HRGPs occurring in the plant cell walls (Hijazi et al. 2014; Shpak et al. 1999). The use of the EGFP reporter protein in this study greatly facilitated the detection and quantification of the target protein. The results from this study demonstrated the profound impacts of the medium composition on the Hyp-*O*-glycosylation of the HypGP tag and subsequent secretion of the conjoined protein.

## Materials and methods

### Transgenic BY-2 cell line and cell cultures

Expression vector *pBI121-SS$^{tob}$-$(SP)_{32}$-EGFP* encoding $(SP)_{32}$-EGFP fusion protein (Shpak et al. 1999; Xu et al. 2008) was obtained from the Kieliszewski lab at Ohio University. *SS$^{tob}$* denotes the tobacco extensin signal sequence (De Loose et al. 1991). Gene expression is under the control of the *35S cauliflower mosaic virus* promoter (*CaMV*35S). Tobacco bright yellow-2 (BY-2) cell line expressing $(SP)_{32}$-EGFP was created by re-transformation of the expression vector into the BY-2 cell with the *Agrobacterium*-mediated method (An 1985). Transformed BY-2 cells were maintained in Schenk and Hildebrandt medium (SH) (Schenk and Hildebrandt 1972) containing 2.1 mg/L *p*-chlorophenoxyacetic acid, 0.4 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/L kinetin, and 34 g/L sucrose. The suspension cells were grown in shake flasks and rotated at 95 rpm on a gyratory shaker under continuous illumination at room temperature (22–24 °C). Subcultures were carried out every week with a 5 % (v/v) inoculum density.

### Manipulation of medium composition

Different types of basal medium, including SH, MS (Murashige and Skoog) (Murashige and Skoog 1962), B5 (Gamborg's B-5) (Gamborg et al. 1968) and BDS (B-5 modified by Dunstan and Short) (Dunstan and Short 1977), and the media with modified composition as described in details in the Results and Discussion section, were used to grow the transgenic BY-2 cell. For all the experiments, about 1.5 g/L (dry weight) of 6-day-old BY-2 cells were inoculated in 80 ml medium contained in 250 ml flasks and cultured for 8–10 days for the determination of fresh weight (FW), dry weight (DW) and secreted EGFP yields. Cultured cells and media were also collected and frozen at −80 °C for Western blotting analysis later.

### Protein extraction and Western blotting analysis

Harvested culture media were directly used for assay of the secreted proteins. Intracellular proteins were extracted from cultured cells as described earlier (Dolan et al. 2014). Briefly, frozen BY-2 cell samples (~0.5 g) were ground by mortar and pestle in liquid $N_2$, and then supplemented with SDS extraction buffer (150 mM Tris–HCl, pH 6.8, 30 % glycerol, 6 % SDS, 5 mM EDTA) at a ratio of 1:2 (w/v). Samples were centrifuged at 13,000×*g* (4 °C) for 15 min and the supernatants collected.

For Western blotting assay, harvested media or cell extraction samples were mixed with an equal volume of 2 × reducing sample buffer, heated at 90 °C for 10 min, cooled and resolved on a 10 % Tris–HCl gel (Bio-Rad, CA, USA) or a 10 % NuPAGE® Novex® Bis–Tris gel (Invitrogen, CA, USA). Protein bands were then electro-blotted onto a 0.2 µm nitrocellulose membrane using a Mini Trans-Blot® Cell (Bio-Rad, CA, USA) or an XCell™ Blot Module (Invitrogen, CA, USA). Immunoblot detection of EGFP was carried out with a primary antibody, rabbit anti-EGFP (Thermo Fisher Scientific, IL, USA), and a secondary antibody, goat anti-rabbit IgG (H + L)-peroxidase conjugated (Bio-Rad, CA, USA). Protein blots were then detected using the CDP-Star and Tropix Nitroblock Enhancer II reagents (Roche, CA, USA) in accordance with the manufacturers' procedures.

### Determination of cell biomass yield

Cultured cells were harvested by vacuum filtration on a coarse sintered funnel and washed three times with distilled water before the fresh weight (FW) determination. The cells were then dried in an oven at 70 °C for 48 h and weighed to determine the dry weight (DW). Cell biomass yield was expressed as gram of cell dry weight per liter medium (gDW/L).

### Quantification of recombinant EGFP

EGFP *equivalent* of the secreted $(SP)_{32}$-EGFP or EGFP was assayed by determining the Relative Fluorescence Unit (RFU) of the culture media on a Modulus™ Single Tube Multimode Reader with a Modulus™ Blue Fluorescence Optical Kit (Turner BioSystems Inc., CA, USA). The EGFP contents of media were then estimated by comparison of the RFU with those of EGFP standard (BioVision, CA, USA) diluted to different concentrations in spent SH medium collected from the culture of wild-type BY-2 cells. EGFP *equivalent* of the intracellular fusion protein was quantified by densitometry based on Western blotting. Briefly, samples and EGFP standard were electrophoresed on a same gel. After immunoblot detection with the anti-

EGFP antibody, the contents of EGFP were estimated by comparison of the band intensities on a VersaDoc 4000 imaging system and analyzed using the Quant-1 software (Bio-Rad, CA, USA).

### Statistical analysis

Assays of biomass and secreted protein yields were carried out with three replicates unless indicated in text, and data are presented as the mean with accompanying standard deviation (SD). One-way analysis of variance (ANOVA) followed by a Tukey's post hoc range test was used to determine differences among treatments with $p < 0.05$ considered to be significant.

## Results and discussions

### Basal medium type impacts Hyp-*O*-glycosylation and secretion of $(SP)_{32}$-EGFP

The transgenic BY-2 cell expressing $(SP)_{32}$-tagged EGFP was initially grown in SH medium, which produced high secreted protein yields up to 125 mg/L EGFP *equivalent* (Zhang et al. 2016). However, the cell biomass accumulation was low (~7.5 gDW/L) compared to those of 10–13 gDW/L normally obtained in plant cell cultures (Wilson and Roberts 2012; Xu et al. 2011). This prompted us to investigate the medium effects on the cell growth and protein production to further improve the secreted protein yields. The transgenic BY-2 cells were grown in three other types of basal medium: MS, B5 and BDS medium, each supplied with the same levels of growth regulators and sucrose as in SH medium, to assess the cell biomass accumulation and secreted protein yields. The nutrient composition of all the four types of basal medium is shown in Supplementary Table 1.

As shown in Fig. 2a, b, both SH and BDS medium supported the dramatic secretion of the recombinant $(SP)_{32}$-EGFP with comparable secreted EGFP yields (125 mg/L in SH vs. 112 mg/L in BDS) and turned the media to bright green, though the cell biomass accumulated in BDS medium (12.1 gDW/L) was 55 % higher than that in SH medium (7.8 gDW/L). The culture in B5 medium accumulated the similar cell biomass to that in BDS medium, but the secreted protein yield (46 mg/L) was only ~1/3 of those achieved in SH and BDS medium. It was very interesting to see that while MS medium produced the highest cell biomass (13.5 gDW/L) among the four types of basal medium, however, a very low level of secreted EGFP was detected in the culture medium (~1.5 mg/L), similar to that obtained in the expression of EGFP control (without a HypGP tag) (Zhang et al. 2016). In fact, low protein secretion of the cell cultures in MS medium was also observed in the expression of other proteins, e.g., human growth hormone and interferon α2b, with a HypGP tag comprised of $(SP)_{10}$ or $(SP)_{20}$, though high secreted yields of these two proteins were detected in SH medium

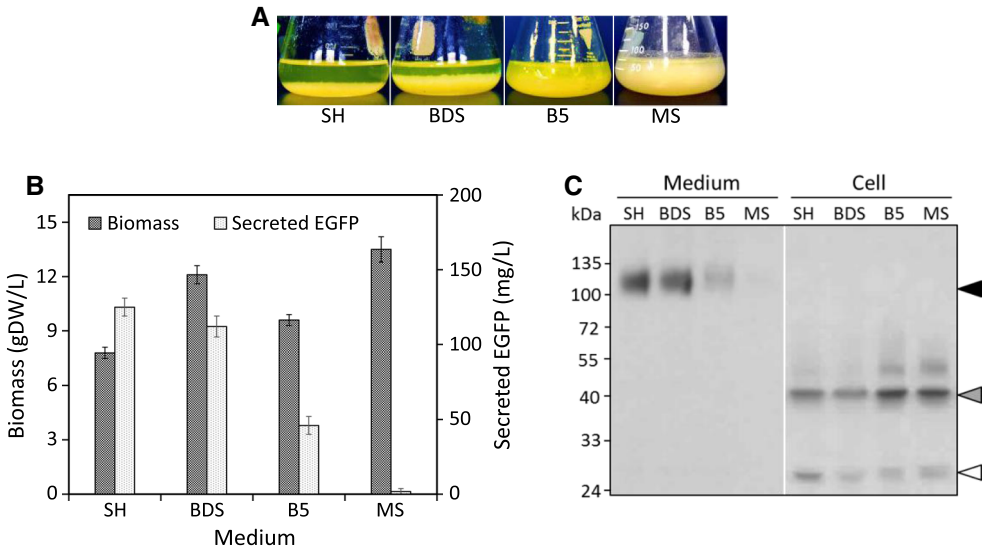


**Fig. 2** Cell growth and EGFP production of the transgenic BY-2 cell cultures in different types of basal medium. **a** Images of BY-2 suspension culture in four different types of media; **b** cell biomass and secreted EGFP yields; each value represents the mean of three replicates ± standard deviation; different letters above each bar indicate a significant difference ($p < 0.05$); **c** anti-EGFP Western blotting detection of the $(SP)_{32}$-*EGFP* transgene products secreted into media and accumulated inside cells. The cells and media were harvested after 10 days of culture. Fully and partially glycosylated $(SP)_{32}$-EGFP, and the EGFP domain cleaved from the $(SP)_{32}$-EGFP fusion are indicated by the *dark arrow*, *gray arrow* and *white arrow*, respectively. The dim up-bands above 40 kDa are presumably the partially glycosylated $(SP)_{32}$-EGFP subject to further glycosylation

(Supplementary Table 2), which was unexpected and worth further investigating.

The extracellular and intracellular protein products of the cell cultures in different media were analyzed by anti-EGFP Western blotting. Similar to that observed earlier (Zhang et al. 2016), the *(SP)$_{32}$-EGFP* transgene products appeared as totally different sizes and were completely segregated between the medium fractions and the cells (Fig. 2c). While the high molecule weight product, termed *fully glycosylated* fusion protein, migrated as a broad band at ~115 kDa in the medium fractions (almost absent in MS medium), the *partially* glycosylated intermediates (~40 kDa) retained only inside cells. This implies the $(SP)_{32}$ tag is subject to two-stage Hyp-*O*-glycosylation in the plant cells with the first stage involving constitutive addition of one galactose to each Hyp, followed by the rate-limiting second stage triggering the cascade for addition of the other Hyp-glycan residues (Zhang et al. 2016). Since only the fully glycosylated protein product was detected in the culture media, the second-stage glycosylation must be essential in directing the efficient secretion of the recombinant proteins. Thus, the low protein secretion observed in MS medium could be attributed to the absence of the second-stage glycosylation of the $(SP)_{32}$ tag in the fast-growing BY-2 cells.

## Macronutrients play a critical role in Hyp-*O*-glycosylation and secretion of $(SP)_{32}$-EGFP

To identify the critical component(s) in the growth media, important in mediating the Hyp-*O*-glycosylation of $(SP)_{32}$-EGFP, three major groups of components comprising a cell culture medium—inorganic salts, vitamins, and growth regulators from different medium were mixed and matched to create eight different medium combinations and their relative effect on the $(SP)_{32}$-EGFP secretion was investigated. In this study, the inorganic salts and vitamins from MS and SH medium were chosen because these two medium supported the highest cell biomass accumulation and the highest secreted protein yield, respectively. The growth regulators were chosen as the one used in current SH medium (2.1 mg/L *p*-chlorophenoxyacetic acid, 0.4 mg/L 2,4-D, 0.1 mg/L kinetin) and the other used in the NT-1 medium (0.2 mg/L 2,4-D) (Mayo et al. 2006).

The determined secreted EGFP yields distinctly sorted the eight media into two groups: the media containing SH salts (medium $M_1$, $M_2$, $M_3$ and $M_4$) generated remarkably high levels of secreted EGFP while the MS salts-containing media (medium $M_5$, $M_6$, $M_7$ and $M_8$) showed little or no secreted EGFP production (Fig. 3a). Anti-EGFP western blotting analysis also confirmed presence of the 115 kDa product in the SH salt-containing media, and absent of which from the MS salt-containing media (Fig. 3a). This

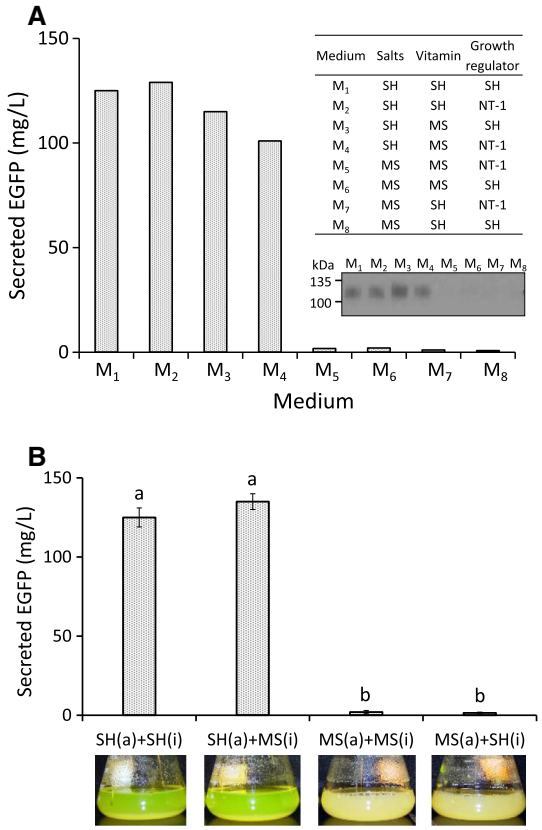


**Fig. 3** Medium mixing and matching experiments to identify the key components contributing to full Hyp-*O*-glycosylation of the $(SP)_{32}$ tag and subsequent protein secretion. **a** The effects of mixing and matching of three major groups of a cell culture medium (inorganic salts, vitamins and growth regulators) on the secreted protein yields. The table outlines the eight medium combination matrices tested. The cell cultures in each medium were carried out in duplicate and the means of secreted protein yields were presented. The fully glycosylated fusion proteins secreted into media (10 days) were identified by the anti-EGFP Western blotting assay (inserted image). **b** The effects of mixing and matching of the macronutrients and micronutrients of MS and SH medium on the secreted protein yields. The *up panel* shows the secreted EGFP yields; the *lower panel* shows the cell suspension culture in four different media for 10 days. *SH(a)* SH macronutrients, *MS(a)* MS macronutrients, *SH(i)* SH micronutrients, *MS(i)* MS micronutrients. Different letters indicate significant difference in secreted EGFP yields ($p < 0.05$)

finding implies the important component(s) contributing to the full glycosylation of the $(SP)_{32}$ tag are most likely associated with the *inorganic salts* of the medium. The intracellular products, including the partially glycosylated $(SP)_{32}$-EGFP (~42 kDa) and the cleaved EGFP domain (27 kDa), were detected in all the cultured cells with similar levels (Supplementary Figure 1), suggesting the production of the intermediate forms of $(SP)_{32}$-EGFP were not impacted by the inorganic salts.

The basal salts are divided into two groups of compounds: macronutrients and micronutrients. Their effects on the Hyp-*O*-glycosylation and protein secretion were further investigated. Again, the macronutrients and

micronutrients of MS and SH medium were mixed and matched to create four medium combinations. The other components, vitamins and growth regulators remained the same as in SH medium. As shown in Fig. 3b, the two SH macronutrients-containing media promoted the secretion of high yields of $(SP)_{32}$-EGFP, turning the media to bright green. The SH macronutrients combined with the MS micronutrients even produced slightly more secreted EGFP (136 mg/L) than the original SH medium. In contrast, very low secreted EGFP yields was detected in the two MS macronutrients-containing media, though these media produced high levels of cell biomass (>13.0 gDW/L). The micronutrient salts did not show significant impact on the secretion of the $(SP)_{32}$-tagged EGFP. This narrowed down to the macronutrients as the crucial components contributing to the full Hyp-*O*-glycosylation of the $(SP)_{32}$ tag.

The observed difference could be due to positive components in the SH macronutrient salts that promote the full Hyp-*O*-glycosylation and secretion of the $(SP)_{32}$-tagged protein or components in the MS macronutrients that inhibits/blocks the second-stage glycosylation resulting in absence of the fully glycosylated $(SP)_{32}$-EGFP. About four to five different inorganic salts make up the macronutrient of SH or MS medium (Supplementary Table 1). In an effort to identify the specific salt component(s) playing the decisive role in the Hyp-*O*-glycosylation of the $(SP)_{32}$ tag, we continued to compare the macronutrient compositions of SH and MS medium, along with the relative concentration of each component. BDS medium supporting the high secretion of $(SP)_{32}$-EGFP as SH medium was used as a secondary reference for the comparison. While these media containing similar salt ions, including $NH_4^+$, $NO_3^-$, $H_2PO_4^-$, $SO_4^{2+}$, $Ca^{2+}$, $K^+$ and $Mg^{2+}$ with some variations in their concentration, the total salt content of SH medium (3.15 g/L) is significantly lower than MS medium (4.53 g/L) with BDS medium falling between (3.85 g/L) (Supplementary Table 1). This is mainly due to the great difference in the nitrogen content (both $NH_4^+$ and $NO_3^-$) of these media (Table 1). In addition, the $Ca^{2+}$ and $Mg^{2+}$ contents of the SH macronutrients were found substantially

**Table 1** Nitrogen sources and concentrations of different plant cell culture media

| Medium | $KNO_3$ (mM) | $NH_4H_2PO_4$ (mM) | $NH_4NO_3$ (mM) | $(NH_4)_2SO_4$ (mM) | $NO_3^-$ (mM) | $NH_4^+$ (mM) | Total N (mM) | $NO_3^-/NH_4^+$ |
|---|---|---|---|---|---|---|---|---|
| Basic medium type | | | | | | | | |
| SH | 24.7 | 2.6 | | | 24.7 | 2.6 | 27.3 | 9.5 |
| BDS | 25.0 | | 4.0 | 1.8 | 29.0 | 7.6 | 36.6 | 3.8 |
| B5 | 24.7 | | | 1.8 | 24.7 | 3.6 | 28.3 | 6.7 |
| MS | 18.8 | | 20.7 | | 39.5 | 20.6 | 60.1 | 1.9 |
| MS medium with reduced $NH_4NO_3$ (N) | | | | | | | | |
| MS-1/2N[a] | 18.8 | | 10.4 | | 29.2 | 10.4 | 39.6 | 2.8 |
| MS-1/3N | 18.8 | | 6.9 | | 25.7 | 6.9 | 32.6 | 3.7 |
| MS-1/5N | 18.8 | | 4.1 | | 22.9 | 4.1 | 27.0 | 5.6 |
| MS-0N | 18.8 | | 0 | | 18.8 | 0 | 18.8 | – |
| SH medium with $KNO_3$ (K) as nitrogen supplement | | | | | | | | |
| SH + 2.5K[b] | 27.2 | 2.6 | | | 27.2 | 2.6 | 29.8 | 10.5 |
| SH + 5K | 29.7 | 2.6 | | | 29.7 | 2.6 | 32.3 | 11.4 |
| SH + 10K | 34.7 | 2.6 | | | 34.7 | 2.6 | 37.3 | 13.3 |
| SH + 20K | 44.7 | 2.6 | | | 44.7 | 2.6 | 47.3 | 17.2 |
| SH medium with $NH_4H_2PO_4$ (P) as nitrogen supplement | | | | | | | | |
| SH + 2.5P[c] | 24.7 | 5.1 | | | 24.7 | 5.1 | 29.8 | 4.8 |
| SH + 5P | 24.7 | 7.6 | | | 24.7 | 7.6 | 32.3 | 3.3 |
| SH + 10P | 24.7 | 12.6 | | | 24.7 | 12.6 | 37.3 | 2.0 |
| SH + 20P | 24.7 | 22.6 | | | 24.7 | 22.6 | 47.3 | 1.1 |
| SH medium with $NH_4NO_3$ (N) as nitrogen supplement | | | | | | | | |
| SH + 2.5N[d] | 24.7 | 2.6 | 1.25 | | 26.95 | 3.85 | 29.8 | 7.0 |
| SH + 5N | 24.7 | 2.6 | 2.5 | | 27.2 | 5.1 | 32.3 | 5.3 |
| SH + 10N | 24.7 | 2.6 | 5 | | 29.7 | 7.6 | 37.3 | 3.9 |
| SH + 20N | 24.7 | 2.6 | 10 | | 34.7 | 12.6 | 47.3 | 2.8 |

[a] MS-1/2N denotes modified MS medium supplied with 1/2 original amount of $NH_4NO_3$ and so forth

[b,c,d] SH + 2.5K, SH + 2.5P and SH + 2.5N denotes modified SH medium supplemented with 2.5 mM nitrogen in the form of $KNO_3$, $NH_4H_2PO_4$ and $NH_4NO_3$, respectively, and so forth

lower than those in MS medium. However, BDS medium contains the similar $Ca^{2+}$ and $Mg^{2+}$ levels to MS medium, indicating these salts are not important in promoting the second-stage glycosylation.

### Nitrogen supply determines Hyp-*O*-glycosylation and secretion of $(SP)_{32}$-EGFP

Nitrogen is a dominant nutrient in the macronutrient salts, and significant difference in the nitrogen levels was found between SH and MS medium. As seen in Table 1, MS medium contains not only much more total nitrogen than SH medium (60.1 mM in MS vs. 27.3 mM in SH), but also a high level of $NH_4^+$ resulting in a much lower $NO_3^-$/$NH_4^+$ ratio than SH medium (1.9 in MS vs. 9.5 in SH). This is due to the presence of a large amount of $NH_4NO_3$ (1650 mg/L) in MS medium and absence of this salt in SH medium or in a low concentration in BDS medium (320 mg/L). The observed low cell biomass accumulation in SH medium was likely caused by insufficient supply of nitrogen in the "nitrogen-starvation" SH medium. To understand the nitrogen effects on the cell growth and secretion of the $(SP)_{32}$-tagged protein, four modified MS media with reduced amounts of $NH_4NO_3$ to 1/2, 1/3 and 1/5 of its original level and without $NH_4NO_3$, designated MS-1/2N, MS-1/3N, MS-1/5N and MS-0N, respectively, were made for the culture of the transgenic BY-2 cell. The nitrogen contents of these media (18.8–39.6 mM) are close to that in SH medium (27.3 mM) or BDS (36.6 mM) medium (Table 1).

With reduced nitrogen supply in MS medium, the cell cultures accumulated less biomass (9.6–11.4 gDW/L) than in original MS medium, but still more than in SH medium (except in MS-0N medium lacking $NH_4^+$). Interestingly, high secretion of EGFP was observed in all the modified MS media, even in MS-0N medium with very low cell biomass accumulated (4.5 gDW/L) (Fig. 4a, b). In fact, the cell cultures in MS-1/2N and MS-1/3N medium produced significantly higher yields of secreted EGFP than in SH medium, reaching 155 and 168 mg/L, respectively (Fig. 4b). The Western blotting assay indicated the fully glycosylated $(SP)_{32}$-EGFP products were exclusively detected in all the modified MS media, as found in SH medium, and the culture in MS-1/3N medium accumulated the highest EGFP yield both extracellularly and intracellularly (Fig. 4c).

Our results indicated the high level of nitrogen contained in MS medium, while promoting rapid cell growth and high biomass accumulation, inhibited the second-stage glycosylation or full glycosylation of the $(SP)_{32}$ tag and subsequent secretion. However, the full glycosylation could be trigged by the reduced nitrogen levels in MS medium, or in other words, the nitrogen-starvation stress as it slowed down the growth of cultured cells and reduced cell biomass accumulation. While the fully glycosylated $(SP)_{32}$ tag boosted the secretion of the conjoined EGFP in the modified MS media, the secreted protein yields were

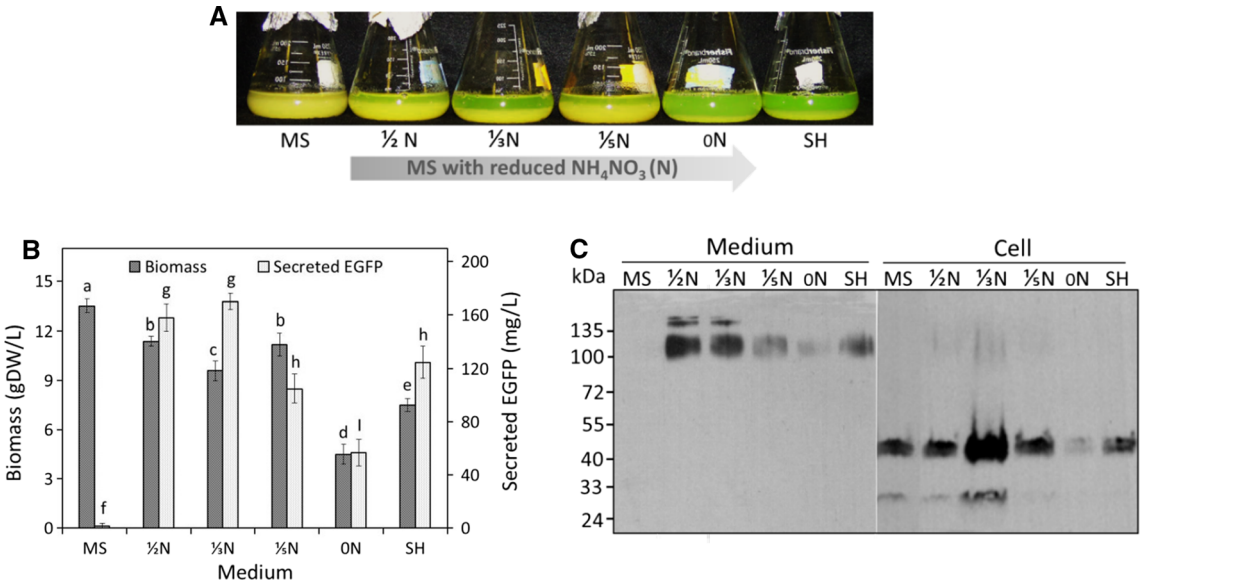


**Fig. 4** BY-2 cell cultures in modified MS medium with reduced amounts of $NH_4NO_3$. **a** Images of BY-2 cell suspension culture in different media for 10 days. **b** Yields of secreted EGFP and cell biomass; each value represents the mean of six replicates ± standard deviation. Different letters indicate significant difference ($p < 0.05$) in biomass yields (*a–e*) or secreted EGFP yields (*f–h*); **c** Anti-EGFP Western blotting detection of the *(SP)$_{32}$-EGFP* transgene products secreted into the media and accumulated inside the cells. 1/2N, 1/3N, 1/5N and 0N denote the modified MS medium with reduced amounts of $NH_4NO_3$ to 1/2, 1/3 and 1/5 of its original level and without $NH_4NO_3$, respectively

not proportional to the cell biomass accumulation, as the culture in MS-1/3N yielded the highest secreted EGFP, but accumulated less cell biomass than in MS-1/2N and MS-1/5N medium (Fig. 4b, c). This is because the secreted protein yield was determined by two factors that has an opposite effect on the protein synthesis/secretion: (1) the extent to which the $(SP)_{32}$ tag was fully glycosylated (major one); and (2) the cell growth and biomass accumulation. The greater extent of full glycosylation of the $(SP)_{32}$ tag (thus more efficient as a molecular carrier to secrete the protein) was always associated with slower cell growth and lower biomass accumulation (thus less protein production). Therefore, the secreted protein yields did not increase proportionally to the cell biomass accumulation. This is also consistent with the observation with the cultures in low-nitrogen SH medium, where high yields of secreted protein came with low cell biomass production.

On the other hand, because low cell biomass was generated in SH medium, it became interesting to know if the secreted EGFP yields would be further improved by increasing the nitrogen levels in SH medium. Three nitrogen sources: $KNO_3$, $NH_4NO_3$ and $NH_4H_2PO_4$ that contain nitrate only, nitrate in combination of ammonium and ammonium only, respectively, were each supplemented to the standard SH medium at the supplemented levels of nitrogen from 2.5 to 20 mM. Of these three sources of nitrogen supply, $NH_4NO_3$ is absent in original SH medium.

As shown in Fig. 5, supplementation of $KNO_3$, which increased the $NO_3^-/NH_4^+$ ratio of SH medium to 17.2, did not improve the biomass accumulation and caused the reduction in the secreted EGFP yields. In contrast, supplementation of $NH_4H_2PO_4$ and $NH_4NO_3$, which reduced the $NO_3^-/NH_4^+$ ratio to 1.1 and 2.8, substantially enhanced the biomass accumulation to 13.5 and 12.2 gDW/L, respectively. However, the secreted EGFP yields constantly decreased with the increasing supply of $NH_4H_2PO_4$, and slightly increased to 151 mg/L with the addition of 5 mM $NH_4NO_3$. These results indicate that it is possible to substantially improve the cell biomass production in the low-nitrogen SH medium through supplementing nitrogen with a suitable $NO_3^-/NH_4^+$ ratio (e.g., <10), however, limited increase in the secreted EGFP yields could be achieved. In fact, high cell biomass production caused the reduction in secreted protein yields because the rate-limiting step boosting the secretion of recombinant protein—full glycosylation of the $(SP)_{32}$ tag—was inhibited by rapid cell growth in media with a high nitrogen supply.

Nitrogen source and availability has been regarded as the critical factors for the productivity of plant cell cultures. Control of nitrogen supply was earlier reported to be a key to improving protein accumulation in plant cell cultures. For example, increased amounts of nitrogen in the culture medium had a dramatic impact on soluble 2G12

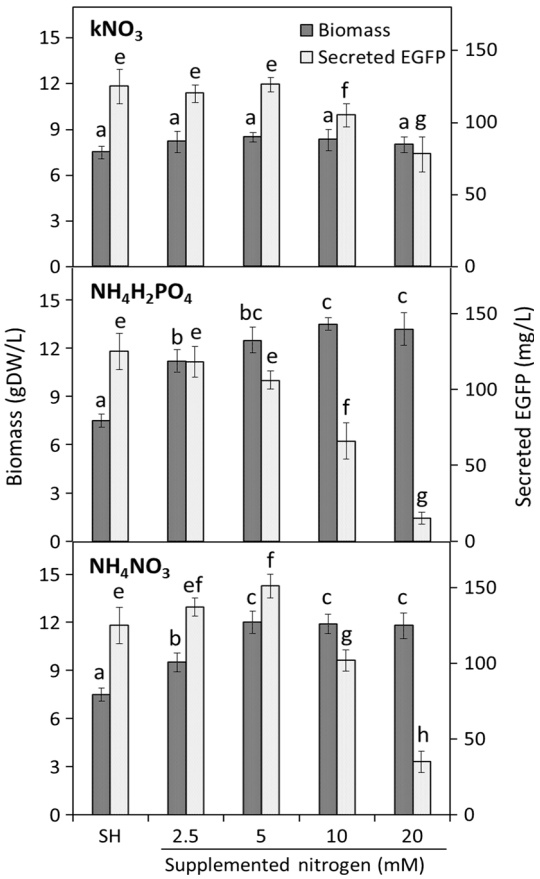


**Fig. 5** Cell biomass accumulation and secreted EGFP yields of the BY-2 cell cultures in SH medium supplemented with different sources of nitrogen: $KNO_3$, $NH_4H_2PO_4$ and $NH_4NO_3$. The *error bars* represent the standard deviation of three parallel cultures ($n = 4$). *Different letters* indicate significant difference ($p < 0.05$) in biomass yields (*a–c*) or secreted EGFP yields (*e–h*)

antibody yields in BY-2 cell culture, resulting in a 10-fold to 20-fold increase in product accumulation as well as stabilizing the secreted product (Holland et al. 2010). Supplementation of medium with additional ammonium doubled the amounts of recombinant GFP and influenza hemagglutinin protein produced by BY-2 cells (Ullisch et al. 2012). This is not surprising considering the fact that nitrogen plays a pivotal role in plant cell metabolism and is directly connected to amino acid and protein biosynthesis (Grimes and Hodges 1990). However, in the HypGP engineering technology, the effect of nitrogen on the secreted yields of the HypGP-tagged protein was not directly through the metabolic regulation of the protein synthesis as those reported earlier. Instead, the nitrogen supply (both the nitrogen levels and the $NO_3^-/NH_4^+$ ratio) significantly impacted the Hyp-*O*-glycosylation of the HypGP tag that functions as a molecular carrier to promote the secretion of the conjoined proteins. Dramatic secretion of HypGP-tagged proteins has a close correlation with the triggering of the second-stage glycosylation of the HypGP

tag leading to a fully glycosylated tag. Therefore, it is interesting to see a high secreted protein yield achieved even in the cultures with low cell biomass accumulation, e.g., the culture in MS-0N medium, as long as the HypGP tag could be fully glycosylated.

It is worth noting that B5 medium has low total nitrogen and a high $NO_3^-/NH_4^+$ ratio similar to SH and BDS medium (Table 1), however, the secreted protein yield obtained (46 mg/L) was significantly lower compared to BDS and SH medium (Fig. 2b), which was unexpected. Apparently, full glycosylation of the $(SP)_{32}$ tag was triggered in B5 medium, because the $(SP)_{32}$ could function in promoting the secretion of tagged EGFP. However, we noticed the cell culture in B5 medium appeared cloudy with many dead cells or cell debris present after the cell suspension was left still for 5 min, allowing the cells to settle down. In contrast, the cells grown in SH or BDS medium settled down fast and the supernatant quickly became clear, as can be seen in Fig. 2a. Some unknown factors in B5 medium possibly affected the physiological status and/or protein synthesis of the cultured cells, leading to lower secreted protein yields in B5 medium than in SH or BDS medium.

The HypGP tag used in this study—$(SP)_{32}$ represents a typical glycosylation module of AGPs occurring in the plant cell wall and plasma membrane. So far, the precise process of Hyp-*O*-glycosylation of AGPs in plant cells, presumably undergoing two-stage glycosylation as implied by our results, has been largely unknown. This is the first report demonstrating the profound effects of medium compositions, particularly nitrogen supply, on the Hyp-*O*-glycosylation of an AGP module and subsequent extracellular secretion in plant cells. However, we do not think the nitrogen supply has direct impact on the glycosylation of the AGP module. Instead, the nitrogen supply affects the metabolism and physiological status of the cultured cells, which in turn impacts the *O*-glycosylation of the $(SP)_{32}$ module. The key mechanism with which, presumably through a stress response of the cultured cells (e.g., nitrogen-starvation stress), has yet been fully understood. However, it appears that plant cell cultures expressing a signature AGP motif, e.g., $(SP)_{32}$, can serve as an ideal model for carrying out mechanism studies to better understand the *O*-glycosylation process of AGPs. Such understanding may provide valuable insight into more fully leveraging the designer HypGP engineering technology for enhanced plant cell-based recombinant protein production.

## Conclusion

The dramatic secretion of the $(SP)_{32}$-tagged EGFP correlates with the full Hyp-*O*-glycosylation of the $(SP)_{32}$ tag. The macronutrients of culture media, specifically, nitrogen supply has a profound impact on the Hyp-*O*-glycosylation of the $(SP)_{32}$ tag and subsequent secretion of the conjoined protein (EGFP). Modification of the nitrogen-rich MS medium with reduced $NH_4NO_3$ supply (from 1/2 of its original level to $NH_4NO_3$-free) triggered high-yield secretion of the $(SP)_{32}$-tagged EGFP up to 168 mg/L. This study not only provides an essential basis for proper medium designing to achieve the highest secreted protein yields with the HypGP engineering technology, but also improves our understanding of the Hyp-*O*-glycosylation process of AGPs in plant cells.

**Author contribution statement** JX, MD and GP conceived and designed research. NZ and DW conducted experiments. JX, NZ and MD analyzed data. JX and MD wrote the manuscript. All authors read and approved the manuscript.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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