Biopharmaceutical Protein Production under Controlled Environments: Growth, Development, and Vaccine Productivity of Transgenic Tomato Plants Grown Hydroponically in a Greenhouse

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Abstract. Biopharmaceutical protein production is a new application of plant biotechnology. Nevertheless, there is limited information for potential protein productivity in commercial production operation. The objective of this study was to characterize the growth and development as well as fruit and protein productivities of transgenic tomato (*Solanum lycopersicum*) plants in comparison with two nontransgenic reference cultivars under greenhouse conditions with commercially adopted cultural practice. Transgenic tomatoes expressing a predominant antigen fusion protein, F1-V, against plague were used as a model system. Three types of tomatoes were grown for this study: 1) a transgenic T_2 line, 'F1-V'; 2) the background wild-type cultivar, TA234; and 3) a commercial greenhouse cultivar well adopted in North America, Durinta. All plants were grown hydroponically in a greenhouse equipped with heating and evaporative cooling systems for 24 to 30 weeks. When comparing 'F1-V' with 'TA234', there were no significant differences in growth, cumulative fruit yield, fruit total soluble protein (TSP) concentration, nor cumulative TSP production between the two genotypes. Although there was a difference in plant leaf morphology, this suggests that the transformation event did not affect the key traits of biopharmaceutical protein production. When comparing 'F1-V' with 'Durinta', 'Durinta' yielded more fruit than did 'F1-V', although final vegetative biomass of the two genotypes was not significantly different. Cumulative fruit yield per plant of 'Durinta' for 13 weeks of harvest was almost twice that of 'F1-V'. However, TSP concentration of fruits of 'Durinta' was only 12% to 34% of that of 'F1-V', making the estimated cumulative TSP production by fruits approximately half that of 'Durinta'. Our results suggest that biomass productivity is not necessarily the high-priority trait in selecting cultivars for high-value protein production and that protein concentration of fruits may be an important factor.

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Transgenic plants that express high-value pharmaceutical proteins such as vaccines and antibodies are attracting considerable interest as an efficient protein production system. Compared with traditional fermentation- or bioreactor-based protein production systems using recombinant microbes or transformed animal or mammalian cells, transgenic plant systems have advantages, including a lower cost, a higher scalability, less downstream manufacturing processes, and lower contamination risk of potential human pathogens or toxins (for reviews, see Daniell et al., 2001; Mason and Arntzen, 1995; Mason et al., 2002; Thanavala et al., 2006; Twyman et al., 2003).

Greenhouse tomato production is one of the most suitable production systems using

plants for such high-value proteins. First, tomatoes are an effective species for high-value protein production. A relatively efficient transformation system has been established in tomato (Mason et al., 2002), and several biotechnologists have selected tomato so far for expression of various subunit vaccines, including those against respiratory syncytial virus (Sandhu et al., 2000), cholera (Jani et al., 2002), Norwalk virus (Huang et al., 2005; Zhang et al., 2006), plague (Alvarez et al., 2006, 2008), hepatitis B virus (Lou et al., 2007), and human immunodeficiency virus type 1 (Ramírez et al., 2007) and also for expression of a taste-modifying high-value protein (Sun et al., 2007). In addition, a fruit of such transgenic tomatoes can be orally administered after minimum processing (e.g., freeze-drying). Second, greenhouse tomato production is advantageous over open-field production in terms of its high biomass yield. Improvement of technology has almost doubled greenhouse tomato productivity in the past two decades (Ho, 2004; Jones, 2007). In the United States, for example, average annual tomato yield per unit area under greenhouse conditions was 15 times higher than that under open-field conditions in 2003 (Cook and Calvin, 2005). Greenhouse tomato productivity is still increasing to nearly 100 kg·m⁻²/year (Ho, 2004; Peet and Welles, 2005). Third, greenhouses are able to function as containment to prevent transgene flow to the outside (Twyman et al., 2003). Fourth, greenhouses possess environmental control capacity to manipulate plant growth, development, and the accumulation of nutritional compounds (Kubota et al., 2006) and thus could create the environmental conditions for steering the plant growth to maximize the protein productivity with minimum input of available resources.

Despite intensive studies introducing valuable genes into tomato, there have been no studies characterizing growth, fruit or protein yield, and productivity of transgene product of the transgenic tomato under greenhouse conditions. In particular, such transgenic plants have not been grown using the cultural practice required for high fruit yield over an extended period of time, including the commercial standard hydroponic, high-wire production system. Moreover, many studies on gene transformation and protein expression only briefly report growing environmental conditions and overlook information on potential environmental and agronomic factors affecting the transgenic plants (e.g., Lou et al., 2007; Ramírez et al., 2007).

The objective of this study was to evaluate the growth, development, and vaccine productivity of selected transgenic plants under the environmental conditions and cultural practices known to be optimum for enhancing the fruit yield in commercial hydroponic greenhouse (Jones, 2007). The transgenic model plant system used in this study was tomato transformed with the *Yersinia pestis* *fl-v* gene (Alvarez et al., 2006) expressing a predominant antigen fusion protein, F1-V, against plague. An elite T_1 plant, '22.11',

which had a very high F1-V expression level of 11% relative to total soluble protein (TSP) (Alvarez et al. 2006), was selected and its seeds (T_2 progeny) were used for this study. Plague, caused by *Y. pestis*, is a deadly infectious disease and has the potential to cause large-scale outbreaks, especially in developing countries (Alvarez et al., 2006), and further could be used as a bioterrorism or biological warfare agent (Inglesby et al., 2000). A previous study showed that oral administration of a freeze-dried transgenic tomato fruit to mice successfully immunized them (Alvarez et al., 2006), indicating that it can be used as an orally delivered vaccine. In addition to the transgenic line, two non-transgenic cultivars were grown. One was 'TA234', the background wild-type cultivar of the transgenic plants. Another was 'Durinta', a greenhouse cultivar used in commercial hydroponic tomato production in Europe and North America. They were used as reference to evaluate the overall biomass and protein productivities and physiological characteristics of the transgenic plants.

Materials and Methods

Plant material. Three types of tomatoes were grown for this study: 1) a transgenic T_2 line, 'F1-V' (third generation after primary transforming event); 2) the background wild-type cultivar, TA234; and 3) a commercial greenhouse cultivar, Durinta (Western Seed Americas Inc., Westport, CT). The primary transformant (T_0) was obtained by *Agrobacterium*-mediated transformation of 'TA234' with the *Y. pestis fl-v* gene driven by the CaMV 35S promoter (Alvarez et al., 2006). One plant of its T_1 progeny, '22.11', showing the F1-V expression of 11% to TSP in fruits was selected and allowed to self-fertilize, and the seeds (T_2) were used as 'F1-V' plants. 'Durinta' was used as a high-yielding reference cultivar. In a local commercial greenhouse in Arizona, 'Durinta' has the potential to yield 75 kg·m⁻² annually (EuroFresh Farms, personal communication).

Growth conditions and experimental setup. 'F1-V' plants were compared with 'Durinta' or 'TA234' plants in separate experiments (Expt. 1 and Expt. 2). All plants were grown hydroponically with a high-wire system in an acrylic greenhouse (BSL2) on top of a six-story building on campus of the University of Arizona in Tucson, AZ (long. 32.2° N, lat. 111.0° W) for 30 and 24 weeks for Expts. 1 and 2, respectively. Seeds were sown into 4 × 4-cm rockwool cubes (Grodan BV, Roermond, The Netherlands) covered with a thin layer of vermiculite on 15 Aug. and 27 Sept. 2007 for Expts. 1 and 2, respectively, and were germinated in the greenhouse. The rockwool cubes were kept moist by subirrigation with water. After cotyledons were fully unfolded, the seedlings were transplanted to 10 × 10-cm rockwool blocks (Grodan BV) and were subirrigated once a day with a half-strength modified Hoagland's nutrient solution described subsequently. On 20 Sept. and 16 Oct. 2007 for

Expts. 1 and 2, respectively, seedlings having more than four fully expanded true leaves were transplanted onto rockwool slabs at a plant density of 2.4 plants/m². Eighteen and six plants were subjected per genotype for Expts. 1 and 2, respectively, and each plant was dealt with as an experimental unit. The full-strength modified Hoagland's nutrient solution described subsequently was automatically supplied using a drip irrigation system with a feeding rate of 100 mL per irrigation event per plant. The irrigation frequency and irrigation period in a day were adjusted depending on plant growth so as to maintain a minimum of 30% efflux of the nutrient solution and avoid salt accumulation. Based on plant growth, the irrigation frequency was increased from once per 60 min up to once per 20 min and the irrigation period in a day was increased from 7.5 h up to 9 h during the experiment for both Expts. 1 and 2. The basal composition of the full-strength modified Hoagland's nutrient solution except nitrogen (N) was prepared according to Wu and Kubota (2008) with slight modifications in major element concentrations (mg·L⁻¹): 47 phosphorus, 350 potassium, 200 calcium, and 60 magnesium. The concentration of N, all derived from NO₃-N, was varied depending on plant growth as follows: 90 mg·L⁻¹ from transplants to the stage in which the second truss was fruiting (the second truss stage), 145 mg·L⁻¹ from the second to fifth truss stage, and 190 mg·L⁻¹ after the fifth truss stage. The inflow full-strength nutrient solution had 2.3 ± 0.4 dS·m⁻¹ electrical conductivity (EC) with 6.2 ± 0.3 pH, and the efflux full-strength nutrient solution had 2.6 ± 0.5 dS·m⁻¹ EC with 6.9 ± 0.6 pH. Average efflux percentage over the inflow nutrient solution measured in Expt. 1 was 75% and 60% for 'F1-V' and 'Durinta', respectively. Common greenhouse plant maintenance, including leaf pruning and removing side shoots, took place on a weekly basis. Fruits were pruned to five per truss following the commercial practice in the same climate region. Flowering trusses were vibrated mechanically for 1 sec every other day using an electric device to promote pollination.

The greenhouse was north-south-oriented and frontage width and ridge length were 6.1 m and 11.0 m, respectively. The height from the floor to the roof peak and gutter were 5.2 m and 3.7 m, respectively. A fan-and-pad evaporative cooling system and an overhead heating system were used to control air temperature of the greenhouse when it was greater than 25 °C during the day or cooler than 15 °C at night. Air temperature, relative humidity, and photosynthetic photon flux (PPF) inside the greenhouse were measured and the averages of every 15 min were recorded with a CR-10X data logger (Campbell Scientific, Logan, UT) throughout the experiment.

Day and nighttime air temperatures were controlled within the range considered as optimum for fresh tomato production (Jones, 2007). Specifically, mean, maximum, and

minimum daytime air temperatures were ≈21, 24, and 17 °C, respectively. Mean, maximum, and minimum nighttime temperatures were ≈18, 20, and 17 °C, respectively. Spatial variations of daytime and nighttime temperatures within the greenhouse were less than 2 and 1 °C, respectively. Daily mean relative humidity was maintained above 60% throughout the experiment. Daily PPF integral measured over plant canopy on sunny days was ≈25 mol·m⁻²·d⁻¹ in October, decreased to 15 mol·m⁻²·d⁻¹ in December, and then increased to 24 mol·m⁻²·d⁻¹ at the end of February.

Growth analysis and fruit harvest. Stem length and leaf number were measured once a week from 2 Nov. 2007. The rates of stem elongation and leaf development of each plant were calculated as the slopes of the first-order regression equation of time courses of stem length and leaf number, respectively. Fruit trusses were harvested three times a week. Because F1-V in fruits rapidly decreases with ripening (Alvarez et al., 2006), only green fruits were selected from the harvested fruits for each of the three genotypes and were used for subsequent biochemical analyses. On 4 Mar. 2008, shoot (leaves and stem, without fruits, above the base of the 10th youngest leaf) was detached, and leaf area and dry weight (DW) of leaves and stem were measured. Leaf area measurement was made with an area meter (LI-3100; LI-COR Inc., Lincoln, NE).

Gas-exchange measurements. Measurements of net photosynthetic rate (P_n), transpiration rate, and stomatal conductance (g_s) in young, fully expanded leaves were carried out using a portable photosynthesis system equipped with a halogen light source (CIRAS-2; PP Systems Inc., Amesbury, MA) between 17 and 31 Oct. 2007 for Expt. 1 and between 7 and 18 Feb. 2008 for Expt. 2. Measurements were made under saturating light conditions at a PPF of 1500 μmol·m⁻²·s⁻¹, an atmospheric CO₂ partial pressure of 37 ± 1 Pa, a leaf temperature of 28 ± 1 °C, and a leaf-to-air vapor pressure deficit of 1.2 ± 0.2 kPa.

Biochemical assay. Biochemical analysis of leaves was made on young, fully expanded leaves collected between 11 and 14 Nov. 2007 for Expt. 1 and on 4 Mar. 2008 for Expt. 2. Biochemical analysis of fruits was made on green fruits collected between 2 and 16 Dec. 2007 for Expt. 1 and between 6 and 18 Jan. 2008 for both Expts. 1 and 2.

For determination of leaf TSP concentration, a leaf sample was homogenized with a chilled mortar and pestle in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.2) containing 2 mM Na-iodoacetate, 1 mM phenylmethylsulfonyl fluoride, 5% (v/v) glycerol, 1% (w/v) polyvinyl pyrrolidone, and 0.8% (v/v) 2-mercaptoethanol. For determination of fruit TSP concentration, a fruit sample was first powdered in liquid N₂ with a chilled mortar and pestle and then homogenized in the same buffer. After centrifugation of this homogenate at 15,000 g_n for 15 min at 4 °C, TSP, which included all proteins soluble in the

previously described buffer, was retrieved from the supernatant as a trichloroacetic acid precipitate. The precipitated TSP content was determined by the method of Lowry et al. (1951). Calibration curves were made with bovine serum albumin.

For determination of fruit F1-V concentration, fruits were kept overnight at -20°C , freeze-dried for at least 72 h, and pulverized to powder. TSP fraction was extracted from the powder and then subjected to enzyme-linked immunosorbent assay using a rabbit polyclonal anti-F1-V antibody as described in Alvarez et al. (2006).

Data analysis. Significant differences between genotypes were tested by *t* test at $P \leq 0.05$ using JMP statistical software (SAS Institute Inc., Cary, NC).

Results

Plant growth and development. Typical canopy structure at final harvest and leaf samples of the three genotypes are shown in Figure 1. The transgenic plants 'F1-V' exhibited somewhat abnormal morphology such as excessive proliferation of small flower buds within a truss and downhanging, curly, distorted leaves (Figs. 1A and D), whereas 'Durinta' (Figs. 1B and F) and 'TA234' (Figs. 1C and G) had relatively flat, expanded leaves. Some 'F1-V' plants also showed magnesium deficiency-like symptom of chlorosis and necrosis on leaves (Fig. 1E), although such symptom was not observed for 'Durinta' (Fig. 1F) or 'TA234' (Fig. 1G) leaves.

'F1-V' plants were compared with 'Durinta' or 'TA234' plants in separate experiments (see "Materials and Methods"). Table 1 shows growth and developmental characteristics. In Expt. 1, the final shoot DW of 'F1-V' tended to be lower than that of

'Durinta', although the difference was not statistically significant. Similarly, leaf area of 'F1-V' was 54% lower than that of 'Durinta'. Leaf area ratio of the shoot, the ratio of leaf area to shoot DW, was not significantly different between 'F1-V' and 'Durinta', indicating that they had similar leaf area expansion relative to biomass. We did not determine shoot DW and leaf area in Expt. 2, but 'F1-V' and 'TA234' showed similar biomass and leaf area by appearances. In Expt. 1, the stem elongation rate of 'F1-V' was $\approx 60\%$ of that of 'Durinta', whereas the leaf development rate (the rate of leaf-number increment per day) was almost the same. These results mean that 'F1-V' had shorter internode lengths than 'Durinta'. In Expt. 2, neither stem elongation rate nor leaf development rate was significantly different between 'F1-V' and 'TA234'. Under the present conditions, all three genotypes exhibited indeterminate growth.

Photosynthesis and total proteins in leaves. Table 2 shows photosynthetic gas-exchange characteristics and TSP concentration of young, fully expanded leaves. In Expt. 1, P_n under saturating light conditions was 21% higher in 'Durinta' than in 'F1-V'. Transpiration rate and g_s did not significantly differ between 'F1-V' and 'Durinta'. In Expt. 2, 'F1-V' and 'TA234' showed similar P_n and transpiration rate, although g_s was significantly higher in 'TA234'. Leaf TSP concentration on a fresh weight basis in 'F1-V' was 21% lower than that in 'Durinta' in Expt. 1, whereas it was not significantly different from that in 'TA234' in Expt. 2.

Yields and protein productivity in fruits. First harvest was recorded on 26 Nov. 2007 and 31 Dec. for Expts. 1 and 2, respectively, and continued through 2 Mar. 2008 for both experiments. In Expt. 1, 'F1-V' showed significantly lower fruit yield than 'Durinta'

(Fig. 2A). Cumulative fruit yield per plant at final harvest of 'F1-V' was approximately half of that of 'Durinta'. On the other hand, 'F1-V' had comparable fruit yield with 'TA234' in Expt. 2 (Fig. 2B).

Figure 3 shows fruit TSP concentration on a DW basis. For Expt. 1, we analyzed fruit TSP concentration twice for the second truss (Fig. 3A) and the sixth to eighth trusses (Fig. 3B) in both 'F1-V' and 'Durinta'. Fruit TSP concentration per DW was considerably higher in 'F1-V' than in 'Durinta' irrespective of truss. In each genotype, TSP concentration was not significantly different between different trusses. This implies that plant age did not affect TSP concentration of at least 'F1-V' and 'Durinta'. In Expt. 2, fruit TSP concentration on a DW basis did not differ between 'F1-V' and 'TA234' (Fig. 3C).

From cumulative fruit yield (Fig. 2A–B) and fruit TSP concentration (Fig. 3), cumulative TSP production was calculated (Fig. 2C–D). It was found that, in Expt. 1, cumulative TSP production of 'F1-V' was 2.2 to 3.8 times higher than that of 'Durinta' throughout 13 weeks of the evaluation period (Fig. 2C). This was the result of the higher fruit TSP concentration in 'F1-V' than in 'Durinta' (Fig. 3A–B), although fruit yield was lower in 'F1-V' than in 'Durinta' (Fig. 2A). This indicates that fruit TSP concentration had a greater implication to TSP production rather than fruit yield in Expt. 1. On the other hand, in Expt. 2, 'F1-V' and 'TA234' had similar cumulative TSP production as were the cases in fruit yield (Fig. 2B) and fruit TSP concentration (Fig. 3C).

F1-V expression and productivity. Figure 4 shows the relationships between F1-V concentration (Fig. 4A) or F1-V/TSP ratio (Fig. 4B) and TSP concentration of the 'F1-V' plants. Data from both Expts. 1 and 2 were combined to analyze general trends of the relationships in 'F1-V' plants. For comparison, data of the parental plant (T_1) of 'F1-V' plants, '22.11' (Alvarez et al., 2006), were also included. F1-V concentrations varied from an undetectable level to $347 \mu\text{g}\cdot\text{g}^{-1}$ freeze-dried weight depending on individual plant. Maximum percentage of F1-V to TSP observed was 3.57%. The relationships indicate that a lower F1-V concentration and a lower F1-V/TSP ratio was associated with a lower TSP concentration. This suggests that a decrease in F1-V was associated with an overall decrease in TSP and that F1-V preferentially decreased compared with the other TSP.

Discussion

To our knowledge, this is the first report that transgenic tomato plants expressing a high-value pharmaceutical protein were successfully grown for a long term (6 months) and their fruits were continuously harvested in a temperature-controlled greenhouse with a growing practice adopted by commercial hydroponic tomato growers. In fact, fruit size of 'F1-V' plants was 50 to 90 g in the present

Fig. 1. Typical examples of canopy at final harvest (4 Mar. 2008) (A–C) and leaves (D–G) of 'F1-V' (A, D, E), 'Durinta' (B, F), and 'TA234' (C, G).

Table 1. Shoot dry weight (DW), leaf area, and leaf area ratio (LAR) of the shoot at final harvest and rates of stem elongation and leaf development.

Genotype	Shoot DW (g)	Leaf area (cm ²)	LAR (cm ² ·g ⁻¹)	Stem elongation rate (cm·d ⁻¹)	Leaf development rate (d ⁻¹)
Expt. 1					
F1-V	22.78 ± 5.75 ^z	2,551 ± 538	116 ± 10	2.34 ± 0.10	0.42 ± 0.01
Durinta	34.80 ± 3.31	5,408 ± 218*	158 ± 16	3.95 ± 0.06*	0.43 ± 0.01
Expt. 2					
F1-V	ND ^y	ND	ND	2.51 ± 0.17	0.48 ± 0.03
TA243	ND	ND	ND	2.55 ± 0.06	0.47 ± 0.01

^zMean ± SE (n = 3–6). Means with an asterisk (*) within each column and experiment are significantly different by *t* test at *P* ≤ 0.05.

^yND = Not determined.

Table 2. Net photosynthetic rate (P_n), transpiration rate, and stomatal conductance (g_s) per unit leaf area under saturating light conditions and total soluble protein (TSP) concentration per unit fresh weight (FW) in leaves.

Genotype	P _n ^z (μmol CO ₂ ·m ⁻² ·s ⁻¹)	Transpiration ^z (mmol H ₂ O·m ⁻² ·s ⁻¹)	g _s ^z (mol H ₂ O·m ⁻² ·s ⁻¹)	Leaf TSP [mg·g (FW) ⁻¹]
Expt. 1				
F1-V	18.0 ± 1.1 ^y	5.83 ± 0.63	0.720 ± 0.175	9.3 ± 0.3
Durinta	22.7 ± 0.4*	4.95 ± 0.62	0.548 ± 0.112	11.7 ± 0.6*
Expt. 2				
F1-V	17.1 ± 1.7	3.23 ± 0.26	0.190 ± 0.028	6.2 ± 1.8
TA234	15.8 ± 0.7	4.38 ± 0.64	0.352 ± 0.063*	6.4 ± 1.1

^zGas-exchange measurements were made at a photosynthetic photon flux of 1500 μmol·m⁻²·s⁻¹, an atmospheric CO₂ partial pressure of 37 ± 1 Pa, a leaf temperature of 28 ± 1 °C, and a leaf-to-air vapor pressure deficit of 1.2 ± 0.2 kPa.

^yMean ± SE (n = 3–5). Means with an asterisk (*) within each column and experiment are significantly different by *t* test at *P* ≤ 0.05.

Fig. 2. Time courses of cumulative fruit yield per plant (A–B) and estimated cumulative fruit total soluble protein (TSP) production (C–D) of 'F1-V' (closed square) and 'Durinta' (open circle) in Expt. 1 (A, C) and those of 'F1-V' and 'TA234' (open triangle) in Expt. 2 (B, D). Fruit yield was evaluated from 26 Nov. 2007 through 2 Mar. 2008 for Expt. 1 and from 31 Dec. 2007 through 2 Mar. 2008 for Expt. 2. Cumulative TSP production was calculated from cumulative fruit yield and mean TSP concentration of fruits (Fig. 3) in each experiment. Vertical bars represent SE (n = 18 for Expt. 1; 6 for Expt. 2). Means with different letters on the final week in each panel are significantly different by *t* test at *P* ≤ 0.05.

experimental conditions (data not shown), greater than what was obtained inside a growth chamber (M.L. Alvarez and G.A.

Cardineau, unpublished data). Growth, development, and protein productivity of the transgenic plants were evaluated in com-

parison with those of two nontransgenic cultivars and found several notable characteristics of them as described subsequently.

As expected, 'Durinta', a commercially grown cultivar, exhibited higher fruit biomass productivity than 'F1-V' (Expt. 1; Fig. 2A). However, vegetative biomass productivities of the two genotypes were not significantly different (Table 1). Because photosynthetic capacity per unit leaf area was significantly higher in 'Durinta' (Table 2), the resultant higher amount of carbohydrates should be preferentially allocated to fruits rather than vegetative organs in 'Durinta' compared with 'F1-V'. In addition, 'Durinta' had larger leaf area with flat, expanded leaves, whereas 'F1-V' had smaller leaf area with curly and shrunken ones (Fig. 1; Table 1). Therefore, canopy light interception to drive photosynthesis may be lower in 'F1-V', possibly contributing to its lower fruit productivity. In Expt. 2, in contrast, we did not observe significant differences in P_n (Table 2) or in fruit yield (Fig. 2B) between 'F1-V' and 'TA234', the wild-type cultivar of 'F1-V'. Although we did not determine shoot DW in Expt. 2, 'F1-V' and 'TA234' showed similar rates of stem elongation and leaf development (Table 1) and also visually similar biomass and leaf area. It is therefore likely that the differences between 'F1-V' and 'Durinta' observed in Expt. 1 are related to the difference between the baseline genotypes rather than the transgenic event.

Despite the superior growth and photosynthesis, 'Durinta' showed much lower protein productivity in fruits than 'F1-V' in Expt. 1. TSP concentration of fruits was considerably lower in 'Durinta' than in 'F1-V' (Fig. 3A–B), although TSP concentration of leaves was higher in 'Durinta' (Table 2). As a consequence of the lower fruit TSP concentration, estimated TSP production was significantly lower in 'Durinta' (Fig. 2C) despite its higher fruit yield (Fig. 2A). In cumulative TSP production, we also did not see any significant difference between 'F1-V' and 'TA234' in Expt. 2 (Fig. 2D). We had originally expected that a highly productive cultivar would also show high protein production per plant. However, our results suggest that a tomato cultivar that has high biomass productivity is not necessarily suitable for high-value protein production. We need to carefully select a cultivar based not only on biomass productivity, but on protein productivity for high-value protein production.

In 'F1-V' plants, F1-V concentrations varied from undetectable to 347 μg·g⁻¹ freeze-dried weight. A possible reason for this variation is the phenotypic segregation in 'F1-V' plants, the T₂ progeny. The parental T₁ plant of 'F1-V', '22.11', carried two copies of *fl-v* transgene (Alvarez et al., 2006), both of which were not necessarily homozygous, and the 'F1-V' plants originated from its self-fertilized seeds (T₂). Therefore, 'F1-V' plants could be a mixture of homozygous, heterozygous, or null (pseudo-wild-type) individuals at each of

Fig. 3. Total soluble protein (TSP) concentration per unit dry weight (DW) in fruits in Expt. 1 (A–B) and 2 (C). In Expt. 1, fruit TSP concentration was determined in different timing with different trusses: the second truss harvested between 29 Nov. and 9 Dec. 2007 (A) and the sixth to eighth trusses harvested between 6 and 13 Jan. 2008 (B). Vertical bars represent SE ($n = 13$ –15 for Expt. 1; 4–5 for Expt. 2). Means with different letters in each panel are significantly different by t test at $P \leq 0.05$.

Fig. 4. The relationship between F1-V concentration per unit freeze-dried weight (FDW) (A) or the ratio of F1-V to total soluble protein (TSP) (B) and TSP concentration per unit FDW in fruits of transgenic plants. Closed and open symbols indicate data taken from the transgenic line 'F1-V' grown in this study and from their parental plant '22.11' grown in a previous study (Alvarez et al., 2006), respectively. For A, $y = 0.0154x^2 - 0.147x + 0.355$, $r^2 = 0.998$, $P \leq 0.05$. For B, $y = 0.896x - 3.55$, $r^2 = 0.947$, $P \leq 0.05$.

two *f1-v* loci. Such segregation within 'F1-V' plants can be involved in the observed variation of F1-V concentration. We also noticed that the F1-V concentrations of the transgenic plants grown in the present study were markedly lower than that of their parental plant, '22.11' (Alvarez et al., 2006). Even the highest concentration observed in one 'F1-V' plant was only 16% of that of '22.11'. To find a possible reason of the significant difference

in F1-V between two studies, we compared the relationship between F1-V concentration or F1-V/TSP ratio and TSP concentration (Fig. 4). Results indicate that lower F1-V (Fig. 4A) and F1-V/TSP ratio (Fig. 4B) were associated with lower TSP. Although the number of plots was limited and more complete analysis is required, the preliminary relationships between TSP and F1-V obtained here may suggest that lower F1-V concentrations and lower F1-V/TSP observed in this study resulted not only from specific decrease in F1-V, but also an overall decrease in TSP, including F1-V, under the present experimental conditions.

One possible reason of this low F1-V and TSP in our study is the relatively late harvest timing within the green fruit stage. Fruit F1-V/TSP in the transgenic tomato dramatically decreases while the ripening stage shifts from the green stage to the breakers stage (Alvarez et al., 2006), associated with a decrease in both TSP and F1-V concentrations (Alvarez et al., unpublished data). In the present study, we analyzed fruits almost reaching their maximum size at the end of the green stage, at which time TSP and F1-V concentrations and F1-V/TSP might already have started decreasing.

Another possible reason of the lower F1-V and TSP production is the difference in growth conditions. In Alvarez et al. (2006), plants were grown in an environmentally controlled growth chamber equipped with artificial light sources. Therefore, there were significant differences in environmental conditions, including light intensity, light quality, and air temperature between their study and ours, and such differences may cause the differences in F1-V and TSP. It is not fully understood how environmental factors and their interactions affect growth and high-value protein productivity of transgenic plants. A few studies reported with transgenic tobacco that certain environmental conditions such as low temperature combined with high light intensity (Stevens et al., 2000) and moderate water stress (Stevens et al., 2007)

could enhance the concentration of high-value protein in leaves. It is possible that environmental conditions that maximize high-value protein production differ from those that maximize yield of harvests, because the concentration of high-value protein per unit biomass of the harvests can vary depending on environmental conditions. Further studies on the effects of environmental conditions on protein productivity of the transgenic plants, including tomato, are necessary to optimize growth condition for maximum high-value protein production.

Based on our results, it is estimated that maximum F1-V production of 293 mg·m⁻² per year can be obtained for 'F1-V' plants under the conditions of this study. To enhance this protein productivity, it is needed to develop environmental control techniques and culture practices specialized for the protein production and to select an appropriate cultivar as well as to establish efficient protein expression system using biotechnology. Additional research is needed.

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