Growth and Yield Response of Transgenic Rice Plants Expressing Protoporphyrinogen Oxidase Gene from *Bacillus subtilis*

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ABSTRACT : Transgenic rice plants expressing a *Bacillus subtilis* protoporphyrinogen oxidase (Protox), the last shared enzyme of the porphyrin pathway in the expressed cytoplasm or the plastids, were compared with non-trangenic rice plants in their growth characteristics such as tiller number, plant height, biomass, and yield. Transgenic rice plants of T_3 generation had 8 to 15% and 25 to 43% increases in tiller number compared to non-transgenic rice plants at 4 and 8 weeks after transplanting (WAT); similar values were observed for T_4 generation at 4 and 8 WAT. However, the plant height in both T_3 and T_4 generations was similar between transgenic rice plants and non-transgenic rice plants at 4 and 8 WAT. Transgenic rice plants had 13 to 32% increase in above-ground biomass and 9 to 28% increase in grain yield compared to non-transgenic rice plants, demonstrating that biomass and yield correlate with each other. The increased grain yield of the transgenic rice plants was closely associated with the increased panicle number per plant. The percent of filled grain, thousand grains and spikelet number per panicle were similar between transgenic and non-transgenic rice plants. Generally, the growth and yield of transgenic generations (T_2, T_3, and T_4) and gene expressing sites (cytoplasm-expressed and plastid-targeted transgenic rice plants) were similar, although they slightly varied with generations as well as with gene expressing sites. The transgenic rice plants had promotive effects, indicating that regulation of the porphyrin pathway by expression of *B. subtilis* Protox in rice influences plant growth and yield.

Keywords: *Bacillus subtilis*, Protoporphyrinogen oxidase (Protox), Rice, Transgenic rice plant.

There is a need for greater food production to keep pace with the rapidly increasing world population. The need for increased rice production is particularly urgent because the population of traditional rice-producing countries will require 70% more rice by the year 2025 (International Rice Research Institute, 2002). Most of the suitable land is now used for growing rice and there are severe resource and environmental limitations to exploiting water resources for further irrigation. The additional rice should be produced largely by increased yield per unit area.

The porphyrin pathway is a very important pathway leading to the formation of heme and chlorophylls in plants (Rebeiz and Castelfranco, 1973; Beale and Weinstein, 1990). Of the numerous factors affecting crop yield, the efficiency with which sunlight energy is captured by pigments and transformed into biomass is important (Rebeiz and Castelfranco, 1973). Light energy beyond the plant's capacity causes a decline in the efficiency of light energy conversion. For rice this was estimated to be approximately 17% (Murata and Matsushima, 1975). Accelerated development at high CO_2 levels was responsible for the 42% increase in tiller number at the maximum tillering stage and the 57% enhancement of grain yield at the final harvest (Jilta *et al.*, 1997). In this context, regulation of the porphyrin pathway enhancing chlorophyll biosynthesis might increase light-harvesting capability of plants and thereby their photosynthesis and yield. Theoretically, there will be at least three different approaches for regulating the porphyrin pathway: precursor administration into the pathway, regulation of related enzymes, and introduction of a gene encoding the enzyme.

Actually, it has been reported that the application of 5-aminolevulnic acid (ALA), the precursor of the porphyrin pathway, at low concentrations to radish foliage increased the fixation of CO_2 in light and decreased the release of CO_2 in darkness (Hotta *et al.*, 1997). Furthermore, the ALA application increased growth and yield by 10 to 60% over the control on kidney bean, barley, potato, and garlic plants (Hotta *et al.*, 1997). Since this approach requires high inputs of cost and labor and sometimes causes phytotoxic damage on crops (Chakraborty and Tripathy, 1992; Matsumoto *et al.*, 1994) it has less commercial incentive for increasing crop yield. Although there have been numerous inhibitors for each enzymatic step of the porphyrin pathway (Duke *et al.*, 1994), no enhancers for the pathway have been found yet. Thus the second approach is not possible at the present time.

The third approach, introduction of a gene encoding the

enzyme related to the pathway into plants, might be a feasible way to enhance the pathway. If the gene is successfully expressed in target plants in a manner of increasing chlorophyll biosynthesis, the plants could have enhanced photosynthetic capacity and thereby higher biomass and yield.

Protox, which catalyzes the oxidation of Protogen IX to Proto IX, is the last common enzyme in the biosynthesis of hemes and chlorophylls (Beale and Weinstein, 1990). To date, a dozen Protox genes have been cloned and partially characterized from bacteria, yeast, human, and plants, each of which shares low amino acid identities among different organisms, but high homology between closely related families (Adomat and B ger, 1999; Dailey and Dailey, 1996; Dailey *et al.*, 1994; Lemontova *et al.*, 1997; Narita *et al.*, 1996; Nishimura *et al.*, 1995). Especially, the Protox gene from *Bacillus subtilis* has been cloned and sequenced (Corrigall *et al.*, 1998; Dailey *et al.*, 1994). Although the *B. subtilis* Protox has similar kinetic characteristics to the eukaryotic enzyme which possesses a flavin and employs molecular oxygen as an electron acceptor, it is capable of oxidizing multiple substrates, such as Protogen IX and coproporphyrinogen III. Furthermore, the *B. subtilis* Protox gene has successfully been expressed in *Escherichia coli* (Dailey *et al.*, 1994) and in tobacco (Choi *et al.*, 1998) and in rice plants (Lee *et al.*, 2000). Expression of *Arabidopsis* Protox gene into tobacco plants has also been attempted (Lermontova and Grimm, 2000). Since the *B. subtilis* Protox gene has relatively less homology than the plant gene, accumulation of photodynamic porphyrins could be avoided in the transgenic plants containing the *B. subtilis* Protox gene. Otherwise the porphyrins would be accumulated due to overexpression of a highly homologous Protox gene and act as photodynamic compounds in the presence of light.

In an effort to modify photosynthetic pigment level so as to achieve increases in growth and yield, we have successfully introduced the Protox gene of *B. subtilis* into rice plants using *Agrobacterium*-mediated gene transformation (Lee *et al.*, 2000). The non-transgenic and the transgenic rice plants were compared with respect to their growth and yield under paddy field conditions.

MATERIALS AND METHODS

Plant transformation and growth

Two types of *B. subtilis* Protox gene constructs (Lee *et al.*, 2000) were developed. A pGA1611 : PPC was constructed to express *B. subtilis* Protox in the cytoplasm. *B. subtilis* Protox was amplified by PCR, digested with SacI and *Kpn*I and ligated into the pGA1611 binary vector digested with the same restriction enzymes; the procedure creates a plasmid in which the Protox gene is under the control of the maize ubiquitin promoter. The other construct (pGA1611: PPP), with transit sequence in front of *B. subtilis* Protox gene, expresses the *B. subtilis* Protox in the plastid. *A. tumefaciens* LBA4404 harboring pGA1611 : PPC or pGA1611 : PPP were grown overnight at 28°C in YEP medium supplemented with 5 µg ml^{-1} tetracyclin and 20 µg ml^{-1} hygromycin. The cultures were spun down and pellets were resuspended in an equal volume of AA medium containing 100 µM acetosyringone. Calli were induced from scutellum of rice (*Oryza sativa* cv. Nackdong) seeds on N6 media as previously described (Rashid *et al.*, 1996, Hiei *et al.*, 1997). The calli were transferred to a co-culture medium and cultured for 2-3 days in darkness at 25°C. After hygromycin selection for 3-4 weeks, the calli were transferred to a regeneration media for shoot and root development. After the roots had sufficiently developed, the transgenic plants were transferred to a greenhouse and grown to maturity. The transgenic rice has been selected continuously based on DNA and RNA blot analysis. In this experiment, we used the selected lines (PPC17, PPC89, PPC93, PPC94, PPP32, PPP40, and PPP72) for transgenic plants of T_2, T_3, and T_4 generations that have shown a stable expression of the Protox gene.

Plant culture

Seeds of selected transgenic lines (T_2, 2000; T_3, 2001; and T_4, 2002) and their counterpart non-trasngenic seeds were soaked in water for 4 days at 25°C. The seeds germinated were sown 40 g seeds in trays (58×28×3 cm) containing commercial soil substrate (Boo-Nong Soil, Seoul Korea). The trays were transferred to a greenhouse. The greenhouse was maintained at 25°C day/15°C night temperature and 10-h photoperiod. Seedlings were watered daily. This experiment was conducted in paddy fields with sandy loam soils for three cropping seasons (2000-2002). Paddies were located at Gwangju, farm of Chonnam National University in the southwestern part of Korea. For transgenic rice plants of T_2 generation and their counterpart non-transgenic rice plants, 18-day-old seedlings were transplanted by hand (one seedling per hill) at a distance of 30 cm between plants and 30 cm between rows (11 hills m^{-2}) on 23 May, 2000. For transgenic rice plants of T_3 and T_4 generations, and non-transgenic rice plants, 15-day-old seedlings were transplanted by hand (one seedling per hill) at a distance of 15 cm between plants and 30 cm between plants and 30 cm between rows (22.2 hills m^{-2}) to simulate the configuration for machine transplanting on 16 May, 2001 for T_3 generation and 6 June, 2002 for T_4 generation. In addition, for transgenic rice plants of T_3 and T_4 generations, 15-day-old seedlings were transplanted at the same year (6 June, 2002)

by hand (one seedling per hill) at a distance of 15 cm between plants and 30 cm between plants and 30 cm between rows (22 hills m^{-2}) to compare year-to-year variations between the T_3 and T_4 generations of transgenic rice plants. To compare biomass and yield in transgenic rice plants of T_4 generation with non-trangenic rice plants under standard rice cultivation method (three seedlings per hill, distance 15×30 cm), 15-day-old seedlings were also transplanted by hand on 6 June, 2002. Plot size was 10×7 m. In all experiments, nitrogen (N) was used as urea, and applied pre-plant incorporated (PPI), 50% of the total (11 g N m^{-2}, 30% at 2 weeks after transplanting (WAT), and 20% at panicle initiation stage. Phosphorus and potassium were also PPI at 4.5 g and 5.7 g m^{-2}, respectively. Other cultural management practices were carried out in accordance with the standard rice cultivation method of the Rural Development Administration of Korea (RDA, 1998).

Measurements of growth and yield parameters

The plant height and tiller number were recorded 4 and 8 WAT. At grain maturity, the plants were cut at the soil sur-
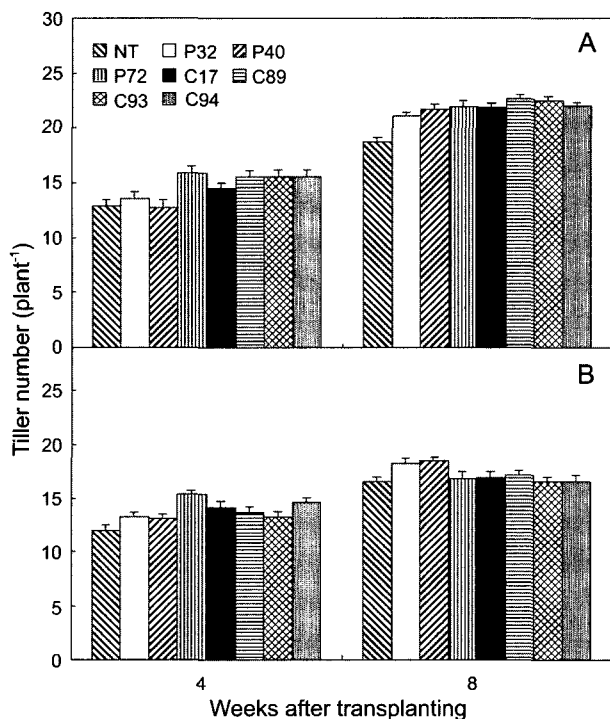
face, oven-dried for one week at 60°C, and above-ground biomass was recorded. Rice yield samples were harvested from 40 hills from three locations per plot. Yield parameters for the number of panicles, spikelets per panicle, ripened grain (%), and 1000-grain weight were determined from these samples. Rough rice yield was calculated based on grain weight adjusted to 14% moisture. In addition, harvest index (HI) was calculated based on the grain yield. Data were analyzed using the analysis of variance (ANOVA) procedure in the SAS program (2000). Treatment means were separated using Fisher's protected LSD test (P=0.05).

Results

Tiller number and plant height

Transgenic rice plants expressing the *B. subtilis* Protox

Fig. 1. Tiller number of non-transgenic and transgenic rice plants expressing a *Bacillus subtilis* protoporphyrinogen oxidase gene. A, T_3 generation; B, T_4 generation; WAT, weeks after transplanting; NT, non-transgenic rice plants; PPP32, PPP40, and PPP72, plastsid targeted transgenic rice plants; PPC17, PPC89, PPC93, and PPC94, cytoplasm targeted transgenic rice plants. Values represent means SE of forty individual plants.
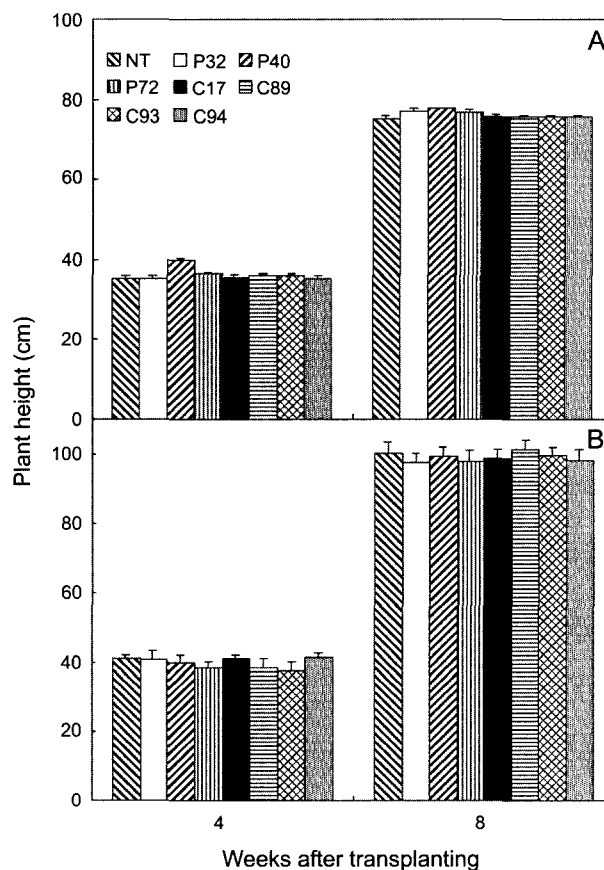

Fig. 2. Plant height of non-transgenic and transgenic rice plants expressing a *Bacillus subtilis* protoporphyrinogen oxidase gene. A, T_3 generation; B, T_4 generation; WAT, weeks after transplanting; NT, non-transgenic rice plants; PPP32, PPP40, and PPP72, plastid targeted transgenic rice plants; PPC17, PPC89, PPC93, and PPC94, cytoplasm targeted transgenic rice plants. Values represent means SE of forty individual plants.

exhibited higher levels of *B. subtilis* Protox mRNA, protein, and activity, and also had a greater capacity to synthesize ALA and a slight increase in chlorophyll and quantum yield of photosystem over non-transgenic rice plants at early vegetative stages (Jung *et al.* 2003). In the present study, the influence of the altered porphyrin pathway on plant growth and yield was investigated in the transgenic rice expressing the *B. subtilis* Protox.

The development of tillers is crucial for determining plant leaf area, which in turn affects photosynthesis and biomass production. For T_3 and T_4 generations, tiller number per plant was greater in transgenic rice plants than in non-transgenic rice plants (Fig. 1). However, there was no difference in tiller number per plant between plastid-targeted transgenic rice plants and cytoplasm-targeted transgenic rice plants at 4 and 8 WAT. Transgenic rice plants of T_3 generation at 4 and 8 WAT had 8 to 15% and 25 to 43% increase in tiller number, which is closely related to assimilated supply (data not shown), when compared to non-transgenic plants, respectively; similar values were observed for T_4 generation at 4 and 8 WAT. However, the plant height in both T_3 and T_4 generations was similar between transgenic rice plants and non-transgenic rice plants at 4 and 8 WAT (Fig. 2).

Biomass, yield, and yield components

Increased tiller number is associated with increased grain yield for rice (Seneweera *et al.*, 1994; Jitla *et al.*, 1997). This is consistent with the observation that transgenic rice plants of T_3 generation had significantly more biomass than non-transgenic rice plants (Table 1). The biomass was 13 to 32% higher in transgenic rice plants than in non-transgenic rice

plants, and greater in plastid-targeted rice plants than in cytoplasm-targeted rice plants (Table 1). Similar results were obtained for T_4 (2002), with biomass increases of 14 to 24% for PPC lines and 16 to 32% for PPP lines (Table 2). There was no significant difference in biomass accumulation between T_3 and T_4 generations (Table 1 and 2).

As with above-ground biomass, grain yields were greater in the transgenic plants compared to non-transgenic rice plants in both T_3 (2001) and T_4 generations (2002); the increased yield in the transgenic rice plants of T_3 generation ranged from 10 to 17% (av. 13%) and 9 to 26% (av. 19%) in PPC and PPP lines, respectively (Table 1), and the increased yield in the transgenic rice plants of T_4 generations ranged from 3 to 15% (av. 9%) and 1 to 23% (av. 14%) on PPC and PPP lines (Table 2). Similarly, panicle number per plant was greater in the transgenic rice plants compared to non-transgenic rice plants. For T_3 generation, when averaged across transgenic lines, the increased panicle number was about 29% for PPC lines and about 30% for PPP lines (Table 1). For T_4 generation, however, it was only 7% and 6% for PPC lines and PPP lines, respectively (Table 2). Although there was no significant difference in the magnitude of increases in panicle number between PPC and PPP lines, it tended to decrease with each generation of the trasgenic rice plants when they were grown in different years. At any rate, the increased grain yield of the transgenic rice plants was closely associated with the increased panicle number per plant. The percentages of filled grain, thousand grains, and spikelet number per panicle were similar in both T_3 and T_4 generations, although it slightly varied with generations as well as with gene expressing sites (Table 1 and 2). For all plants tested, most yield parameters, except panicle number

Table 1. Biomass, yield, and yield components of the non-transgenic and the transgenic rice plants of T_3 generation (2001) expressing a *Bacillus subtilis* protoporphyrinogen oxidase gene.

Line	Biomass (g plant^{-1})	Panicles plant^{-1}	Spikelets panicle^{-1}	Ripened grain (%)	1,000 grain (g)	Yield (g plant^{-1})	Yield (g m^{-2})	Yield index
NT	62.8[d]	14.6[b]	86.3[a]	88.8[a]	24.9[ab]	31.9[c]	701[c]	100
PPC17	71.3[cd]	18.0[ab]	87.9[a]	89.2[a]	24.3[ab]	36.2[abc]	797[abc]	113
PPC89	72.2[bc]	19.3[a]	81.7[a]	87.8[a]	25.1[ab]	35.2[bc]	775[bc]	110
PPC93	73.4[bc]	20.3[a]	80.6[a]	86.9[a]	24.4[ab]	37.2[ab]	818[ab]	117
PPC94	70.9[cd]	18.0[ab]	86.7[a]	87.5[a]	25.5[a]	36.5[abc]	803[abc]	114
PPP32	78.1[ab]	19.9[a]	88.5[a]	87.8[a]	23.4[b]	38.6[ab]	850[ab]	121
PPP40	82.8[a]	18.8[a]	91.1[a]	88.0[a]	23.4[b]	40.3[a]	886[a]	126
PPP72	74.9[ab]	18.7[ab]	83.7[a]	87.1[a]	26.3[a]	34.7[bc]	764[bc]	109

[a]NT, non-transgenic rice plants; PPC17, PPC89, PPC93, and PPC94, cytoplasm targeted transgenic rice plants; PPP32, PPP40, and PPP72, plastid targeted transgenic rice plants.
[b]Planting distance (15×30 cm), transplanting (one seedling per hill).
[c]Yield index was calculated based on yield (g m^{-2}) and represents percent of non-transgenic rice plant.
[d]Means within a column followed by the same letter are not significantly different at the 5% according to the LSD test.

Table 2. Biomass, yield and yield components of the non-transgenic and the transgenic rice plants of T_4 generation (2002) expressing a *Bacillus subtilis* protoporphyrinogen oxidase gene.

Line	Biomass (g plant⁻¹)	Panicles plant⁻¹	Spikelets panicle⁻¹	Ripened grain (%)	1,000 grain (g)	Yield (g plant⁻¹)	Yield (g m⁻²)	Yield index
NT	55.1ᵇ	16.3ᵇ	100.9ᵃᵇ	83.5ᵃ	23.4ᵃᵇ	30.6ᵇ	672ᵇ	100
PPC17	63.4ᵃ	17.7ᵃ	101.6ᵃ	81.0ᵃ	23.1ᵃᵇ	32.2ᵃᵇ	709ᵃᵇ	105
PPC89	68.5ᵃ	18.0ᵃ	88.2ᶜ	79.4ᵃ	23.8ᵃᵇ	35.3ᵃ	776ᵃ	115
PPC93	62.7ᵃ	16.4ᵇ	90.5ᵇᶜ	82.7ᵃ	24.0ᵃᵇ	31.6ᵃᵇ	695ᵃᵇ	103
PPC94	67.8ᵃ	17.7ᵃ	95.3ᵃᵇᶜ	80.6ᵃ	24.6ᵃᵇ	34.7ᵃ	763ᵃ	113
PPP32	72.7ᵃ	19.0ᵃ	100.7ᵃᵇ	80.4ᵃ	23.9ᵃᵇ	37.5ᵃ	826ᵃ	123
PPP40	64.0ᵃ	16.2ᵇ	89.3ᶜ	84.3ᵃ	21.8ᵃᵇ	30.8ᵃᵇ	677ᵃᵇ	101
PPP72	66.8ᵃ	17.0ᵃ	93.3ᵃᵇᶜ	83.0ᵃ	25.5ᵃ	36.6ᵃ	806ᵃ	120

[a]NT, non-transgenic rice plants; PPC17, PPC89, PPC93, and PPC94, cytoplasm targeted transgenic rice plants; PPP32, PPP40, and PPP72, plastid targeted transgenic rice plants.
[b]Planting distance (15×30 cm), transplanting (one seedling per hill).
[c]Yield index was calculated based on yield (g m⁻²) and represents percent of non-transgenic rice plant.
[d]Means within a column followed by the same letter are not significantly different at the 5% according to the LSD test.

Table 3. Variation of biomass, yield, and yield components of the non-trangenic and transgenic rice plants of T_2, T_3 and T_4 generations (2002) expressing a *Bacillus subtilis* protoporphyrinogen oxidase gene.

Line	Gr.	Biomass (g plant⁻¹)	Panicles plant⁻¹	Spikelets panicle⁻¹	Ripened grain (%)	1,000 grain (g)	Yield (g plant⁻¹)	Yield (g m⁻²)	Yield index
NT	–	55.1 b	16.3ᵇᶜ	100.9ᵃ	83.5ᵃ	23.4ᵃᵇᶜ	30.6ᵇ	672ᵇ	100
PPC89	T_2	68.4ᵃ	18.4ᵃᵇᶜ	89.2ᵃ	71.8ᶜ	25.2ᵃ	34.6ᵃ	761ᵃ	113
	T_3	69.7ᵃ	18.8ᵃᵇᶜ	91.2ᵃ	76.5ᵃᵇᶜ	24.7ᵃᵇ	32.5ᵃᵇ	716ᵃᵇ	106
	T_4	68.5ᵃ	18.0ᵃᵇᶜ	88.2ᵃ	79.4ᵃᵇᶜ	23.8ᵃᵇ	35.3ᵃ	776ᵃ	115
PPC94	T_2	65.9ᵃ	18.4ᵃᵇᶜ	97.7ᵃ	76.6ᵃᵇᶜ	23.9ᵃᵇ	33.4ᵃ	734ᵃ	109
	T_3	65.0ᵃ	18.2ᵃᵇᶜ	91.3ᵃ	73.4ᵇᶜ	24.4ᵃᵇ	33.9ᵃ	747ᵃ	111
	T_4	67.8ᵃ	17.7ᵃᵇᶜ	95.3ᵃ	80.6ᵃᵇᶜ	24.6ᵃᵇ	34.7ᵃ	763ᵃ	113
PPP32	T_2	74.9ᵃ	19.6ᵃ	95.3ᵃ	78.5ᵃᵇᶜ	23.0ᵇᶜ	37.8ᵃ	832ᵃ	124
	T_3	74.4ᵃ	19.9ᵃ	98.1ᵃ	80.7ᵃᵇᶜ	24.9ᵃ	38.6ᵃ	850ᵃ	126
	T_4	72.8ᵃ	19.0ᵃ	100.7ᵃ	80.4ᵃᵇᶜ	23.9ᵃᵇ	37.5ᵃ	826ᵃ	123
PPP40	T_2	74.5ᵃ	19.1ᵃᵇ	103.7ᵃ	83.4ᵃᵇ	23.9ᵃᵇ	37.4ᵃ	822ᵃ	122
	T_3	76.0ᵃ	20.1ᵃ	99.2ᵃ	78.3ᵃᵇᶜ	23.7ᵃᵇ	39.6ᵃ	872ᵃ	129
	T_4	64.0ᵃ	16.2ᶜ	89.3ᵃ	84.3ᵃ	21.8ᶜ	30.8ᵃᵇ	677ᵃᵇ	101

[a]NT, non-transgenic rice plants; PPC89 and PPC94, cytoplasm targeted transgenic rice plants; PPP32 and PPP40, plastid targeted transgenic rice plants; Gr, generation.
[b]Planting distance (15×30 cm), transplanting (one seedling per hill).
[c]Yield index was calculated based on yield (g m⁻²) and represents percent of non-transgenic rice plant.
[d]Means within a column followed by the same letter are not significantly different at the 5% according to the LSD test.

and spikelet number per panicle of non-transgenic rice plants, were greater in T_3 generation (2001) compared to T_4 generation (2002) when they were grown in different years. However, when all generations (T_2, T_3, and T_4) of the transgenic rice plants were grown in the same year (2002), no significant differences between generations were observed for grain yield and most yield components (Table 3). As with the results from the experiment in different years, grain

yield and panicle number were greater in transgenic rice plants compared to those of non-trangenic rice plants, with greater increases in PPP lines than in PPC lines for all generations except T_4 of PPP40. When averaged across three generations, the increased grain yield (or panicle number) in transgenic rice plants was about 11% (12%) and 25% (20%) for PPC and PPP lines, respectively. In many cases, spikelet number per panicle tended to reduce in the transgenic rice

Table 4. Biomass and yield of the non-transgenic and the transgenic rice plants of T_4 generation expressing a *Bacillus subtilis* protoporphyrinogen oxidase gene under the standard rice cultivation method of the Rural Development Administration of Korea.

Line	Biomass (kg 10a^{-1})	Index	Yield (kg 10a^{-1})	Index
NT	1.171[c]	100	330[b]	100
PPC89	1.487[a]	127	423[a]	128
PPP40	1.348[b]	115	400[a]	121

[a]NT, non-transgenic rice plants; PPC89, cytoplasm targeted transgenic plants; PPP40, plastid targeted transgenic plants.
[b]Planting distance (15×30 cm), transplanting (three seedlings per hill).
[c]Yield index was calculated based on biomass or yield and represents percent of non-transgenic plant.
[b]Means within a column followed by the same letter are not significantly different at the 5% according to the LSD test.

plants but this was not significant. Similar results were observed for the percent of filled grain (Table 3). When the transgenic rice plants were also grown in accordance with the standard rice cultivation method (distance, 15×30 cm; three seedlings per hill) of the Rural Development Administration of Korea (RDA, 1998), the biomass and yield of the transgenic rice plants were greater than in non-transgenic rice plants, and biomass was greater in cytoplasm-expressed rice plants than in plastid-targeted rice plants, but yield was similar in both cytoplasm and plastid-targeted rice plants (Table 4). In this experiment, however, biomass and yield in both trangenic and non-transgenic rice plants were relatively lower than in other experiments. One reason was a lodging of plants caused by a typhoon.

The growth and yield of transgenic rice plants expressing the *B. subtilis* Protox suggests that these plants produce more tiller number, biomass, and yield than non-transgenic rice plants. Thus, genetic manipulation of porphyrin biosynthesis by expressing the Protox gene appears to be one way to alter plant growth and yield.

Discussion

Previous efforts have been focused on engineering plants to permit more photosynthesis (Cure and Acock, 1986; Poorter, 1993; Hotta *et al.*, 1997). Regulation of the porphyrin pathway enhancing chlorophyll biosynthesis might increase light-harvesting capability of plants and thereby their photosynthesis and crop yield. In our previous study, transgenic rice plants expressed *B. subtilis* Protox mRNA and protein at a much higher level than non-transgenic rice plants; plastid-targeted transgenic rice plants had a higher level of Protox expression than cytoplasm-targeted rice plants (Jung *et al.*, 2003).

The transgenic rice plants expressing the Protox gene in cytoplasm and plastid have greater tiller number per plant compared to their counterpart non-transgenic rice plants (Fig. 1). Tillering activity, which is closely related to assimilated supply, increased approximately 23% in transgenic lines. Increased tiller number is associated with increased

grain yield rice (Seneweera *et al.*, 1994; Jitla *et al.*, 1997). On the other hand, greater tiller number in trasngenic rice plants was most likely associated with the number of emerging tiller buds at the very early vegetative phase (data not shown), showing the influence of Protox expression on tiller differentiation. Accelerated development in rice shoot apex early in development was crucial for obtaining maximum increase in gain yield at elevated CO_2 (Jitla *et al.*, 1997).

The Protox-expressing transgenic plants had a significantly greater above-ground biomass compared to non-transgenic rice plants over transgenic generations (Table 1, 2, 3, and 4). Larger leaf biomass per plant is likely to permit more photosynthesis in these transgenic rice plants. Increased tiller number corresponds to an increase in carbohydrate sink and grain yields (Jitla *et al.*, 1997).

Despite the fact there was no significant difference between cytoplasm-targeted (PPC lines) and plastid-targeted transgenic rice plants (PPP lines) with respect to tiller number, at final harvest biomass accumulation was greater in PPP lines than in PPC lines (Table 1 and 2). This probably suggests an existence of gene expressing site-dependence of biomass accumulation of the transgenic plants. For biomass accumulation, the observation of significant difference between T_3 (2001) and T_4 transgenic generations (2002) was likely due to year-to-year variation (Table 1 and 2), possibly caused by weather conditions such as cumulative solar radiation and air temperatures, rather than due to generation-to-generation variability. In fact, we could not found any differences between transgenic generations with respect to biomass when they were grown in the same year (2002). This indirectly suggests that a Protox gene transferred into two different sites will be highly stable in various environmental conditions.

The transgenic rice plants had a greater grain yield over all generations used in this experiment, with average increases of 11% and 25% for PPC and PPP lines respectively (Table 3). The increases of grain yield in the transgenic rice plants were approximately corresponded to those of above-ground biomass, indicating that the increased yield was largely due to greater biomass. In addition to biomass, the increased

panicle number per plant also largely contributed to increase in grain yield of the transgenic plants. This is consistent with other studies for rice crops; increases in grain yield have been associated with greater tiller number and subsequent increase in panicle number (Ziska *et al.*, 1997; Moya *et al.*, 1998; De Costa *et al.*, 2003). On the other hand, the reductions observed for both spikelet number per panicle and the percent of filled grains were most likely due to strong compensation mechanisms which exist not only between panicle number and spikelet number per panicle but also between total spikelet number and grain filling (Evans, 1993). Of these components, an increase in one component will not necessarily result in an increase in overall grain yield. It can therefore be concluded that the increased grain yield in the transgenic rice plants is due to an increase in both the biomass and panicle number rather than increase in partitioning of biomass to panicles or grains.

Judging from our experiments, we believe that the *B. subtilis* Protox-expressing transgenic rice plants are potentially more productive in tiller number, biomass, and yield. The expression of Protox seems to affect chlorophyll biosynthesis through regulating the porphyrin pathway, enhancing photosynthetic activity, and ultimately leading to biomass and yield increase. Of the numerous efforts to improve productivity, the introduction of a gene encoding the Protox enzyme related to the porphyrin pathway into rice plants is likely to be an efficient way to enhance biomass and yield. It would be worthwhile to examine whether these biomass and yield increases for transgenic rice plants under the Protox gene are also triggered by the possible involvement of other growth factors.

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