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Original Paper

Effect of nitrate concentration in nutrient solution on hemagglutinin content of *Nicotiana benthamiana* leaves in a viral vector-mediated transient gene expression system

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Abstract Viral vector-mediated transient expression enables rapid biopharmaceutical protein production using whole plants. In recent years, plant growth conditions have received close attention as factors influencing the content of recombinant protein. In particular, because nitrogen is an important resource used in protein synthesis, nitrogen supplied to the root zone may influence accumulation of recombinant proteins. We tested whether fertilization of *Nicotiana benthamiana* with high-nitrate nutrient solution during plant growth prior to *Agrobacterium*-mediated delivery (agroinfiltration) of a tobamoviral vector resulted in high recombinant influenza hemagglutinin (HA) content per unit of leaf fresh weight and/or per plant. Nutrient solution containing 60 mM nitrate yielded 40% higher HA content per unit of leaf fresh weight, and comparable HA content per plant, relative to nutrient solution containing 12 or 36 mM nitrate. There were positive correlations among HA, total soluble protein (TSP), and soluble reduced-nitrogen content, suggesting that fertilization with the high-nitrate nutrient solution increased soluble reduced nitrogen and subsequently provided high TSP and HA. Although the increased HA content per unit of leaf fresh weight was offset by reduced leaf fresh weight on a per plant basis, nitrate-enriched fertilization represents an easily applicable technique to obtain a given amount of HA from smaller leaf biomass, thereby potentially reducing downstream processing costs for HA vaccine production using viral vector-mediated transient expression systems.

Key words: Influenza vaccine antigen, nitrogen fertilization, plant growth conditions, plant-made pharmaceutical (PMP), tobacco.

Plants are emerging as promising alternatives to conventional platforms for the production of recombinant proteins, including biopharmaceuticals. Plants can produce a wide range of biopharmaceutical proteins, including vaccine antigens, hormones, growth factors, blood proteins, cytokines, and antibodies; many such plant-made pharmaceuticals (PMPs) have demonstrated their intended biological activities *in vitro* and *in vivo* (De Muynck et al. 2010; Liénard et al. 2007; Rybicki 2010). PMP production systems using whole plants combine efficient protein production at a large scale, low risk of contamination with human pathogens or toxins, and the ability to confer post translational modifications on target proteins, and therefore hold many advantageous features over conventional platforms, such as bacterial, insect, or mammalian cell culture-based systems (Fischer et al. 1999; Fischer and Emans 2000; Houdebine 2009; Ma et al. 2003; Mett et al. 2008).

Transient gene expression with viral or non-viral vectors and stable transformation of the nuclear or chloroplast genome are the major strategies for expressing recombinant proteins in whole plants (Matoba et al. 2011). In particular, viral vector-mediated transient expression systems generally offer high-level accumulation of recombinant proteins on rapid timescales within several weeks. Several biopharmaceuticals, such as influenza subunit vaccine antigens and monoclonal antibodies for various infectious diseases, have been produced via viral vector-mediated transient expression systems (Pogue et al. 2010; Warzecha 2012). Furthermore, the initial clinical safety and efficacy of such biopharmaceuticals have been confirmed in several reports (Girard et al. 2013; McCormick et al. 2008; Yusibov et al. 2011), demonstrating the feasibility of this emerging technology.

A great deal of effort has been devoted to engineering viral vectors to increase expression levels of recombinant PMP proteins (Gleba et al. 2007; Matoba et al. 2011;

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Rybicki 2010). However, less attention has been paid to plant growth conditions. Recombinant proteins are biosynthesized from substances derived from the plant itself; therefore, plant growth conditions, which exert significant effects on plant metabolism and nutritional status, likely influence the level of recombinant protein accumulation. In fact, recent studies have shown that environmental factors, such as light intensity and temperature during plant growth, significantly influence recombinant protein production (Buyel and Fischer 2012; Matsuda et al. 2012). Therefore, we propose that optimizing plant growth conditions should be a fundamental approach to increasing the accumulation of PMP proteins.

The amount of nitrogen (N) supplied to the root zone is a potentially influential parameter of the growth conditions. Because N is an important resource used for protein biosynthesis, the amount of N in the growth medium is predicted to affect the accumulation of recombinant proteins, particularly in transient overexpression systems. In previous studies using transgenic tobacco cell-culture systems, additional nitrate supply was shown to enhance recombinant protein yields. For example, in transgenic *Nicotiana tabacum* BY-2 cell culture, the amounts of 2G12 antibody in supernatant of shake-flask culture and in culture medium of stirred tank bioreactor were increased by addition of 59 mmol l^{-1} (mM) and 100 mM nitrate to the culture media, respectively (Holland et al. 2010). Similarly, intracellular and extracellular accumulation of M12 antibody produced by transgenic *N. tabacum* hairy root cells was increased by additional nitrate supply of up to 12 g l^{-1} (Häkkinen et al. 2013). We hypothesized that fertilization with high-nitrate hydroponic nutrient solution could exert a similar effect on protein accumulation levels upon viral vector-mediated transient expression in whole plants. Assuming that plant biomass may not be significantly affected under such conditions, elevated recombinant protein content per unit of plant biomass should increase the protein yield per plant, which represents an ultimate goal of our research. Meanwhile, independent of overall yield, increasing the recombinant protein content per unit of biomass should improve the cost-effectiveness of downstream processes such as protein extraction and purification, because starting the process with a smaller amount of biomass would reduce the use of downstream consumables (Buyel and Fischer 2012).

The aim of this study was to test whether fertilization of host plants with high-nitrate hydroponic nutrient solution would result in increase in recombinant protein content, per unit of leaf fresh weight (FW) and/or per plant, in a viral vector-mediated transient expression system. As a representative system of this type, we selected the magnICON "deconstructed" tobamovirus vector (Gleba et al. 2005, 2007; Marillonnet et al. 2004; Marillonnet et al. 2005) because of its proven ability to express various biopharmaceutical proteins at high levels in *N. benthamiana* (Hamorsky et al. 2013; Matoba et al. 2011; Rybicki 2010). Hemagglutinin (HA), a vaccine antigen of the influenza virus, was used as a model PMP protein. In order to investigate the effect of nitrate concentration on plant N status, we measured reduced and nitrate-N contents in plant leaves.

The plasmid pNM216 used in this study was described previously (Matsuda et al. 2012). A cDNA encoding HA derived from influenza A virus strain A/California/07/2009 (H1N1), modified to contain an N-terminal secretory signal peptide and a C-terminal endoplasmic reticulum-retention signal peptide, was subcloned into the plasmid vector pICH26212 (magnICON; Icon Genetics GmbH, Halle [Saale], Germany) containing cDNAs encoding tobamovirus-derived RNA dependent RNA polymerase and movement protein. The resulting plasmid, pNM216, was transferred into *Agrobacterium tumefaciens* strain GV3101::pMP90 (Koncz and Schell 1986) by the freeze/thaw method (Holsters et al. 1978).

Seeds of *N. benthamiana* were sown into a plug tray filled with a substrate containing rockwool and peat moss (Bestmix No. 3; Nippon Rockwool Co., Tokyo, Japan), germinated, and grown in a temperature-controlled room under white fluorescent lamps at a photosynthetic photon flux density (PPFD) of 200 μmol m^{-2} s^{-1} during a 16-h day^{-1} photoperiod at temperatures of 25/21°C (day/night). The tray was sub-irrigated once daily with tap water for the first 7 day, and subsequently with modified nutrient solution described previously (Matt et al. 2001), in which the concentrations of major nutrients were as follows: 4.0 mM KNO_3, 8.0 mM $NaNO_3$, 1.0 mM KH_2PO_4, 2.4 mM $MgSO_4$, and 4.0 mM $CaCl_2$. On day 14 after seeding, each seedling was transplanted to a 0.65-l plastic pot filled with the same substrate, irrigated once daily with 50 ml of the nutrient solution, and grown in the same room for an additional 14 days.

Starting on day 28 after seeding, plants were supplied with the nutrient solution described above (12 mM total nitrate: N12) or the same solution supplemented to a total nitrate concentration of 36 mM (N36) or 60 mM (N60). The nitrate concentrations of the N36 and N60 nutrient solutions were achieved, respectively, by adding 24 and 48 mM $NaNO_3$ to the N12 nutrient solution. Molar equivalents (Eq) of all solutes in the N12, N36, and N60 nutrient solutions were 56, 104, and 152 mEq l^{-1}, respectively.

The bacteria harboring the magnICON vector were harvested during the logarithmic growth phase and resuspended in infiltration buffer as previously described (Matoba et al. 2010) to give an absorbance at 600 nm of 0.03. On day 42 after seeding, plants were subjected to

vacuum agroinfiltration with the bacteria as previously described (Matsuda et al. 2012). The agroinfiltrated plants were incubated for 7 days in temperature-controlled growth chambers (MIR-554; Panasonic Co., Osaka, Japan) equipped with white fluorescent lamps at a temperature of 20°C throughout the day at a PPFD of 100 μmol m^{-2} s^{-1} during a 16 h day^{-1} photoperiod (Matsuda et al. 2012). Plants were watered once daily with 50–100 ml of tap water, based on a preliminary experiment showing that no significant difference was found in HA content per unit of leaf fresh weight and a negative effect was found in growth among plants supplied with tap water and N12 and N36 nutrient solutions for the 7-day post-agroinfiltration period (data not shown).

After the 7-day incubation period following vacuum agroinfiltration, parts of the fully expanded seventh through ninth leaves were sampled and subjected to biochemical assays. Sampled fresh leaf parts were homogenized, and the HA content was quantitated by direct enzyme-linked immunosorbent assay (ELISA) using a rabbit polyclonal anti-HA antibody as previously described (Matsuda et al. 2012). HA content per plant was estimated by multiplying HA content per unit of leaf FW by leaf FW per plant at 7-day post infiltration (dpi). Total reduced-N, soluble reduced-N, and total soluble protein (TSP)-N contents of leaves were quantitated with Nessler's reagent after Kjeldahl digestion (Makino et al. 1984). Nitrate was extracted with 80% (v/v) ethanol, and the content was determined colorimetrically (Cataldo et al. 1975). Leaf dry weight (DW) per plant was measured as previously described (Matsuda et al. 2012).

HA content in N60 leaves was significantly greater (approximately 40%) than that in N12 leaves on a FW basis (Figure 1A). Total reduced-N, soluble reduced-N, TSP-N, and nitrate-N contents per unit of leaf FW at 7 dpi did not differ significantly among treatments, although all of these measurements except for total reduced N tended to be slightly higher in N36 and N60 than in N12 (Table 1). There was a positive correlation between HA and TSP-N contents (Figure 1A, Table 1), as previously reported in plants grown at different temperatures post-agroinfiltration (Matsuda et al.

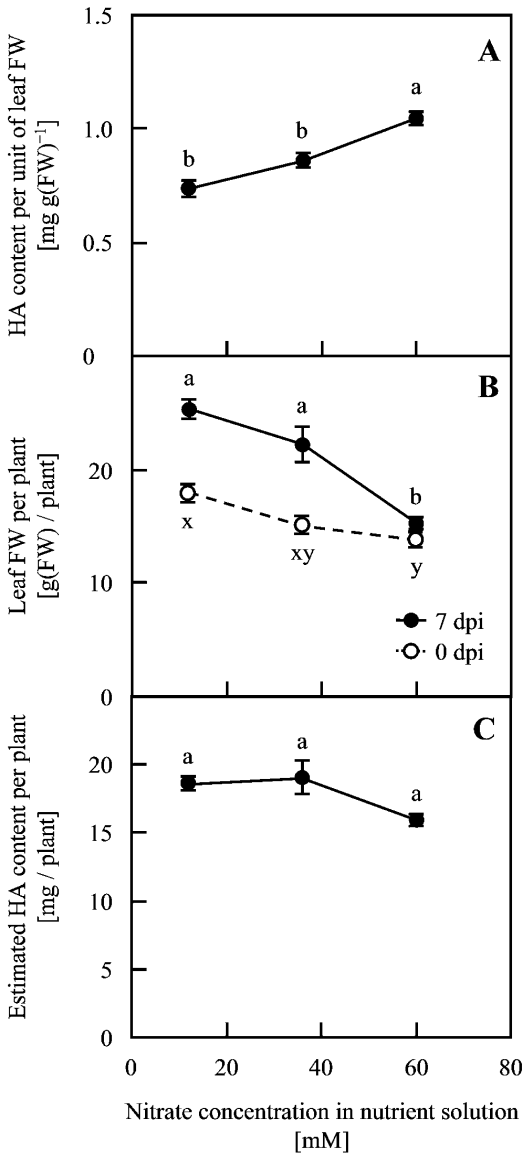

Figure 1. Hemagglutinin (HA) content per unit of leaf fresh weight (FW) at 7 days post infiltration (dpi) (A), leaf FW per plant at 0 and 7 dpi (B), and estimated HA content per plant at 7 dpi (C) in *Nicotiana benthamiana* grown in nutrient solution containing 12, 36, or 60 mM nitrate for 14 days before vacuum agroinfiltration. Vertical bars represent standard errors of the means (n=4). Means labeled with different small letters in each panel and dpi differ significantly from each other at the 5% level, as determined by the Tukey–Kramer's HSD test.

Table 1. Total reduced-nitrogen (N), soluble reduced-N, total soluble protein (TSP)-N, and nitrate-N contents per unit of leaf fresh weight (FW), and the ratio of leaf dry weight (DW) to FW, at 7 days post infiltration in *Nicotiana benthamiana* grown in nutrient solution containing 12 (N12), 36 (N36), or 60 (N60) mM nitrate for 14 day before vacuum agroinfiltration.

Treatment	Total reduced-N [μmol g(FW)$^{-1}$]	Soluble reduced-N [μmol g(FW)$^{-1}$]	TSP-N [μmol g(FW)$^{-1}$]	Nitrate-N [μmol g(FW)$^{-1}$]	Leaf DW to FW [%]
N12	804±10	134±5	65±8	47±3	7.51±0.07
N36	857±56	137±4	70±2	55±2	7.84±0.13
N60	841±39	162±6	84±5	54±4	7.91±0.11
Analysis of variance	N.S.	N.S.	N.S.	N.S.	N.S.

N.S. indicates that there was no significant difference among treatments at the 5% level, as determined by the analysis of variance.

2012), and also between HA and soluble reduced-N contents (Figure 1A, Table 1). These results suggest that fertilization with high-nitrate nutrient solution increased soluble reduced N, which in turn yielded high TSP and HA contents. Interestingly, unlike the soluble reduced-N content, the total reduced-N component was not significantly affected by nitrate concentration in the nutrient solution. The biological mechanism accounting for this observation remains to be determined.

Leaf FW in the N60 treatment at 7 dpi was significantly lower than that in N12, and this tendency was also observed at 0 dpi (Figure 1B). We speculate that the reduction of leaf FW was attributed to osmotic stress induced by the approximately 3-fold higher molar equivalent in the N60 nutrient solution relative to the N12 solution, which may have inhibited water uptake, cell elongation, and/or leaf development (Horie et al. 2012). A slightly higher ratio of DW to FW (or lower water content) of leaves in N60 than in N12 (Table 1) may be an indication of such osmotic stress. Nevertheless, it is worth noting again that such stress did not seem to have a negative effect on the expression and accumulation of HA in leaf tissue; in fact, significantly higher HA content per unit of leaf FW was obtained under such conditions.

In a transgenic cell culture system, osmotic stress induced by adding mannitol to the culture medium is reportedly useful in stabilizing extracellularly-secreted recombinant proteins and thereby enhancing protein yields (Soderquist and Lee 2005). However, in our preliminary study, we observed that HA content per unit of leaf fresh weight was not increased by addition of 12–36 mM NaCl, which corresponded to 24–72 $mEq\,l^{-1}$, to the N12 nutrient solution of 56 $mEq\,l^{-1}$ (data not shown). Hence, osmotic stress simply applied to the root zone may not contribute to increasing protein accumulation levels in transient expression systems using whole plants.

Estimated HA content per plant did not differ significantly among treatments (Figure 1C). Therefore, the N60 solution supplied before agroinfiltration gave a comparable HA content per plant, and a significantly higher HA content per unit of leaf FW, relative to the N12 and N36 solutions. This may offer a significant advantage to the downstream processing of HA vaccine production, given that the volume of buffer used to extract leaf proteins is proportional to the amount of leaf biomass (Matoba et al. 2011). Our results showed that a similar amount of HA could be produced in ~30% less leaf biomass with the high-nitrate nutrient solution compared to conventional conditions, suggesting that ~30% less buffer (hence, significantly reduced column loading time and liquid handling) may be used to obtain a given amount of purified HA vaccine.

The reduction of plant growth in N60 (Figure 1B) was probably due to a detrimental effect of excess nitrate supply as observed in *N. tabacum* (Stöhr 1999). In this regard, CO_2 enrichment may provide a solution to mitigate the reduction of growth without decreasing N and/or protein accumulation per unit of biomass, because such a treatment was reported to promote growth without decreasing protein content per unit of fresh weight in *N. tabacum* (Geiger et al. 1999). Combinatorial effects of nitrate fertilization and CO_2 environment on HA accumulation in transient expression system should be investigated in future work.

In summary, we achieved higher HA content per unit of leaf FW, without decreasing HA content per plant, in a viral vector-mediated transient expression system using *N. benthamiana* by fertilizing the host plants with high-nitrate nutrient solution during plant growth before agroinfiltration. Although increased HA content per unit of leaf FW was offset by reduced leaf FW per plant, the nitrate-enriched nutrient supply enabled "biological concentration" of HA in leaves. Our results suggest that fertilizing plants with high-nitrate nutrient solution represents an easily applicable technique for reducing the biomass required to obtain a given amount of HA, thereby potentially reducing downstream processing costs. An obvious next step is to determine plant growth conditions that can provide high HA accumulation without affecting dry matter production. The negative effects of high nitrate concentration on biomass production might be mitigated by decreasing osmotic stress and/or by controlling physical environment, e.g., modifying the nutrient composition and fertilization schedule, and CO_2 enrichment; this possibility should be addressed in future studies.

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