A Simulation Model for Heterologous Protein Production in Transgenic Lettuce

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A simulation model was developed for heterologous protein production in transgenic lettuce. The model focused on the time course of fresh weight and heterologous protein of each leaf. The total amount of heterologous protein in a head of lettuce was expressed by the summation of all amounts of heterologous protein in all leaves. Soluble rather than heterologous protein concentration was used due to the unavailability of the transgenic lettuce producing specific heterologous protein. The parameters of the model were decided based on lettuce (*Lactuca sativa* L. cv. 'Greenwave') grown in the plant factory at Osaka Prefecture University. The first simulation reproduced the time course of soluble protein production in a head of lettuce grown under conventional environmental conditions. In the second simulation, protein production at 15°C and 25°C was simulated to investigate the effect of the model parameters on protein production. The results indicated the existence of an optimum cultivation cycle and the advantages and disadvantages of protein production at low and high temperature.

Keywords : edible vaccine, modeling, soluble protein, temperature control

INTRODUCTION

Since the antibody from mouse was first expressed in transgenic tobacco (Hiatt et al., 1989), transgenic crop plants have been considered as promising systems for bulk production of antibodies at relatively low cost (Daniell et al., 2001; Smith, 1996; Stevens, 2000; Streatfield et al., 2001). Daniell et al. (2001) also indicated that purification requirements could be eliminated when the antibody-containing plant tissue was used as a food (edible vaccine). Lettuce, one of the most popular leafy vegetables in the world, has already been used for heterologous protein production (Kapusta et al., 1999; Kim et al., 2007; Sun et al., 2006; Li et al., 2007). Lettuce is cultivated at many plant factories in which environmental conditions such as air temperature, relative humidity, light intensity and CO₂ concentration are precisely controlled (Morimoto et al., 1995; Murase, 2002; Shimizu et al., 2004). Since lettuce has a short cultivation cycle from germination to harvest, these systems can be profitable. The short cultivation cycle also makes it easier to explore the relationship between environmental growth conditions and heterologous protein production compared to other crops that require a longer cultivation cycle.

When transgenic lettuce is used to produce heterologous protein, most of the protein will be stored in the leaves. Therefore, maximization of leaf fresh weight seems to be the best solution for

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maximizing the amount of heterologous protein. However, the amount of protein in a leaf decreases during senescence and under stress (e.g. drought, extreme temperature, and shading) (Smart, 1994). Stevens et al. (2000) investigated the relationship among leaf senescence, environmental conditions (temperature and light intensity) and amount of antibodies (IgG) expressed in tobacco. They reported that the top (young) leaves contained about twice the amount of antibodies compared to the base (old) leaves. They also reported that plants grown at 25°C contained less antibodies per amount of leaf tissue than the plants grown at 15°C. These results indicate that the concentration of heterologous protein in a leaf varies depending on leaf age and environmental factors. Smith (1996) mentioned the need to determine how effectively the heterologous protein accumulates in plants before the technology can be used commercially. Therefore, a new methodology is required to evaluate the accumulation amount of heterologous protein.

Conventional environmental control in plant factories or fully equipped greenhouses aims to produce lettuce with sufficient fresh weight, good taste, and good shape based on the commercial standard. However, these factors are not criteria, and the amount of heterologous protein is only important for production of the protein in transgenic lettuce. Therefore, the concept of environmental control must be quite different from the conventional controls. However, the complexity of interactions between environmental factors and protein productivity makes it difficult to determine the optimum environmental control. For these reasons, a new simulation model is necessary to enable us to integrate knowledge and to test hypotheses on how to enhance heterologous protein production in transgenic lettuce.

The objectives of this study were to create a preliminary model for estimating the amount of heterologous protein in transgenic lettuce, and to simulate the effect of environmental factors on protein production.

MATERIALS AND METHODS

Model description

The final goal of the model is to simulate heterologous protein production in the total system. Initial efforts focused on the development of a preliminary model to simulate heterologous protein production in a head of lettuce. When lettuce is utilized to produce heterologous protein, the protein accumulates mainly in the leaf tissue. Consequently, the model focused on the time course of fresh weight and heterologous proteins of each leaf. The total amount of soluble protein in a plant ($P(t_p)$) (mg) on day t_p after transplanting is expressed by the summation of all amounts of heterologous protein in all leaves:

$$P(t_p) = \sum_{i=1}^{L_n} Fw_i \cdot Pc_i \quad (1)$$

where Fw_i is the fresh weight and Pc_i is the heterologous protein concentration of the i th leaf and L_n is the number of leaves. To simplify the model, it was assumed that all leaves have the same time course of fresh weight and heterologous protein concentration.

This study used soluble rather than specific heterologous protein due to the unavailability of the transgenic lettuce producing heterologous protein, and Stevens et al. (2000) reported that the amount of IgG corresponds to the amount of soluble protein.

The parameters of these functions were decided based on lettuce (*Lactuca sativa* L. cv. 'Greenwave') grown in the plant factory at Osaka Prefecture University. Plants were grown at air temperatures of 23/18°C (photo/dark period), relative humidity of approximately 50/80% (photo/dark period), photosynthetic photon flux density of approximately 300 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and with a photo and dark period of 14/10 h·d⁻¹. Light sources were white fluorescent lamps fixed above the culture system. A half-strength nutrient solution (A-type Otsuka solution formula, Otsuka

Chemical Co., Ltd., Japan) at pH 6.0, electric conductivity of $0.12 \text{ S} \cdot \text{m}^{-1}$, was supplied to the plants using a recirculation, deep flow technique. CO_2 level was controlled at approximately $1,000 \mu\text{mol mol}^{-1}$. The parameters used in the model were estimated from measured parameters using the method of least squares.

Leaf fresh weight was measured using a precise weighing scale. The following equation including two logistic functions was employed to describe the leaf fresh weight (FW) (g) at leaf age (t) (day). One logistic function describes the growth and the other describes senescence:

$$FW = \frac{FW_{\max}}{1 + e^{-r_{gr}(t_{gr}-t)}} - \frac{FW_{\max}}{1 + e^{-r_{sen}(t_{sen}-t)}} \quad (2)$$

where $FW_{\max}$ is maximum leaf fresh weight (g), r_{gr} is growth speed (day^{-1}), t_{gr} is related to onset of growth (day), r_{sen} is senescence speed (day^{-1}), and t_{sen} is related to onset of senescence (day^{-1}). Measured data and the approximation are shown in Fig. 1.

Total soluble protein was extracted from samples, each consisting of fresh leaf discs (approximately 200 mg fresh weight) homogenized in 200 μl of ice-cold protein extraction buffer (60 mM Tris/HCl [pH 8.0], 500 mM NaCl, 10 mM EDTA, 30 mM β -mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 4% polyvinylpolypyrrolidone). Samples were centrifuged (12,000 g, 4 min, 4°C) and the supernatants were transferred to new tubes. Total soluble protein concentration of each sample was quantified by dye binding at absorbance of 595 nm (Bradford, 1976) using a commercial preparation of protein reagent (Bio-Rad Laboratories Ltd.), using known concentrations of bovine serum albumin as the protein standard. Soluble protein concentration (Pc) (mg g^{-1}) at leaf age (t) (day) was assumed to be described by a logistic equation:

$$Pc = Pc_{\max} - \frac{Pc_{\deg}}{1 + e^{-r_{deg}(t_{deg}-t)}} \quad (3)$$

where $Pc_{\max}$ is the maximum soluble protein concentration ($\text{mg} \cdot \text{g}^{-1}$), $Pc_{\deg}$ is the maximum degradation of soluble protein concentration ($\text{mg} \cdot \text{g}^{-1}$), r_{deg} is the degradation speed of soluble protein concentration (day^{-1}), and t_{deg} is related to onset of degradation of soluble protein concentration (day). Measured data and the approximation are shown in Fig. 2.

Images of lettuce plants were captured every hour using a network camera (BB-HCM311, Panasonic) installed above the plants from 1 to 17 days after planting and the number of leaves was counted. The leaves were also counted at 21, 25, 30, and 40 days by destructive sampling. A linear relationship ($r=0.978$) was found between days after planting (t_p) and number of leaves (L_n). Therefore, the following linear equation was used to describe the number of leaves:

Fig. 1 Measured leaf fresh weight and its approximation.

Fig. 2 Measured soluble protein concentration and its approximation.

$$L_n = r_{ln} t_p + \alpha \quad (4)$$

where r_{ln} is increasing ratio of number of leaves, and α is a constant for adjustment. Measured data and the approximation are shown in Fig. 3.

Parameters estimated by measured data of lettuce are summarized in Table 1.

Low- and high-temperature growth conditions

As previously mentioned, Stevens et al. (2000) reported that air temperature greatly affected both heterologous protein (antibody: IgG) concentration per leaf fresh weight and growth of transgenic tobacco. Heterologous protein concentration in leaves grown at 15°C was approximately three times higher than that at 25°C. In contrast, the fresh weight and number of leaves of plants grown at 15°C were approximately equivalent to two-thirds of that at 25°C. In the long run, which temperature provides more efficient production of heterologous protein? The simulation was conducted with adjusted parameters based on the results of Stevens et al. (2000). t_{gr} and t_{sen} , which control the time course of leaf fresh weight, were adjusted. t_{gr} and t_{sen} at 15°C were 1.5 times greater than that at 25°C. This means that leaves at 15°C grow and age more slowly than at 25°C (Fig. 4).

T_{deg} and Pc_{deg} , which control the time course of soluble protein concentration of a leaf, were ad-

Fig. 3 Number of leaves at days after transplanting and approximation.

Table 1 Parameters estimated by measured data of lettuce grown under the previously explained environmental conditions.

FW_{max}	Fresh weight of a leaf				Soluble protein concentration				Number of leaf	
	r_{gr}	t_{gr}	r_{sen}	t_{sen}	Pc_{max}	Pc_{deg}	r_{deg}	t_{deg}	Ln	a
12.6	0.29	11.5	0.80	28.0	10.2	7.45	-0.66	13.0	0.72	0.07

Fig. 4 Time course of fresh weight of leaf grown at 25°C (solid line) and 15°C (dotted line).

justed. T_{deg} at 15°C was equivalent to 66% of that at 25°C, and P_{Cdeg} at 25°C was equivalent to 125% of that at 15°C. This means that the soluble protein of the leaf decreased more rapidly at 25°C than at 15°C, and the remaining soluble protein concentration was higher at 15°C than at 25°C (Fig. 5).

Ln , which controls the time course of the number of leaves, was adjusted. Ln at 15°C was equivalent to 66% of that at 25°C. This means that the plant grown at 25°C had a larger number of leaves than the plant grown at 15°C (Fig. 6).

The parameters at 15°C and 25°C are summarized in Table 2.

RESULTS AND DISCUSSION

Simulation for conventional growth conditions

Figure 7 shows the results of the time course of leaf fresh weight, the amount of soluble protein, and the production efficiency for the amount of soluble protein per head of lettuce grown under conventional environmental conditions. The production efficiency for the amount of soluble protein was calculated by dividing the amount of soluble protein by the number of days after transplanting. Leaf fresh weight reached a maximum value of 147.6 g on Day 37 after transplanting. The amount of soluble protein also reached a maximum value of 634.6 mg on Day 37. These results indicate that a cultivation period longer than 37 days would be a waste of time and energy. Production efficiency for the amount of soluble protein reached a maximum value of 18.8 mg·plant⁻¹ on Day 30 after transplanting. This indicates that a 30-day cultivation cycle is the optimum cycle for maximizing productivity per plant. Of course, the cost of seedlings and labor such as transplanting, harvesting, etc. was ignored.

Fig. 5 Time course of soluble concentration in leaf grown at 25°C (solid line) and 15°C (dotted line).

Fig. 6 Time course of number of leaves grown at 25°C (solid line) and 15°C (dotted line).

Fig. 7 Time course of leaf fresh weight (top), amount of soluble protein (center) and production efficiency for amount of soluble protein (bottom) per head of lettuce grown under conventional environmental conditions.

Fig. 8 Time course of amount of soluble protein in leaf grown at 25°C (solid line) and 15°C (dotted line).

Fig. 9 Time course of fresh weight of plant grown at 25°C (solid line) and 15°C (dotted line).

Fig. 10 Time course of amount of soluble protein contained in plant grown at 25°C (solid line) and 15°C (dotted line).

Fig. 11 Time course of production efficiency for soluble protein of plant grown at 25°C (solid line) and 15°C (dotted line).

Simulation for low- and high-temperature growth conditions

The time course of the amount of soluble protein per leaf is shown in Fig. 8. The amount of soluble protein in the leaf grown at 25°C reached a maximum value of 73.9 mg on Day 14 after transplanting. Thereafter, the amount of soluble protein decreased rapidly because of degradation of concentration and decrease of leaf fresh weight due to senescence. The amount of soluble

Table 2 Parameters at 15°C and 25°C adjusted based on the data of Steven et al. (2000).

	Fresh weight of a leaf					Soluble protein concentration				Number of leaf	
	FW_{max}	r_{gr}	t_{gr}	r_{sen}	t_{sen}	PC_{max}	PC_{deg}	r_{deg}	t_{deg}	Ln	a
15°C	12.6	0.29	17.4	0.80	33.9	10.2	7.45	-0.44	19.5	0.47	0.07
25°C	12.6	0.29	11.5	0.80	28.0	10.2	9.31	-0.66	13.0	0.72	0.07

protein in the leaf grown at 15°C increased more slowly than at 25°C. The maximum value was 59.1 mg and the amount remained the same for a longer time than that at 25°C. The amount of soluble protein was still over 30 mg on Day 31. This is because of the high concentration of remaining soluble protein and late senescence. These results indicate that the low temperature greatly benefits the production of heterologous protein.

Figure 9 shows the time course of the fresh weight per plant grown at 25°C and 15°C. On Day 40 after transplanting, the fresh weight of leaves grown at 15°C and 25°C was 88.7 g·plant⁻¹ and 147.6 g·plant⁻¹, respectively. This is because the fresh weight of each leaf at 15°C increased more slowly than at 25°C, and the number of leaves grown at 15°C was fewer than that at 25°C.

Figure 10 shows the time course of the amount of soluble protein per plant at 25°C and 15°C. The amount of soluble protein at 25°C increased much more rapidly than that at 15°C. On Day 20 after transplanting, the amount of soluble protein in the plant grown at 15°C and 25°C was 47.4 and 397.0 mg, respectively. The main reason for the difference in the amount of soluble protein is the difference in the leaf fresh weight (4.7 g at 15°C and 42.1 g at 25°C). On Day 39, the amount of soluble protein at 15°C (507.1 mg) and at 25°C (709.4 mg).

The maximum production efficiency for soluble protein at 15°C and 25°C was 13.1 mg plant⁻¹·day⁻¹ on Day 40 and 22.6 mg plant⁻¹·day⁻¹ on Day 25, respectively. Rapid growth at 25°C contributed to the high production efficiency. After Day 25, production efficiency at 25°C decreased gradually because of the rapid degradation and low concentration of soluble protein in the leaves.

Model improvements

A major concept not incorporated in this model is the interactions between functions. A negative correlation was observed between the leaf fresh weight and the concentration of soluble protein in a leaf. One reason for this is that plant cells absorb water and enlarge by 10 to 100 times their original volume without requiring proportionality of protoplasmic content (Mohr and Schopfer, 1995). This means that the concentration of soluble protein automatically decreases with increasing leaf fresh weight. Therefore, the function for protein concentration should incorporate leaf fresh weight. The second major concept missing from the model is the influence of lighting conditions. In most models, light availability is assumed to be the major factor limiting growth (Aikman and Benjamin, 1994). Stevens et al. (2000) reported that the final amount of heterologous protein (antibodies) in mature leaf tissue could be slightly increased by high lighting conditions. In addition, the shading of lower leaves by the foliage above is considered to be an important factor in determining the pattern of sequential senescence (Smart, 1994). Therefore, lighting conditions should be incorporated in the model as a key factor for simulating heterologous protein production. The third missing concept is the consideration of plant density. This model considered productivity per plant and time. However, the optimal plant density is also a key factor for optimizing the overall design of an industrial crop production system (Seginer and Ioslovich, 1999). Therefore, productivity per area as well as energy should be considered in evaluating heterologous protein production in the total system. Finally, of course, the concentration of targeted heterologous proteins should be used as an indicator for more precise simulation.

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