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AGRONOMIC CHARACTERISTICS OF TRANSGENIC RICE WITH ENHANCED PHOSPHATE UPTAKE ABILITY BY OVER-EXPRESSED TOBACCO HIGH AFFINITY PHOSPHATE TRANSPORTER

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Abstract

This study was conducted to examine phosphate (Pi) accumulation and performance of agronomic traits of transgenic rice overexpressing a tobacco high affinity phosphate transporter (*NtPT1*) gene. Transgenic plants containing one copy of *NtPT1* transgene were selected and advanced to T_3 generation based on the stability of inheritance and expression of the transgene. Performance of major agronomic traits was examined for the 19 selected T_3 transgene-homozygous lines. In the transgenic line 1-7-8, seed phosphorous (P) content, 1,000 grains weight, number of panicles per hill, and number of grains per panicle were increased by 17%, 6%, 3%, and 10%, respectively, compared to those in the control plants. Yield per plant of the lines 1-7-6, 1-7-8, 1-9-3, and 10-2-7 was 2-6% higher than that of the control variety. In the lines with increased yield per plant, number of panicles per hill and number of grains per panicle were increased. Nitrogen content, chlorophyll content and CO_2 assimilation rate increased concurrently with P content in the flag leaf of the transgenic lines. Some *NtPT1* transgenic lines like 1-7-8 showed increased P accumulation with improved or acceptable performance of agronomic and quality traits. In conclusion, these results indicate that the regulation of PT activity at the transcription level can contribute to increase Pi uptake and improve the performance of agronomic traits like seed yield.

Introduction

Major functions of phosphorous (P) include energy storage and transfer, the maintenance of membrane integrity, and regulation of many enzyme reactions (Marschner, 1995). Consequently, changes in phosphate (Pi) content in the cell may affect multiple structural and regulatory functions leading to altered phenotypic expressions. The importance of P as an essential nutrient has been manifested in many cases of P deficiency. Plant growth and tillering is severely reduced under P deficiency. Numbers of leaf, panicles, and grains per panicle are also reduced under P deficiency (Dobermann & Fairhurst, 2000).

Plants aquire P as Pi. Thus, P use efficiency (PUE) in plants is limited at the Pi uptake step. Pi uptake is controlled by the active process requiring energy involving phosphate transporter (PT) activity, especially at the plasma membrane of the epidermal cells of roots. Thus, PTs have been considered as potential targets for improving Pi uptake efficiency. Supporting evidences for this assumption have been provided from the transgenic plant cells overexpressing a PT gene (Mitsukawa *et al*., 1997), the enhanced expression of PT genes under Pi deficiency (Poirier & Bucher, 2002), and the transgenic rice cells overexpressing a barley gene for high-affinity PT (Rae *et al*., 2003), but only at

the cellular levels. Few direct evidences on the role of PT in Pi uptake have been reported at the whole plant level. The rate-limiting role of PT in Pi uptake has not been supported in the transgenic barley overexpressing one of the barley PT genes (Rae *et al*., 2004).

In the previous study, we developed transgenic rice overexpressing a tobacco phosphate transporter gene (*NtPT1*) (Yoo & Yun, 2000), and demonstrated that PT activity can be modulated at the transcription level and the increased PT activity is sufficient to increase Pi uptake (Park *et al*., 2007). As the *NtPT1*-transgenic rice lines had higher Pi uptake activity, it is expected that the increased PT activity should alter Pi content in the cell, causing multiple consequences in growth and development of transgenic rice plants.

Thus, small-scale field studies were conducted with the 19 primary independent transgenic lines to evaluate levels of Pi accumulation and performance of agronomic and yield-related traits in the field condition.

Materials and Methods

1. Confirmation of transgene transmission and expression: *NtPT1*-transgenic lines were grown and advanced by self pollination. Transgenic T_0 plants were self-pollinated and the seeds from each line were harvested separately. T_1 plants grown from a portion of the T_0 seeds were screened for the presence of the transgene by Southern blot analysis and selected for transgene homozygous lines. Seeds of T_0 transgenic plants were surface-sterilized in 0.1% $HgCl_2$ solution for 1 min., then germinated at 25^{o}C for 3 days in the dark (Yoshida *et al*., 1976). Seedlings were transferred to a solution culture system filled with standard Yoshida solution: N (1.43mM), P (0.32 mM), K (0.51 mM), Ca (0.75 mM), Mg (1.64 mM), Fe (59.37 μM), B (18.92 μM), Mn (9.50 μM), Mo (0.10 μM), Zn (0.15 μM), Cu (0.16 μM), and citric acid (70.72 μM) (Yoshida *et al*., 1976). Seedlings were cultured for 4 weeks in a greenhouse with a 16 h photoperiod (16 h day/8 h night) at 25°C/18°C (day/night) and used for Northern blot analyses.

Northern blot analysis was performed with total RNA that was prepared from the selected transgenic lines using Trizol Reagent (MRC, USA) according to manufacturer's protocol. The total RNA (20 μg) was denatured with a mixture of 2.15 M formaldehyde and 50% formamide and fractionated by electrophoresis on a 1.2% formaldehyde-gel. Separated total RNA was transferred onto a nylon membrane (Hybond-N^+; Amersham, UK) and detected using AlkPhos direct DNA labeling and chemiluminescent detection kit (Amersham Pharmacia Biotech Inc, USA) according to manufacturer's protocol.

2. Field performance test: The transgenic lines were grown and advanced to T_2 generation in a greenhouse at Chonbuk National University, Korea. Nineteen transgene-homolozygous T_2 lines were selected based on the agronomic traits and yield performance. Transgenic T_3 and control (non-transgenic) seedlings were established in a greenhouse and transplanted at 15 cm X 30 cm spacing to the plots in the isolated paddy field for the test of genetically modified organism at National Honam Agricultural Research Institute, Korea. Plants were cultivated according to the recommendations of Rural Development Administration (RDA), Korea (Anon., 2000). Plots were arranged in the randomized complete design with three replications. Plots were treated with a slow-releasing granular fertilizer at 90 N, 45 P, and 57 K (kg/ha) before transplanting. Agronomic, yield-related and quality characteristics were measured according to the recommendations of RDA, Korea (Anon., 2000).

3. Chemical analysis: P and nitrogen (N) contents were analyzed for the flag leaves sampled at the heading stage and seeds harvested at maturity. The flag leaves and seeds were dried at 60°C for 3 d, ground into powder in a mixer mill (MM301, Retsch, Germany), then wet-digested in a mixture of H_2SO_4 and H_2O_2 (Lee & Kim, 2001). The digests were dissolved in sterile triple-distilled water and aliquots were used for P analysis using a sequential plasma spectrometer (ICPS-7500, Shimadzu Corp., Japan). N contents in the flag leaves and seeds were analyzed using an automatic elemental analyzer (VarioMax CNS, Elementar Analysensysteme GmbH, Germany) (Bettina *et al*., 2003).

4. Analysis of CO_2 assimilation and chlorophyll contents: CO_2 assimilation rates and chlorophyll contents were measured for fully expanded flag leaves at the heading stage. CO_2 assimilation rates were measured at 10:00-11:00 am when the photosynthetic photon flux densities was over 1,700μmol/m^2/s using a portable infra-red gas analyzer (LCA-4, ADC Ltd., UK). Measurements were taken from at least three different plants. Chlorophyll contents were measured in SPAD units using a chlorophyll meter (Model SPAD-502, Minolta, USA).

5. Statistical analysis: Analysis of variance was conducted with the program Statistix (Statistix 9.0, Analytical Software, USA). Multiple comparisons between lines were performed by the least significance difference method.

Results

1. Development of advanced generation of *NtPT1* transgenic rice: A total of 107 T_0 transgenic lines were established via Agrobacterium-mediated transformation of *NtPT1* into rice (cv. Dongjinbyo) genome (Yoo & Yun, 2000). Nineteen T_2 lines were selected based on the stability of transgene expression in the subsequent generations (Fig. 1). The transgenes were expressed constitutively in all the transgenic plants (Fig. 1). Seedlings at T_3 generation were established using the transgene-homozyous seeds of each line and used for agronomic characterization.

2. Agronomic characteristics: Average plant height for the transgenic lines and the control variety was 87 cm and 96 cm, respectively. Among the transgenic lines, line 1-9-1 had the highest plant height, line 10-3-1 longest panicle, and line 122-14-5 shortest plant height and panicle length, respectively. Plant height and panicle length of line 1-7-8 were reduced by 7% and 20% relative to those of the control variety. Days to heading of the control variety were 107 and those of the transgenic lines ranged from 105 to 107 (Fig. 2).

One thousand grain weight and 1,000 brown grain weights were highest in the control variety as 29.5 g and 24.7 g, respectively, and the values of these traits were about the same in the transgenic lines 59-5-4 and 96-12-4. However, the averages of the transgenic lines for these traits were 7% and 10% lower than those of the control variety. Conversely, number of panicles per hill was higher in the transgenic plants as 11.7 than the control variety by about 3%. The lines 1-7-1, 1-7-4, 1-7-15, 10-2-7, 59-6-6, and 96-12-4 had over 12 panicles per hill. The effective tiller number, number of grains per panicle and 1,000 grains weight of line 1-7-8 were increased by 3%, 10% and 6 %, respectively, relative to those of the control variety. Average yield per plant of the transgenic lines was 30.3 g that is low by 9% than that of the control variety. However, yield per plant of the lines 1-7-6, 1-7-8, 1-9-3, and 10-2-7 was 2 to 6% higher than that of the control variety. Yield increase in these lines is mostly contributed by the increased number of panicles per hill and number of grains per panicle (Table 1).

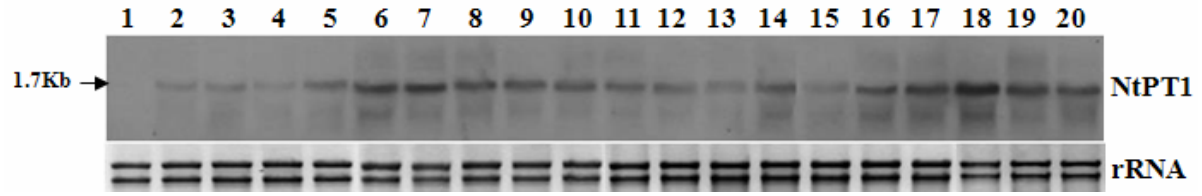

Fig. 1. Northern blot analysis of *NtPT1* transgene in the transgenic T_3 and non-transgenic rice plants. Lane 1, non-transgenic plant; lanes 2 to 28, transgenic lines 1-7-1, 1-7-4, 1-7-6, 1-7-7, 1-7-8, 1-7-15, 1-9-1, 1-9-3, 10-2-7, 10-2-8, 10-7-1, 10-8-1, 10-8-7, 59-5-4, 59-6-6, 96-11-5, 96-12-4, 122-14-5 and 122-18-3, respectively. Total RNA (20 µg) from the roots of transgenic and non-transgenic plants was separated on a 1.2% formaldehyde-gel and detected using AlkPhos direct DNA labeling and chemiluminescent detection kit (Amersham Pharmacia Biotech Inc, USA) according to manufacturer's protocol. Ethidium bromide-stained rRNA bands as an indicator of equal loading (rRNA).

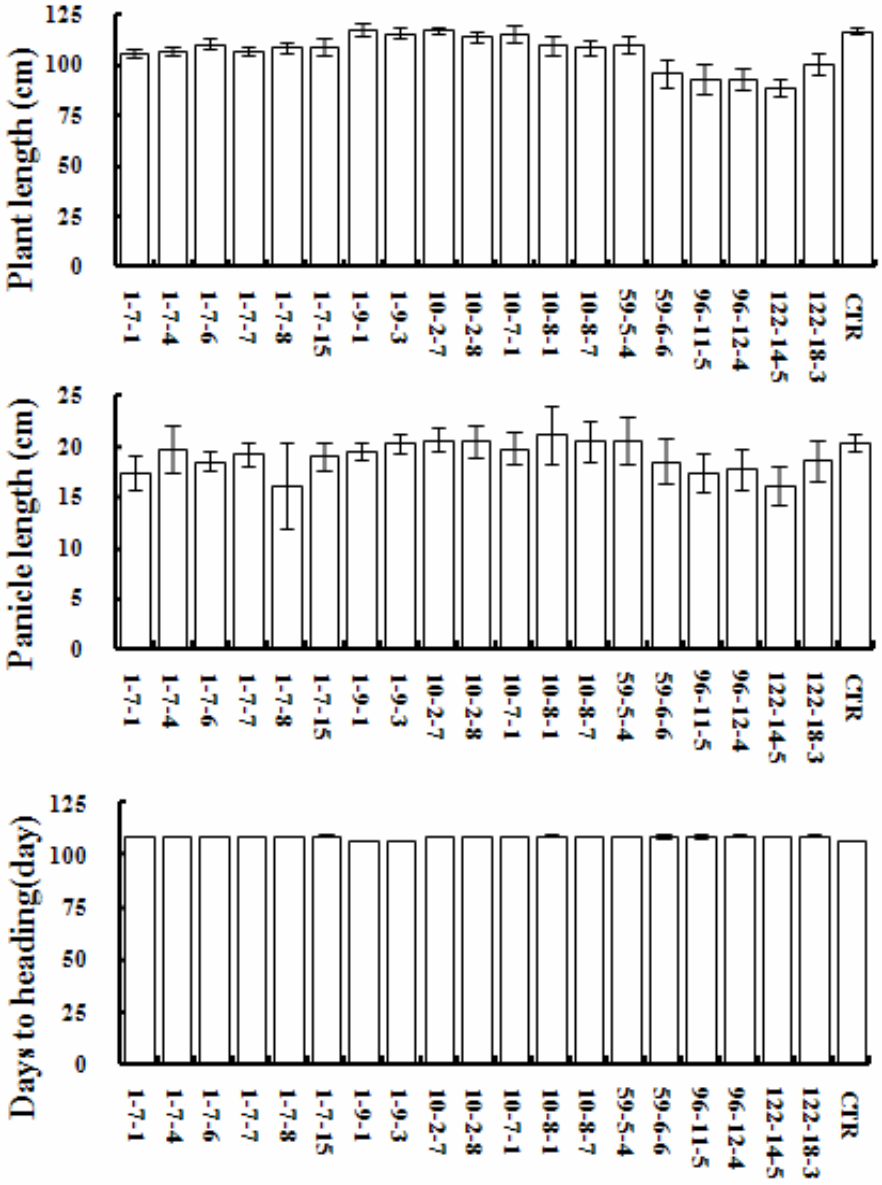

Fig. 2. Performance of the agronomic traits of the nineteen transgenic lines and a control variety in a typical paddy field. Transgenic (1-7-1, 1-7-4, 1-7-6, 1-7-7, 1-7-8, 1-7-15, 1-9-1, 1-9-3, 10-2-7, 10-2-8, 10-7-1, 10-8-1, 10-8-7, 59-5-4, 59-6-6, 96-11-5, 96-12-4, 122-14-5 and 122-18-3) and non-transgenic control (CTR) plants were grown in paddy field containing 31 mg P/Kg. Least significant difference (LSD) at $p<0.05$ was 3.5 and 1.7 for plant height and panicle length, respectively.

Table 1. Yield-related characteristics of the T_3 transgenic lines and the control variety, Dongjinbyo.

Line	1,000 grain weight (g)	1,000 brown grain weight (g)	No. of panicle/hill	No. of grain/panicle	Yields (g)/plant
1-7-1	27.0	22.3	12.1	94.14	30.7
1-7-4	25.6	21.1	12.5	94.24	30.2
1-7-6	27.0	22.1	11.8	106.29	34.0
1-7-7	27.9	22.7	10.9	103.78	31.4
1-7-8	27.6	22.4	11.7	108.65	35.1
1-7-15	25.3	21.0	12.8	96.06	31.4
1-9-1	25.6	21.0	12.0	102.85	31.4
1-9-3	26.8	22.2	11.2	112.93	33.9
10-2-7	28.8	23.5	12.0	100.60	34.5
10-2-8	27.8	23.1	11.2	94.20	29.2
10-7-1	26.8	22.1	11.8	95.80	30.2
10-8-1	27.2	22.2	11.3	91.07	27.9
10-8-7	26.8	21.8	11.5	88.77	27.3
59-5-4	29.4	24.3	10.6	105.56	32.8
59-6-6	27.8	22.7	12.1	90.88	30.8
96-11-5	29.3	23.2	11.7	78.36	27.5
96-12-4	29.3	24.6	12.6	68.50	25.8
122-14-5	26.1	20.8	10.7	84.00	22.8
122-18-3	26.8	21.5	11.8	88.93	28.2
Mean of transgenic lines	27.3	22.3	11.7	95.03	30.3
CTR	29.5	24.7	11.4	98.58	33.2
Transgenic lines ratio relative to CTR	-7%	-10%	3%	-4%	-9%

CTR, control variety

3. Mineral nutrients contents and CO_2 assimilation: P content in the flag leaf at maturity was 67.8 μmole/g DW in the control variety. The content is similar with the range reported in rice (Dobermann & Fairhurst, 2000). The content increased by about 2 to 31% in the transgenic lines. P content per grain was also increased in all the transgenic lines than in the control variety by 6 to 50%. P content per grain of line 1-7-8 is increased by about 10%. P content in the flag leaf at maturity increased by about 13% in the transgenic lines compared to the control variety. The seed P contents of all the transgenic plants increased by 27% on average compared to that of the control variety. N contents in the transgenic lines increased about 17% in the flag leaves but unchanged in the seeds (Table 2).

CO_2 assimilation rates at heading stage were higher in the transgenic lines by about 11% than in the control variety. Similarly, chlorophyll contents at the heading stage were higher in the transgenic lines by about 10% than in the control variety (Fig. 3).

Discussion

P content in flag leaves and grains of the *NtPT1*-transgenic lines increased up to 27~50% due to increased PT expression. In the previous study, we confirmed that PT activity is regulated at the transcriptional level and an important regulatory role of PT in Pi uptake (Park *et al*., 2007). Therefore, the expression of *NtPT1* transgene in rice could increase Pi uptake from the root and eventually Pi accumulation in plants grown in the field condition.

Table 2. P and N contents in the T_3 transgenic lines and the control variety, Dongjinbyeo.

Line	P contents		N contents	
	Seed (µmol P/seed)	Flag leaf (µmol P/g DW)	Seed (%)	Flag leaf (%)
1-7-1	15.5	77.35	1.02	1.24
1-7-4	16.9	76.86	1.07	1.61
1-7-6	17.6	76.37	1.00	1.44
1-7-7	18.2	68.95	1.01	1.47
1-7-8	16.0	71.46	1.02	1.57
1-7-15	16.3	76.58	1.04	1.40
1-9-1	16.4	72.63	1.05	1.60
1-9-3	18.3	71.93	1.09	1.56
10-2-7	18.8	76.10	1.04	1.41
10-2-8	16.9	82.23	1.05	1.26
10-7-1	18.0	72.38	1.09	1.27
10-8-1	19.3	70.66	1.11	1.32
10-8-7	21.2	75.60	1.09	1.35
59-5-4	19.6	79.50	1.03	1.24
59-6-6	21.7	81.15	1.05	1.40
96-11-5	20.4	84.83	1.08	1.39
96-12-4	18.8	88.95	1.02	1.30
122-14-5	20.8	80.06	1.10	1.33
122-18-3	21.9	71.71	1.07	1.22
Mean of transgenic lines	18.6	76.60	1.05	1.39
CTR	14.6	67.76	1.04	1.19
Transgenic lines ratio relative to CTR	27.4	13.0	1.0	16.8

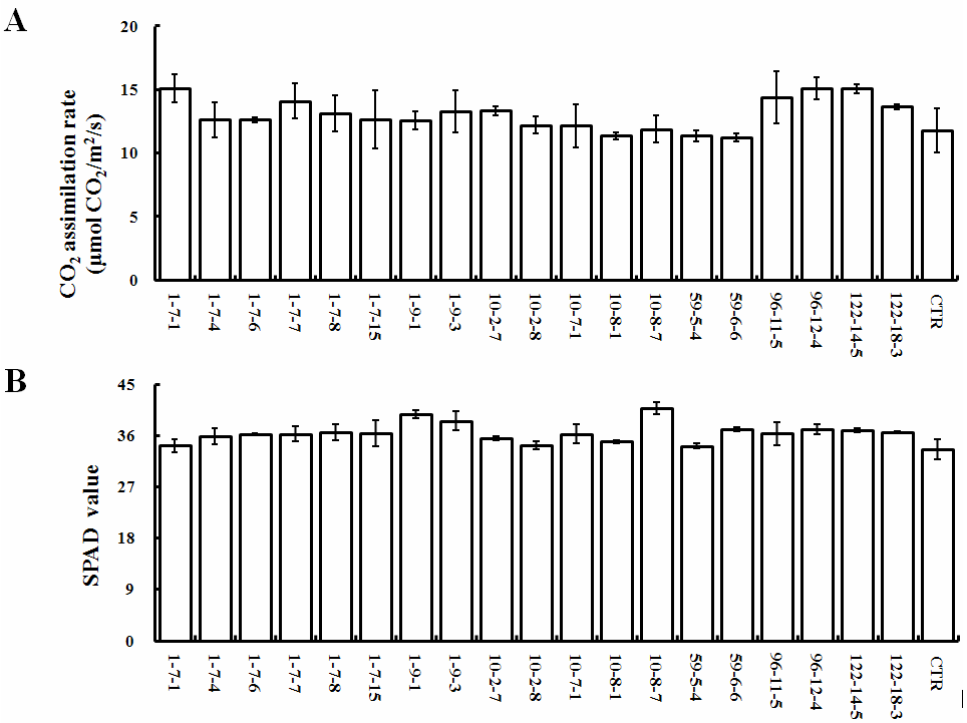

Fig. 3. CO_2 assimilation and chlorophyll contents of the nineteen transgenic lines and the control variety at heading stage in a typical paddy field. Transgenic (1-7-1, 1-7-4, 1-7-6, 1-7-7, 1-7-8, 1-7-15, 1-9-1, 1-9-3, 10-2-7, 10-2-8, 10-7-1, 10-8-1, 10-8-7, 59-5-4, 59-6-6, 96-11-5, 96-12-4, 122-14-5 and 122-18-3) and non-transgenic (CTR) plants were grown in paddy field containing 31 mg P/Kg. LSD

at $p<0.05$ was 2.00 and 2.31 for CO_2 assimilation and chlorophyll contents at heading period, respectively.

The increased P contents in the transgenic plants could cause multiple consequences as P plays diverse roles both as a structural and regulatory element in the growth and development of plants (Marschner, 1995). Increased P can stimulate plant growth and development. For example, a distinct stimulation in growth rate is achieved when Pi supply is increased (Dietz & Foyer, 1986). P also affects various aspects of photosynthesis (Terry & Rao, 1991). The rate of photosynthesis in chloroplasts is dependent on the Pi concentration (Cockburn *et al*., 1967; Rao & Terry, 1989; Jacob & Lawlor, 1991; Qiu & Israel, 1994). The transgenic lines with increased CO_2 assimilation rates like line 1-9-1 and 1-9-3 had the highest N content in the flag leaves. These results indicated a significantly consistent relationship between the increased P, N, and CO_2 assimilation rates in the transgenic lines. P is an essential element for the development of root, tiller and leaf at the early growing stage (Chang, 1983). Rice shows stage-specific interaction in uptake of P and N. At maximum tillering stage, common rice variety show a negative correlation between P and N uptake, but a strong positive correlation at harvest stage between P and N uptake (Islam *et al*., 2008). Also, there is a positive correlation between N and P content in rice straw (Kanareugsa, 1980). Furthermore, chlorophyll content in plants can increase by Pi application (Jiang *et al*., 2007). Thus, increased P in the transgenic plants might have induced increase in N uptake and accumulation. It is well documented that N increase chlorophyll contents and CO_2 assimilation. There is a high correlation between the N and chlorophyll contents (Park & Lee, 2003). Also, CO_2 assimilation is positively correlated with the leaf chlorophyll content (Park & Lee, 2003). Therefore, the increased P uptake of the transgenic lines from the early seedling stage should contribute to the development of healthy root system that may help adsorb mineral nutrients including N. Increased N content facilitates plants better growth and development and to maintain higher chlorophyll content in leaves, eventually contributing to the increased CO_2 assimilation rates. Consequently, it would be generally expected that the increased assimilation in the transgenic lines could result in the increased yield.

Nevertheless, the increased P not necessarily entail increased yield. There was a significant positive correlation between apparent photosynthetic rate per unit leaf area and grain yield only at the ripening stage in N top-dressed plots with rather a negative correlation at the heading stage (Ishii, 1988). Moreover, a decrease in rice yield due to P application has been also observed in certain soils (Place *et al*., 1970; Anon., 1966). Average grain yield per plant decreased by 9% in the transgenic lines though number of panicle per plant increased. The two main causes of decreased yield in the transgenic lines were decreased number of grains per panicle and 1,000 grains weight. Consistently, there was a negative correlation between the seed P contents and the yield per plant among the transgenic lines. Yield decrease was greater in the lines with higher Pi uptake rates like line 122-14-5. However, yield of lines 1-7-6, 1-7-8, 1-9-3, and 10-2-7 was 2~6% higher than that of the control variety. The Pi uptake rates of these lines were higher than that of the control variety but lower than those of the transgenic lines with very high uptake rates like line 122-14-5. These lines had higher number of panicles per plant but lower 1,000 grains weight. These results may suggest multiple implications including the following: 1) in general, though P higher than the optimal range can promote tillering at the vegetative stage and CO_2 assimilation at maturing stage, it may not necessarily contribute to increase yield, 2) yield increase at higher P levels could be achieved by the increased panicle numbers per plant.

From the production point of view, it is clear that yield increase achieved by enhanced uptake of Pi is through increase in grain numbers but not in grain weight. As numbers of grains are primarily determined by numbers of flowers, the transgenic plants could be useful in elucidating the molecular mechanisms underlying the formation of flower and the development of grains. Some *NtPT1* transgenic lines like 1-7-8 showed increased Pi uptake efficiency with improved or acceptable performance of agronomic and quality traits. Therefore, it is apparent that the differential responses are directly or indirectly related to the increased P content in the cells caused by the expression of the *NtPT1* transgene.

In summary, the *NtPT1*-induced increase in Pi uptake and accumulation had diverse effects on many agronomic and yield-related traits. Though grain yield decreased in many transgenic lines, it increased in several transgenic lines like 1-7-8, indicating that the transgenic lines with higher yield potential could be developed by the increased PUE via the regulation of PT activity.

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