Research Report

Effect of Nitrogen Deficiency on Recombinant Protein Production and Dimerization and Growth in Transgenic Plants

Yangjoo Kang[1], Yong Kyoo Shin[2], Sang-Won Park[3], and Kisung Ko[1*]

[1]Department of Medicine, College of Medicine, Chung-Ang University, Seoul 06974, Korea
[2]Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 06974, Korea
[3]Resarch Policy Bureau, R&D Performance Evaluation & Management Division,
Rural Developement Administration, Jeonju 54875, Korea

*Corresponding author: ksko@cau.ac.kr

Abstract. This study investigated the effects of nitrogen (NH_4^+) in the soil on the expression of prostatic acid phosphatase (PAP)-IgA Fc and PAP-IgA FcK recombinant proteins in transgenic plants, and on plant growth in the greenhouse. Plant height and leaf length were greater in soil A (NH_4^+: 51 mg·L^{-1}) than in soil B (NH_4^+: 1 mg·L^{-1}). In soil B, the plant leaves turned yellow, with chlorophyll concentrations that were significantly lower than the chlorophyll concentration of leaves from plants grown in soil A. The expression level of the PAP-IgA Fc and PAP-IgA FcK proteins and the protein dimerization rates were lower in soil B when compared with soil A. In addition, PAP-IgA Fc and PAP-IgA FcK expression and dimerization levels were significantly lower in transgenic plants grown in soil B, when compared with transgenic plants grown in soil A. Soil characteristics such as hydrogen ion concentration (pH), electric conductivity (EC), and bulk density did not differ between soil A and B. Thus, appropriate soil NH_4^+ concentrations are essential for optimized plant growth and enhanced expression of dimerized PAP-IgA Fc and PAP-IgA FcK recombinant proteins in transgenic plants.

Additional key words: ammonia nitrogen, chlorophyll concentration, recombinant fusion proteins, prostatic acid phosphatase

Introduction

Plant expression systems have several advantages when compared with mammalian expression systems. For example, there is a low risk of contamination with infectious human pathogens, easy post-translational adjustment, and the production of recombinant proteins is cost-effective (Daniell et al., 2009; Lim et al., 2015b; Rigano and Walmsley, 2005). An additional advantage is the capacity of plant for large-scale production of biomass using only the inputs of light, water, soil, and nutrients (Lim et al., 2015b; Ma et al., 2003). Due to these advantages, a wide range of recombinant bio-therapeutic proteins, industrial enzymes, and protein polymers have been produced in plants (Ma et al., 2003).

The production of highly valuable recombinant therapeutic proteins in plants is affected by environmental factors, such as temperature, light, insects, pests, salinity, nutrition, and other soil conditions (Elbers et al., 2001; Jamal et al., 2012; Lim et al., 2015b; Passioura, 2002) and plant species (Song

et al., 2015). Changes in any of these factors can influence the large-scale production of plant biomass and eventually lead to a decreased efficiency of recombinant protein production. For example, changes in environmental factors such as temperature and day length, in combination with soil conditions, have been shown to affect the expression level of recombinant therapeutic proteins including plant growth (Britto et al., 2001; Britto and Kronzucker, 2002; Oh, 2015; Passioura, 2002).

Prostatic acid phosphatase (PAP) is a prostate cancer antigen that is highly expressed by malignant prostate cell tissue and is often used as a therapeutic glycoprotein (Lim et al., 2015b; McNeel et al., 2009; Saif et al., 2014; Tarassoff et al., 2006; Vihko et al., 1988). Previous studies showed that fusion of the Fc fragment of human IgG_1 to the glycoprotein GA733 could facilitate the purification of the protein, increase its half-life, and enhance the effectiveness of vaccination by targeting antigen-presenting cells (Czajkowsky et al., 2012; Larrick et al., 2001; Ma et al., 2003; Park et al., 2015), as
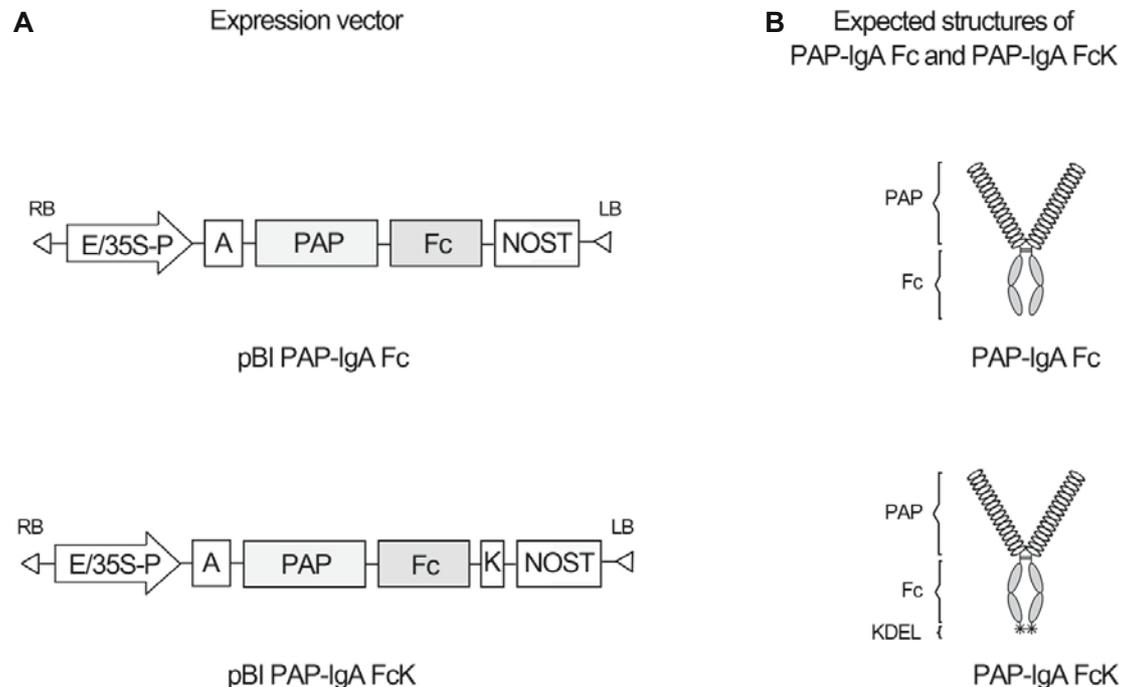
Fig. 1. Plant expression vectors PAP-IgA Fc and PAP-IgA Fc with the ER retention signal KDEL (PAP-IgA FcK). (A) Gene expression cassettes for PAP-IgA Fc and PAP-IgA FcK. E/35S-P, the cauliflower mosaic virus 35S promoter with a duplicated enhancer region; A, untranslated leader sequence of each tobacco virus; K, ER retention signal KDEL; and NOST, terminator of cauliflower mosaic virus 35S gene. (B) Predicted structures of recombinant fusion proteins. PAP, spring shape region; IgA Fc, gray oval region. The asterisk highlights the KDEL ER retention signal

well as enhance expression levels in plants (Lim et al., 2015b). In addition, the fusion of the KDEL endoplasmic reticulum (ER) retention signal to the recombinant GA733-Fc protein resulted in increased expression and oligomannose glycosylation levels (Lu et al., 2012).

In this study, plant growth, chlorophyll concentration, and recombinant protein expression patterns were investigated in transgenic plants expressing PAP-IgA Fc and PAP-IgA FcK fusion proteins, after growth in soil with different NH_4^+ concentrations, to determine whether NH_4^+ affects the quantity and quality of the expressed recombinant proteins including plant growth (Britto et al., 2001; Jang et al., 2014; Passioura, 2002).

Materials and Methods

Construction of the Plant Expression Vector

The N-terminus of the synthetic DNA sequence encoding prostatic acid phosphatase (PAP) (GenBank accession No. M34840.1) was extended with a 30-amino acid plant ER signal peptide (MATQRRANPSSLHLITVFSLLAAVVSAEVD). The recombinant fusion protein PAP-IgAFc was generated by fusing PAP and the Fc fragment of human IgA_1 (GenBank accession No. AY647978.1). PAP-IgA FcK was tagged at the C-terminal with an ER retention signal KDEL. The genes

were expressed under the control of the enhanced cauliflower mosaic virus (CaMV) 35S promoter and the tobacco viral 5′-leader sequence (TEV). The PAP-IgA Fc and PAP-IgA FcK expression cassettes were subcloned into the *Hin*dIII and *Eco*RI restriction enzyme sites of the binary plant expression vector pBI121 to yield pBI PAP-IgA Fc and pBI PAP-IgA FcK, respectively (Fig. 1).

Plant Transformation

Plant expression vectors pBI PAP-IgA Fc and PAP-IgA FcK were transferred into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Transgenic tobacco (*Nicotiana tabacum*) plants were generated by *Agrobacterium*-mediated transformation. Transgenic plant lines were selected on Murashige and Skoog medium (Duchefa biochemie, Haarlem, Netherlands) containing 100 mg·L^{-1} kanamycin and 250 mg·L^{-1} cefotaxime.

Measurement of Plant Growth

Transgenic plantlets were transferred to soil and grown in a growth chamber with a constant temperature of 23°C, 70% humidity and a 16:8-h light-dark cycle. Transgenic plants were grown in a greenhouse under controlled conditions. Ten transgenic plants expressing the PAP-IgA Fc and PAP-IgA FcK recombinant therapeutic proteins that were originally

Table 1. Analysis of the components of soil A and B, including pH (hydrogen ion concentration), EC (electrical conductivity), NH_4^+ (ammonia nitrogen)

Component	Soil A	Soil B
pH (1:5, v/v)	6.1	6.3
EC (1:5, v/v, ds·m^{-1})	0.39	1.20
NH_4^+ (mg·L^{-1})	51	1
Bulk density (g·cm^{-3})	0.27	0.22

established in an in vitro culture, were transplanted to soil A and B and grown for 4 weeks in a greenhouse (Table 1). Plant height and the leaf length of the top (T), middle (M), and basal (BA) leaves were measured.

Determination of Bulk Density

The bulk density of the soil was determined using a plunger compaction method. The soil was placed in a metal cylinder and the collar added without any pressure on the soil. Then, 500 g of weight was placed on top of the soil and allowed to stand for 3 min. The soil was flattened to the top of the cylinder using a spatula. The bulk density was calculated after weighing the cylinder containing soil. The bulk density of the samples was calculated as follows: bulk density (g/cm^3) = cylinder (g) - cylinder weight (g)/cylinder volume (cm^3). The bulk density analysis was repeated three times.

Measurement of the soil pH

The pH meter was calibrated with standard pH 4.01 and pH 7.00 solutions. 100 mL of distilled water was added to x (x = 20 × bulk density) grams of soil in a 250-mL Erlenmeyer flask, and the soil sample was shaken using a Multi Bio Shaker (Dasol Scientific, Seoul, Korea) for 1 h. The mixture was then allowed to stand without agitation for 1 h. The pH was measured using a pH meter (Thermo Scientific Orion, Waltham, MA) in less than 60 s.

Measurement of the soil Electrical Conductivity (EC)

X (x = 20 × bulk density) grams of soil were added to 100 mL of distilled water in a 250 mL Erlenmeyer flask and shaken using a Multi Bio Shaker (Dasol Scientific, Seoul, Korea) for 1 h. The mixture was filtered using 5-μm filter paper (Advantec, Japan), and the EC of the filtrate was measured directly using a conductivity meter (Thermo Scientific Orion, Waltham, MA).

Measurement of the Ammonia Nitrogen (NH_4^+) content

Ammonia nitrogen (NH_4^+) concentrations were determined using a Kjeldahl distiller (JISICO, Seoul, Korea). X (x = 10 × bulk density) grams of soil were added to 100 mL of 2 M KCl solution in a 100 mL Erlenmeyer flask and shaken for 30 min. The sample was filtered through 5-μm filter paper (Advantec, Japan). Boric acid solution (2%) was added to the 100 mL Erlenmeyer flask as a standard. A fixed quantity of filtrate was added into a semi-micro Kjeldahl flask, and the distillate was added to MgO (approximately 0.2 - 0.3 g). The NH_4^+ concentration was calculated using the following formula: NH_4^+ (mg·L^{-1}) = (T-B) × N × F × 14 × (1/1,000) × (Quantity of extracting solution/the sample solution) × (1/W) × 1,000,000. The letters are defined as follows: T, standard solution of sulfuric acid (mL); B, standard solution of sulfuric acid used as a blank (mL); N, normality of the standard solution of sulfuric acid; F, factor of standard solution of sulfuric acid; and W: weight of the soil (g).

Statistical Analysis

All values are presented as the mean ± SD. Comparisons of transgenic plant growth and chlorophyll concentration were analyzed using the unpaired t-test, where $p < 0.01$ was considered statistically significant. Statistical significance was determined using Microsoft Excel statistical software (Microsoft Corporation, Microsoft Office Excel 2013, Redmond, WA).

Chlorophyll Concentration

In vitro transgenic plants were transplanted to pots containing soil A or B and grown in the greenhouse for 4 weeks. Five PAP-IgA Fc and PAP-IgA FcK transgenic plants were used for the analysis of chlorophyll content. Ten-millimeter diameter leaf pieces were randomly sampled from the leaves of each transgenic plant in soil A and B pots using a 10-mm cork borer. All samples were subsequently incubated in 95% ethanol for 48 h at 37°C with continuous shaking. The samples were centrifuged at 12,470×g for 2 min at room temperature and transferred to 96-well plates (Nunc, Roskilde, Denmark). The chlorophyll content in the clarified extract was calculated from absorbance measurements at 644 and 648 nm, using the following formula (Ch a+b = 5.24 × A_{644} + 22.24 × A_{648}), where Ch is the chlorophyll concentration in micrograms per milliliter and A is absorbance at the given wavelength (Lim et al., 2015a). Each measurement was repeated four times.

PCR Amplification of Genomic DNA

Genomic DNA was extracted from fresh leaf tissue of transgenic and non-transgenic plants using a DNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. At least five plants of each PAP-IgA Fc and PAP-IgA FcK transgenic line were used for PCR analysis. PCR amplification of genomic DNA was used to confirm the presence of the recombinant genes using the following primer pairs: for PAP-IgA Fc, forward primer 5′-GCC CTC GTT TTC AAG AAC TTG-3′, reverse primer 5′- CGG GAT CCT TAG TAA CAT GTG CCA TCA ACC TC- 3′; for PAP-IgA FcK, forward primer 5′-GCC CTC GTT TTC AAG AAC TTG-3′, reverse primer 5′-GGA TCC TTA TAA TTC GTC CTT G-3′. The PCR conditions were as follows: initial denaturation at 94°C for 3 min; 27 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 30 s; and a final extension at 72°C for 7 min. A non-transgenic plant was used as a negative control. The elongation factor 1-α (*EF-1α*) gene, which is involved in plant growth was used as a reference gene. The expected size of the DNA products for PAP-IgA Fc and PAP-IgA FcK were 1,332 and 1,334 bp, respectively.

Immunoblot Analysis

Five plants were used for immunoblot analysis. All samples had the same fresh weight of leaves. For each sample, 100 mg of leaf was homogenized in 300 μL 1× phosphate buffer saline (137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl and 2 mM KH_2PO_4) solution to extract the total protein content. A volume of 20 μL of extracted sample was mixed with 5 μL of loading buffer (1 M Tris-HCl, 50% glycerol, 10% SDS, 5% 2-mercaptoethanol, 0.1% bromophenol blue), loaded in 10% SDS-PAGE, and transferred to a nitrocellulose membrane (Millipore Corp, Billerica, MA). Membranes were blocked with 5% skim milk (Sigma, St. Louis, MO) in 1× PBS buffer for 1 h 30 min at room temperature. The blots were incubated for 2 h at room temperature with a 1:4,000 diluted rabbit anti-human PAP monoclonal antibody (Abcam Inc., Cambridge, MA). After 30 min of rinsing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti rabbit IgG heavy and light chain polyclonal antibody (Bethyl Laboratories, Montgomery, TX) for 2 h. Antibody binding was detected using chemiluminescence substrate (Bio-Rad Laboratories Inc., Hercules, CA) and visualized by exposing the membrane to an X-ray film (Fuji, Tokyo, Japan). A non-transgenic plant was used as the negative control and the immunoblot analysis was repeated three times. The detected protein bands were digitized to an electronic image and the band intensity was measured using Image J software (National Institutes of Health, Bethesda, MD).

Results

Generation of PAP-IgA Fc and PAP-IgA FcK

PCR and western blot analyses confirmed the gene insertion and protein expression of PAP-IgA Fc and PAP-IgA FcK in 10 randomly selected in vitro transformants (Fig. 2A and 2B). The PCR analysis confirmed that all transgenic plant lines contained either PAP-IgA Fc or PAP-IgA FcK and showed the expected bands at 1,964 bp and 1,976 bp, respectively (Fig. 2C and 2D). Expression of PAP-IgA Fc and PAP-IgA FcK proteins in all transgenic plant lines except for line #7 was confirmed by western blotting. Transgenic lines expressing PAP-IgA Fc or PAP IgA FcK showed a protein band of approximately 72 kDa (Fig. 2E and 2F). Lines #6 (E) and #4 (F) were selected for further study (Fig. 2E and 2F).

Stem Height and Leaf Length in Soils A and B

Transgenic plants expressing PAP-IgA Fc or PAP-IgA FcK were transplanted to pots containing soils A or B and grown in a greenhouse for 4 weeks. There were significant differences in plant height and leaf length between plants grown in soil A and B (Fig. 3A and 3B). The height of plants grown in soil A (PAP-IgA Fc: 42.3 cm, PAP-IgA FcK: 35.0 cm) was six times greater than that of plants grown in soil B (PAP-IgA Fc: 7.9 cm, PAP-IgA FcK: 6.4 cm). The leaf length of transgenic plants grown in the soil A (PAP-IgA Fc: 19.0 cm, PAP-IgA FcK: 17.0 cm) was three times longer than the leaf length of plants grown in soil B (PAP-IgA Fc: 5.98 cm, PAP-IgA FcK: 5.17 cm). Overall, growth in plants growing in soil A was increased when compared with plants growing in soil B (Fig. 3C and 3D).

Chlorophyll Concentration in Leaves of PAP-IgA Fc and PAP-IgA FcK Transgenic Plants in Soils A and B

A spectrophotometer was used to determine the chlorophyll concentration in leaf samples harvested from PAP-IgA Fc and PAP-IgA FcK transgenic tobacco plants 4 weeks after transplantation to soils A or B. The chlorophyll content in leaves of transgenic plants grown in soil A (PAP-IgA Fc: 14.2 mg·g^{-1}FW, PAP-IgA FcK: 16.0 mg·g^{-1}FW) was higher in both PAP-IgA Fc and PA-IgA FcK than in plants grown in soil B (PAP-IgA Fc: 4.0 mg·g^{-1}FW, PAP-IgA FcK: 4.4 mg·g^{-1}FW) (Fig. 4B). The color of leaves from transgenic plants grown in soil A and B was green and yellow, respectively (Fig. 4A).

Confirmation of the Presence of PAP-IgA Fc and PAP-IgA FcK Genes in Transgenic Plants Grown in Soil A and B

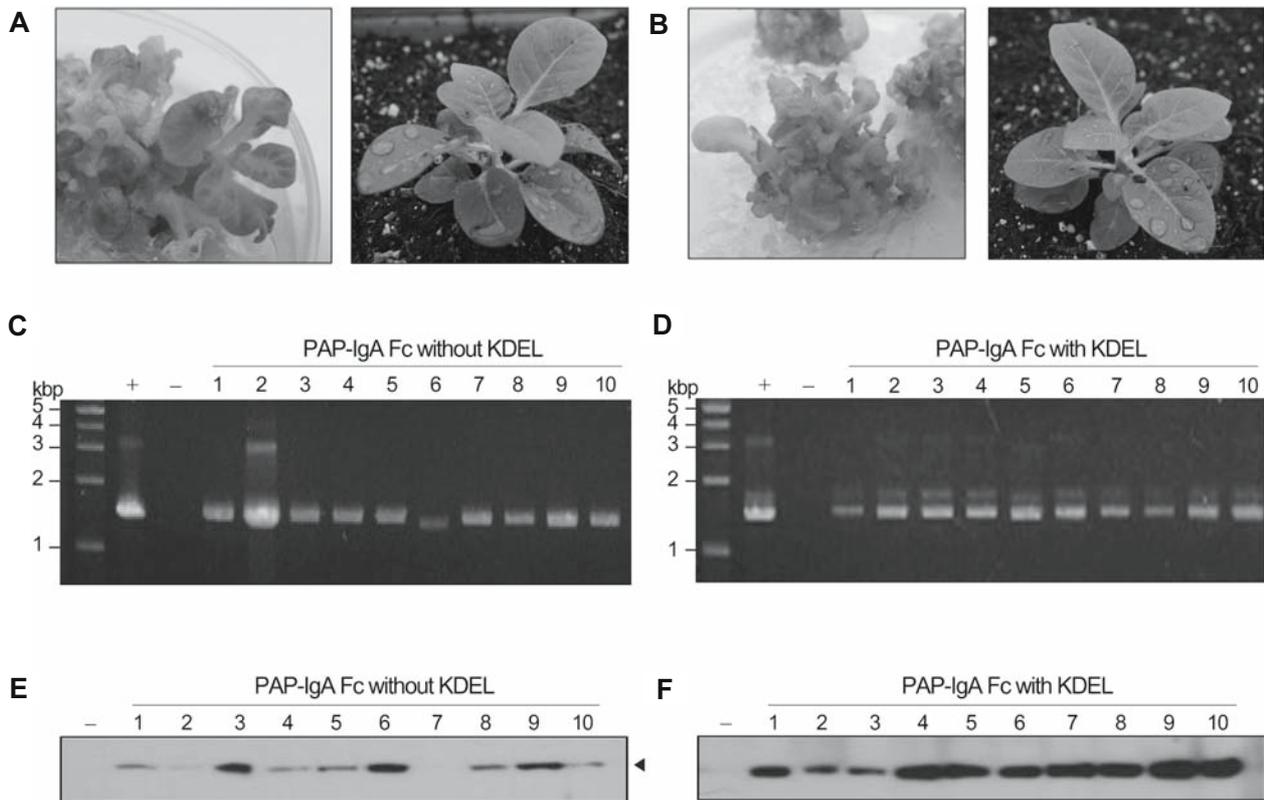PCR was used to verify the presence of PAP-IgA Fc and

Fig. 2. PCR and Western blot analyses to select transformants. Transgenic shoots generated from PAP-IgA Fc (A left) and PAP-IgA FcK (B left) transformed tobacco callus on selection medium containing kanamycin (100 mg·L^{-1}) and cefotaxime (250 mg·L^{-1}) and selected transgenic tobacco plants PAP-IgA Fc (A right) and PAP-IgA FcK (B right). Confirmation of PAP-IgA Fc (1,964 bp) (C) and PAP-IgA FcK (1,976 bp) (D) gene insertion in transgenic plants. A positive control (+) indicates PCR amplified DNA from the vector pBI121 PAP-IgA F*c*; A negative control (-) indicates the genomic DNA of a non-transgenic tobacco plant. Conformation of PAP-IgA Fc (71.7 kDa) (E) and PAP-IgA FcK (72 kDa) (F) protein expression in transgenic plants. A negative control (-) indicates a non-transgenic tobacco plant.

PAP-IgA FcK genes in leaf samples harvested from transgenic plants 4 weeks after transplantation to soils A or B from an in vitro tissue culture. The presence of the transgene in the plant genomic DNA was confirmed in five randomly selected transgenic lines. The transgenic plant lines containing the PAP-IgA Fc or PAP-IgA FcK transgenes showed the expected amplified bands at 1,332 up and 1,344 bp, respectively (Fig. 5). The EF-1α band was used as a standard control and was detected at 67 bp.

Expression of PAP-IgA Fc or PAP-IgA FcK Proteins in Transgenic Plants Grown in Soils A or B

Expression of PAP-IgA Fc and PAP-IgA FcK proteins in leaves of transgenic plants 4 weeks after transplanting to soil A or B was investigated by western blotting. Protein expression levels of PAP-IgA Fc and PAP-IgA FcK were significantly lower after transplanting to soil B than after transplanting to soil A (Fig. 6A). In addition, protein polymerization (Fig. 6B), dimerization (Fig. 6C), and monomerization (Fig. 6D) increased in both PAP-IgA Fc and PAP-IgA FcK transgenic plant lines that were grown in soil

A when compared with transgenic plants grown in soils B.

Discussion

This study demonstrates that in transgenic plants, the characteristics of the soil, particularly the NH_4^+ concentration, affect plant growth and the expression of prostatic acid phosphatase (PAP)-IgA Fc and PAP-IgA FcK fusion proteins. PAP-IgA Fc and PAP-IgA FcK were generated by fusing PAP to the Fc fragment of IgA1 type human immunoglobulin, with and without the KDEL ER retention signal. The signal peptide sequence (MATQRRANPSSLHLITVFSLLAAVVSAEVD) of the N-terminus of PAP from *Nicotiana plumbaginifolia* was modified to be correctly targeted to the ER, and C-terminus of the Fc was fused to the KDEL of the ER retention signal (Lu et al., 2012; Lim et al., 2015b). The PAP-IgA Fc and PAP-IgA FcK genes were under the control of a duplicated CaMV 35S promoter.

PAP-IgA Fc and PAP-IgA FcK transgenic plants were planted in different soils (A and B) and grown for more than one month. The soil characteristics such as the hydrogen ion
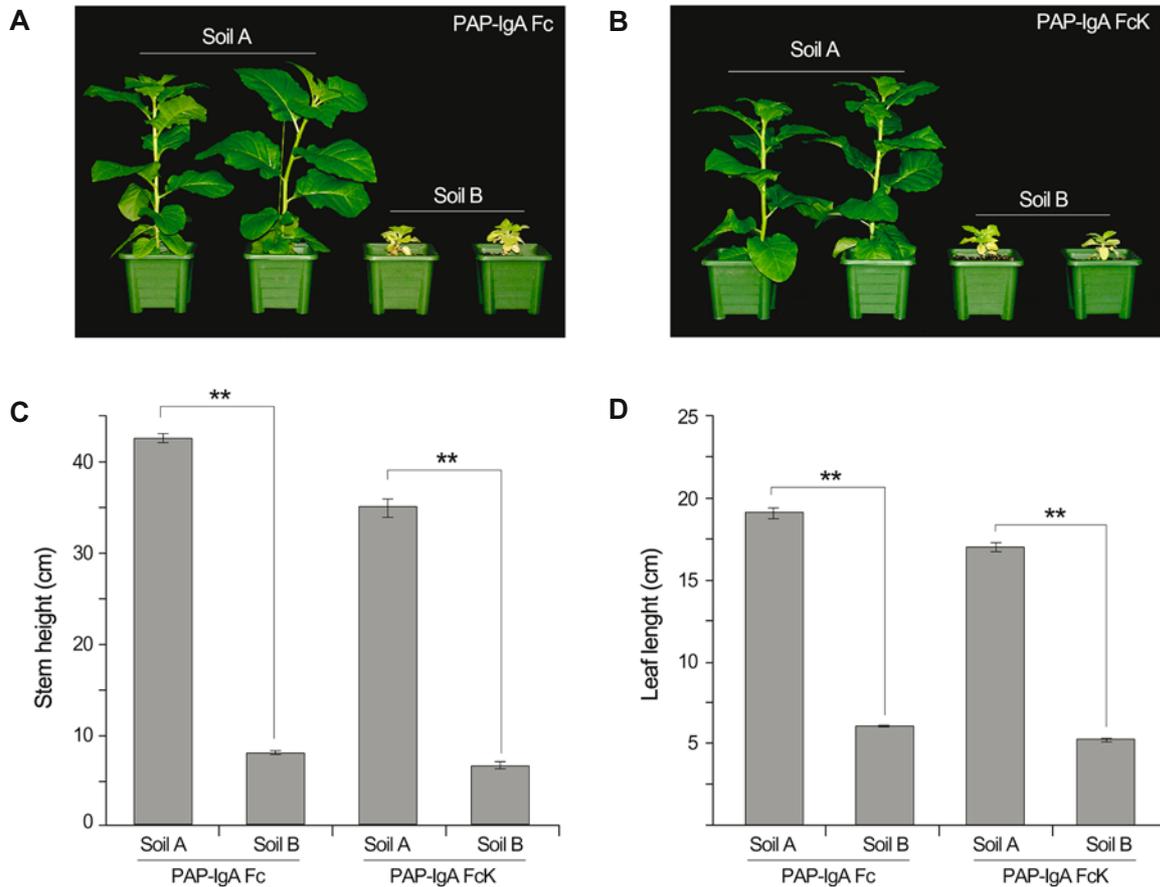
Fig. 3. Plant growth patterns (stem height and leaf length) of PAP-IgA Fc and PAP-IgA FcK transgenic plants in soil A and B. Comparison of plant growth of the PAP-IgA Fc (A) and PAP-IgA FcK (B) transgenic plants grown for 4 weeks in soil A (NH_4^+: 51 mg·L^{-1}) or soil B (NH_4^+: 1 mg·L^{-1}). Stem height (C) and the leaf length (D) of PAP-IgA Fc and PAP-IgA FcK transgenic plants are shown where asterisks indicate significant differences (**$p < 0.01$, t-test).
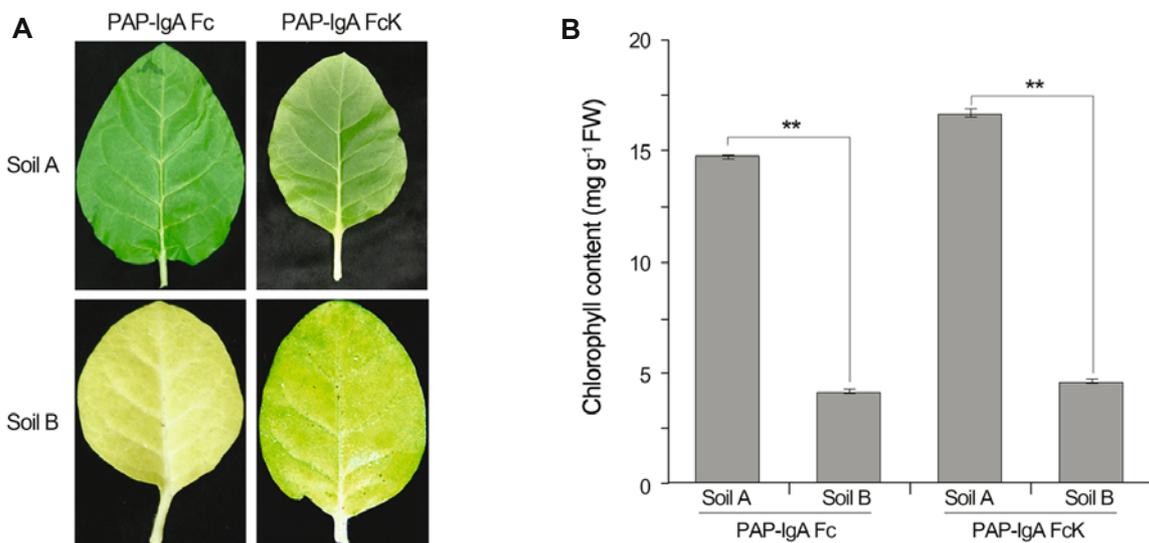

Fig. 4. Chlorophyll concentration of PAP-IgA Fc and PAP-IgA FcK plant leaves grown in soil A or B. (A) Comparison of leaf color of the PAP-IgA Fc and PAP-IgA FcK transgenic plants grown for 4 weeks in soil A (NH_4^+: 51 mg·L^{-1}) or B (NH_4^+: 1 mg·L^{-1}). (B) Chlorophyll content of leaves from PAP-IgA Fc and PAP-IgA FcK transgenic plants in soil A or B. The asterisks indicate significant differences (**$p < 0.01$, t-test).
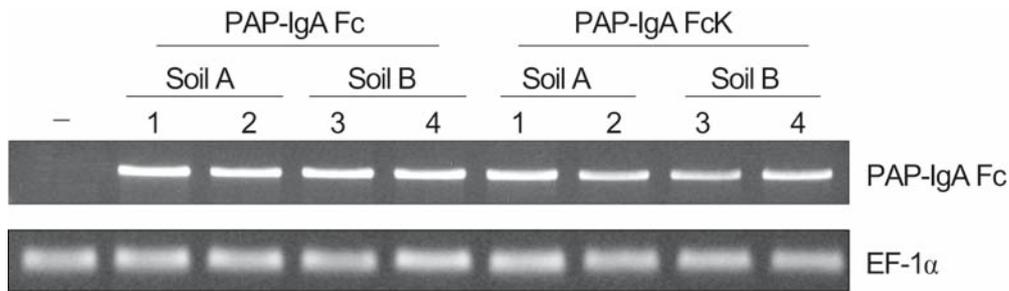
Fig. 5. Confirmation of PAP-IgA Fc and PAP-IgA FcK gene insertion in transgenic plants. Genomic DNA of PAP-IgA Fc (1,964 bp) and PAP-IgA FcK (1,976 bp) transgenic plants in soil A or B. The negative control (−) consists of genomic DNA of a non-transgenic tobacco plant. The EF-1α gene (67 bp) was used as a control gene.
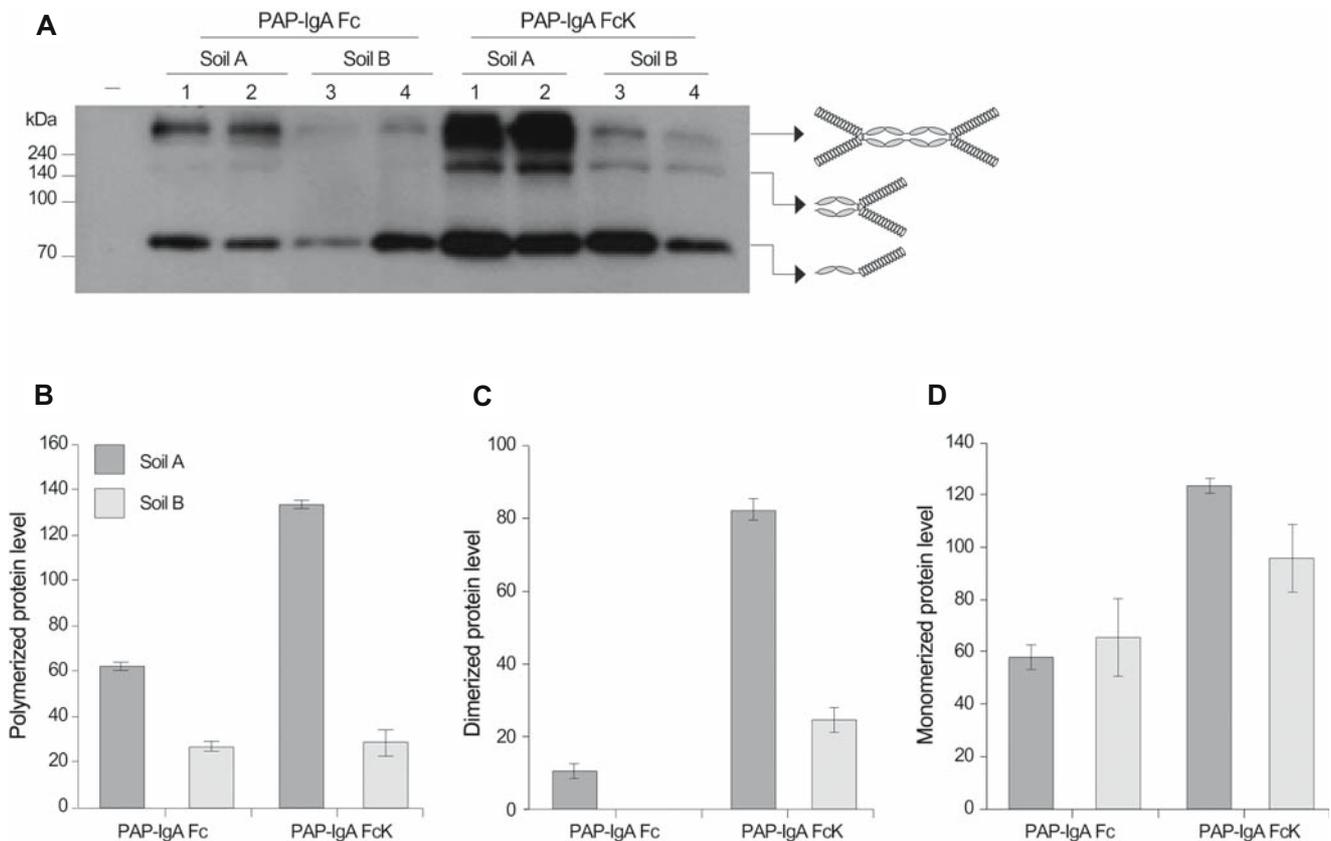

Fig. 6. Effect of soil components on the expression level of polymerized, dimerized, and monomeric PAP-IgA Fc and PAP-IgA FcK in plant leaves. (A) Western blot revealing the PAP-IgA Fc and PAP-IgA FcK expression levels after transplantation of the in vitro-grown plants to soil A or B in a greenhouse. In the protein schematics, PAP is indicated as the spring-shaped region; IgA Fc, gray oval region. The region designated with an asterisk indicates the KDEL ER retention signal. The expression level of the polymerized (B), dimerized (C), and monomeric (D) PAP-IgA Fc and PAP-IgA FcK proteins in transgenic plants after transplanting to soil A or B. −, negative control, NT, non-transgenic plant. The PAP-IgA Fc and PAP-IgA FcK proteins were detected using a rabbit anti-human PAP antibody.

concentration (pH), electric conductivity (EC), ammonia nitrogen (NH_4^+) concentration, bulk density, and water content were determined (Table 1). These analyses showed that soil A and B only significantly differed in the NH_4^+ concentration (51 mg·L^{-1} in soil A, and 1 mg·L^{-1} in soil B) (Table 1). Plants in soil A grew well, whereas the plants in soil B did not, although all plants grew well under in vitro culture conditions. We therefore hypothesize that the growth of transgenic

plants and the expression of PAP-IgA Fc and PAP-IgA FcK depends on the presence of nitrogen components in the soil. Nitrogen components are especially important for plant growth and nicotine production (Xi et al., 2008). Environmental factors such as soil, sunlight, water, and nutrients are essential for plants, and affect the large-scale production of plant biomass (Elbers et al., 2001; Jamal et al., 2012; Lim et al., 2015b; Passioura, 2002). Therefore, these factors can limit plant

growth (Converti et al., 2009; Kovalchuk et al., 2001; Poorter and Nagel, 2000; Smith and Stitt, 2007). The goal of our study was to determine whether soil components affect plant growth as well as the expression of recombinant fusion proteins and the dimerization of PAP-IgA Fc and PAP-IgA FcK recombinant proteins.

PAP-IgA Fc and PAP-IgA FcK transgenic plants were transplanted and grown in soil A and soil B pots for 4 weeks in a greenhouse. Growth analysis revealed a significant difference between soil A and B. PAP-IgA Fc and PAP-IgA FcK transgenic plants were taller when grown in soil A than when grown in soil B. In addition, leaf phenotypes of plants grown in soil A and B were different. The leaf color of transgenic plants grown in soil B changed to yellow, whereas the plants in soil A had green leaves (Çakmakçi et al., 2006). The chlorophyll concentration was measured in leaves of transgenic plants in both soil A and B and showed that PAP-IgA Fc and PAP-IgA FcK plants in soil B harbored significantly lower chlorophyll values. Chlorophyll has an important role in photosynthesis as it captures light energy of the sun, and changes it to chemical energy to ultimately create a carbohydrate (De Graaff et al., 2006; Zhao et al., 2005). Indeed, it has been shown that NH_4^+ in the soil affects the chlorophyll content of the plant, photosynthesis, and the growth of plants (Baryla et al., 2001; Daughtry et al., 2000; Shibghatallah et al., 2013).

In this study, soils A and B were analyzed to determine which components affect the growth of the transgenic plants. Indeed, the two soils harbored significantly different concentrations of NH_4^+. The NH_4^+ concentration in the soil was previously reported to affect the growth of the plant (Allen and Smith, 1986; Azarmi and Esmaeilpour, 2010; Britto and Kronzucker, 2002; Jang et al., 2014; Loubet et al., 2002; Park et al., 2014). The length of the leaves and the height of stems were affected when NH_4^+ concentrations were low in the soil (Azarmi and Esmaeilpour, 2010; De Graaff et al., 2006; Zhao et al., 2005). However, a high concentration of NH_4^+ in the soil may lead to toxicity (Britto et al., 2001; Britto and Kronzucker, 2002). The NH_4^+ concentration was measured to be 51 mg·L^{-1} and 1 mg·L^{-1} in soils A and B, respectively. These results indicate that the NH_4^+ concentration in the soil affects plant growth.

The PCR results confirmed that the genes for PAP-IgA Fc and PAP-IgA FcK were successfully inserted in the genome of the transgenic plants. PAP-IgA Fc and PAP-IgA FcK genes were stably inserted 4 weeks after transplantation from in vitro conditions to soil A or B. These results indicate that the insertion of the PAP-IgA Fc and PAP-IgA FcK genes is not affected by the NH_4^+ concentration.

Immunoblot analysis detected the predicted PAP-IgA Fc and PAP-IgA FcK fusion protein bands in extracts from the leaves of the transgenic tobacco plants. All leaf soluble protein samples with identical volumes revealed a similar PAP-IgA Fc or PAP-IgA FcK protein band pattern; however, the intensity of the bands was variable. The dimerization bands of the PAP-IgA Fc and PAP-IgA FcK recombinant protein decreased 4 weeks after transplanting of the in vitro cultured plant to soil B in a greenhouse. These results indicate that components of the soil do not lead to any changes in the fusion protein patterns of PAP-IgA Fc and PAP-IgA FcK, but protein expression levels vary.

The advantage of utilizing a plant expression system for the production of highly valuable recombinant proteins is that it is a cost-effective option for large-scale production (Ko, 2014; Lee et al., 2013; Lim et al., 2015b). The large-scale production of highly valuable plant-derived recombinant therapeutic proteins in plants depends on various environmental factors including temperature, light, salinity, nutrients, insects, pests, and general soil conditions (Elbers et al., 2001; Jamal et al., 2012; Lim et al., 2015b; Passioura, 2002).

In this study, we confirmed that the leaf tissue of plants grown in soil A (NH_4^+: 51 mg·L^{-1}) harbored high expression levels of PAP-IgA Fc and PAP-IgA FcK and a high level of dimerized proteins when compared with plants grown in soil B (NH_4^+: 1 mg·L^{-1}). In addition, the chlorophyll concentrations of the PAP-IgA Fc and PAP-IgA FcK transgenic plants in soil A were three times higher when compared with plants grown in soil B. In conclusion, we characterized the effect of ammonia nitrogen (NH_4^+) concentration in the soil on plant growth, chlorophyll concentration in leaves, and recombinant protein expression levels. These results indicate the importance of soil components for the growth of transgenic plants and recombinant protein expression. Thus, soil conditions should be carefully optimized to enhance the cultivation of transgenic plants for the production of biologically active therapeutic proteins.

Literature Cited

Allen S, Smith JAC (1986) Ammonium nutrition in *Ricinus communis*: Its effect on plant growth and the chemical composition of the whole plant, xylem and phloem saps. J Exp Bot 37:1599-1610

Azarmi R, Esmaeilpour B (2010) Effect of NO$_3^-$ to NH$_4^+$ ratio on growth, yield and element composition of cucumber (*Cucumis sativus L.*). J Food Agric Environ 8:607-610

Baryla A, Carrier P, Franck F, Coulomb C, Sahut C, Havaux M (2001) Leaf chlorosis in oilseed rape plants (*Brassica napus*) grown on cadmium-polluted soil: Causes and consequences for photosynthesis and growth. Planta 212:696-709

Hortic. Environ. Biotechnol. 57(3):299-307. 2016.

307

Britto DT, Kronzucker HJ (2002) NH$_4^+$ toxicity in higher plants: A critical review. J Plant Physiol 159:567-584

Britto DT, Siddiqi MY, Glass ADM, Kronzucker HJ (2001) Futile transmembrane NH$_4^+$ cycling: ellular hypothesis to explain ammonium toxicity in plants. Proc Natl Acad Sci USA 98:4255-4258

Çakmakçi R, Dönmez F, Aydin A, Şahin F (2006) Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions. Soil Biol Biochem 38:1482-1487

Converti A, Casazza AA, Ortiz EY, Perego P, Del Borghi M (2009) Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chem Eng Process 48:1146-1151

Czajkowsky DM, Hu J, Shao Z, Pleass RJ (2012) Fc-fusion proteins: New developments and future perspectives. EMBO Mol Med 4:1015-1028

Daniell H, Singh ND, Mason H, Streatfield SJ (2009) Plant-made vaccine antigens and biopharmaceuticals. Trends Plant Sci 14:669-679

Daughtry CST, Walthall CL, Kim MS, De Colstoun EB, McMurtrey Iii JE (2000) Estimating corn leaf chlorophyll concentration from leaf and canopy reflectance. Remote Sens Environ 74:229-239

De Graaff MA, Van Groenigen KJ, Six J, Hungate B, Van Kessel C (2006) Interactions between plant growth and soil nutrient cycling under elevated CO$_2$: A meta-analysis. Global Change Biol 12:2077-2091

Elbers IJW, Stoopen GM, Bakker H, Steven LH s, Bardor M, Molthoff JW, Jordi WJRM, Bosch D, Lommen A (2001) Influence of growth conditions and developmental stage on N-glycan heterogeneity of transgenic immunoglobulin G and endogenous proteins in tobacco leaves. Plant Physiol 126:1314-1322

Jang HH, Lee HS, Chiwon W, Choi JM (2014) Influence of the composition and concentration of fertilizer solution on growth and nutrient uptake of 'Maehyang' strawberry during vegetative growth. Korean J Hortic Sci Technol 32:484-492

Jamal A, Lee JH, Lee KJ, Oh DB, Kim DS, Lee KK, Choo YK, Hwang KA, Ko K (2012) Chimerism of multiple monoclonal antibodies expressed in a single plant. Hortic Environ Biotechnol 53:544-551

Ko K (2014) Expression of recombinant vaccines and antibodies in plants. Monoclon Antib Immunodiagn Immunother 33:192-198

Kovalchuk O, Titov V, Hohn B, Kovalchuk I (2001). A sensitive transgenic plant system to detect toxic inorganic compounds in the environment. Nat Biotechnol 19:568-572

Larrick JW, Yu L, Naftzger C, Jaiswal S, Wycoff K (2001) Production of secretory IgA antibodies in plants. Biomol Eng 18:87-94

Lee JH, Park DY, Lee KJ, Kim YK, So YK, Ryu JS, Oh SH, Han YS, Ko K (2013) Intracellular reprogramming of expression, glycosylation, and function of a plant-derived antiviral therapeutic monoclonal antibody. PLoS One 8. e68772

Lim CW, Lim S, Baek W, Lee SC (2015a). The pepper late embryogenesis abundant protein CaLEA1 acts in regulating abscisic acid signaling, drought and salt stress response. Physiol Plant 154:526-542

Lim CY, Lee KJ, Oh DB, Ko K (2015b). Effect of the developmental stage and tissue position on the expression and glycosylation of recombinant glycoprotein GA733-FcK in transgenic plants. Front Plant Sci 5:778

Loubet B, Milford C, Hill PW, Sim Tang Y, Cellier P, Sutton MA (2002) Seasonal variability of apoplastic NH$_4^+$ and pH in an intensively managed grassland. Plant Soil 238:97-110

Lu Z, Lee KJ, Shao Y, Lee JH, So Y, Choo YK, Oh DB, Hwang KA, Oh SH, Han YS, Ko K (2012) Expression of GA733-Fc fusion protein as a vaccine candidate for colorectal cancer in transgenic plants. J Biomed Biotechnol 2012. 364248

Ma Julian KC, Drake PMW, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4:794-805

McNeel DG, Dunphy EJ, Davies JG, Frye TP, Johnson LE, Staab MJ, Horvath DL, Straus J, Alberti D, et al (2009) Safety and immunological efficacy of a DNA vaccine encoding prostatic acid phosphatase in patients with stage D0 prostate cancer. J Clin Oncol 27:4047-4054

Oh W (2015) Effects of Temperature, Photoperiod and Light Intensity on Growth and Flowering in Eustoma grandiflorum. Korean J Hortic Sci Technol 33:349-355

Park EY, Choi JM (2014) Development of root media containing ppine bark for cultivation of horticultural crops. Korean J Hortic Sci Technol 32:499-506

Park SR, Lim CY, Kim DS, KO K (2015) Optimization of ammonium sulfate concentration for purification of colorectal cancer vaccine candidate recombinant protein GA733-FcK isolated from plants. Front Plant Sci 6:1040

Passioura JB (2002) Soil conditions and plant growth. Plant Cell Environ 25:311-318

Poorter H, Nagel O (2000) The role of biomass allocation in the growth response of plants to different levels of light, CO$_2$, nutrients and water: A quantitative review. Aust J Plant Physiol 27:595-607

Rigano MM, Walmsley AM (2005) Expression systems and developments in plant-made vaccines. Immunol Cell Biol 83:271-277

Saif JMS, Vadakekolathu J, Rane SS, McDonald D, Ahmad M, Mathieu M, Pockley AG, Durrant L, Metheringham R, et al (2014) Novel prostate acid phosphatase-based peptide vaccination strategy induces antigen-specific T-cell responses and limits tumour growth in mice. Eur J Immunol 44:994-1004

Shibghatallah MAH, Khotimah SN, Suhandono S, Viridi S, Kesuma T (2013) Measuring leaf chlorophyll concentration from its color: A way in monitoring environment change to plantations. AIP Conf Proc 1554:210-213

Smith AM, Stitt M (2007) Coordination of carbon supply and plant growth. Plant Cell Environ 30:1126-1149

Song I, Kim DS, Kim MK, Jamal A, Hwang KA, Ko K (2015) Comparison of total soluble protein in various horticultural crops and evaluation of its quantification methods. Hortic Environ Biotechnol 56:123-129

Tarassoff CP, Arlen PM, Gulley JL (2006) Therapeutic vaccines for prostate cancer. Oncologist 11:451-462

Vihko P, Virkkunen P, Henttu P, Roiko K, Solin T, Huhtala ML (1988) Molecular cloning and sequence analysis of cDNA encoding human prostatic acid phosphatase. FEBS Lett 236:275-281

Xi X, Li C, Zhang F (2008) Tobacco plants can use nitrogen taken up before mechanical wounding to synthesize nicotine afterwards. Plant Signal Behav 3:87-90

Zhao D, Reddy KR, Kakani VG, Reddy VR (2005) Nitrogen deficiency effects on plant growth, leaf photosynthesis, and hyperspectral reflectance properties of sorghum. Eur J Agron 22:391-403