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Unintended effects of genetic transformation on photosynthetic gas exchange, leaf reflectance and plant growth properties in barley (*Hordeum vulgare* L.)

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Abstract

Characterization of different component processes of photosynthesis is useful to understand the growth status of plants and to discover possible unintended effects of genetic modification on photosynthesis in transgenic plants. We focused on the changes in photosynthetic gas-exchange properties, reflectance spectra, and plant growth traits among groups of different transgenic barley T1 ($\mathrm{Tol_{T1}}$) and its isogenic controls ($\mathrm{Tol_{NT1}}$), $\mathrm{Tol_{T1}}$, and group of its own transgenic progenies T2 ($\mathrm{Tol_{T2}}$), $\mathrm{Tol_{NT1}}$ and its wild type (WT), respectively. Gas-exchange measurements showed that only the net photosynthetic rate (P_N) and the light-use efficiency (LUE) differed significantly between $\mathrm{Tol_{T1}}$ and $\mathrm{Tol_{T2}}$ with no obvious changes of other characteristics. Reflectance measurements indicated that the reflectance ratio was sensitive to identify the differences between two barley groups. Differences in reflectance expressed on an index basis depended on barley groups. The relationship between LUE and the photochemical reflectance index (PRI) at a leaf level among different barley groups of WT, $\mathrm{Tol_{NT1}}$, $\mathrm{Tol_{T1}}$ and $\mathrm{Tol_{T2}}$ did not changed obviously. The differences in the total leaf area per plant (LA) between WT and $\mathrm{Tol_{NT1}}$ as well as between $\mathrm{Tol_{T1}}$ and $\mathrm{Tol_{T2}}$ were significant. This study finally provided a plausible complex explanation for the unintended effects of genetic transformation on photosynthesis-related properties in barley at different levels. Furthermore, it was concluded that the photosynthesis-related properties of transgenic plants based on gas exchange, leaf reflectance, and plant growth measurements responded to the same environment in a more different way between two subsequent generations than to the processes of the gene insertion by *Agrobacterium* and associated tissue culture.

Additional key words: genetic modification; leaf area; light-use efficiency; reflectance index; reflectance spectra; stomatal limitation; transgenic plants; water-use efficiency.

Introduction

There has been a significant debate concerning the unintended effects of plant genetic transformation (Conner and Jacobs 2000, Kabouw *et al.* 2012). Although the methods used to produce transgenic plants are continually improving, it is not possible at present to control the exact expression and location of the transgenes inserted into the plant genomes. Thus, unintended effects characterized by undesired and unpredictable alterations in the phenotype, response, or composition, can arise among transgenic plants generated even under identical conditions and using the same transgene construct through a disruption of host gene functions or through a somaclonal variation (Cellini *et al.* 2004, Shrawat and Lörz 2006).

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Abbreviations: C_a – atmospheric CO_2 concentration; C_i – intercellular CO_2 concentration; DM – dry mass; E – transpiration rate; g_s – stomatal conductance; LA – total leaf area per plant; L_s – stomatal limitation; LUE – light-use efficiency; $\mathrm{NIRR_{800}}$ – near-infrared reflectance at 800 nm; PAR – photosynthetically active radiation; PH – plant height; P_N – net photosynthetic rate; PRI – photochemical reflectance index; R_{FR} – mean value of reflectance in the far red spectra; R/FR – ratio of reflectance in the red and far red spectra; R_R – mean value of reflectance in the red spectra; R_{Red}/R_{Green} – ratio of reflectance in the red and green spectra; R_V – mean value of reflectance in the visible spectra; SIPI – structure-independent pigment index; T_{leaf} – leaf temperature; WUE – water-use efficiency; λ_{RE} – red edge λ.

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In terms of agronomy (Tian and Yang 1999, Zhao *et al*. 2002, Guo *et al*. 2007) and physiology (Saxena and Stotzky 2001, Dong *et al*. 2006, Sun *et al*. 2007), some studies have been conducted to take a closer look at the photosynthesis-related, unintended effects in transgenic plants with exogenous genes, which are not relating to the improvement of photosynthetic performance. Duan *et al.* (2006) founded that the nontargeted agronomic properties, including a plant height, were different among *bar*-transgenic rice. Hebbar *et al.* (2007) reported that the heavy boll load altered the growth and physiological processes, and as a result, transgenic *Bt* cotton had higher stomatal conductance (g_s), transpiration rate (E), and net photosynthetic rate (P_N) than nontransgenic *Bt* counterparts up to 110 days after sowing. Also, our former study confirmed that the photosynthesis of transgenic *Bt* cotton could be affected both directly and indirectly through influencing either foreign genes expression or the growth and physiological processes (Sun *et al*. 2009).

Photosynthesis represents the final result of the complex interaction of numerous processes, any of which may be influenced by various environmental factors either directly or indirectly. Light plays a key role in photosynthesis and productivity of crops by providing the energy needed for assimilatory power, an activation of enzymes involved in photosynthesis, promoting the stomata opening, and regulating the development of the photosynthetic apparatus (Stirling *et al*. 1993). However, availability of photosynthetically active radiation (PAR) at the cellular level, the penetration of light through plant canopies, and a variation in the partitioning of incoming radiation into a reflectance, absorption and transmittance are influenced by the optical properties of plant leaves (Slaton *et al*. 2001, Kakani *et al*. 2004). More recently, nondestructive optical methods, based on the absorbance and/or reflectance of light by the intact leaf, have been developed (Markwell *et al*. 1995, Gamon and Surfus 1999). Leaf reflectance spectra determined by the surface properties of the leaf, as well as internal structure and biochemical components, have been used to probe the physiological status, growth and development specially linked to photosynthetic performance of plants (Peñuelas and Filella 1998, Carter and Knapp 2001, Slaton *et al*. 2001, Sims and Gamon 2002).

Whether and how these characteristics of transgenic barley (*Hordeum vulgare* L.), which is the fourth most abundant cereal crop in the world and also provides a useful model for the study of wheat, respond to the gene manipulation by *Agrobacterium* has not yet been reported. The objectives of our study were: (*1*) to investigate possible alterations in a gas exchange, reflectance, and plant growth properties between transgenic and nontransgenic barley plants to detect possible unintended effects of genetic modification; (*2*) to provide a plausible integrative explanation for the possible unintended effects of genetic modification on photosynthesis in terms of the gas exchange, reflectance, and plant growth; and (*3*) to distinguish the unintended effects of genetic modification from the foreign gene insertion to those due to tissue culture variation.

Materials and methods

Barley lines and growth: Transgenic barley (*Hordeum vulgare* L.) seeds of segregating T1 and homozygous T2, transformed with the BRACT vector pBRACT 216 by *Agrobacterium tumefaciens* strain AGL1 used in this study, were kindly provided by Dr. Wendy Harwood, the Department of Crop Genetics, John Innes Centre, Norwich, UK. The pBRACT 216 plasmid is based on pGreen and is GatewayTM compatible containing the *hpt* gene (conferring hygromycin resistance) under the control of CaMV 35s promoter and the firefly luciferase gene (*luc*) under the control of the maize ubiquitin promoter (*ubi* 1) and the maize *RpoT* intron 4 at position +165 (Fig. 1*A*). Four different transgenic barley lines of T1 (segregating, BRACT No's: 81-05-1, 84-13-1, 87-09-1 and 90-03-1) and T2 (homozygous, BRACT No's: 81-05-1-8, 84-13-1-15, 87-09-1-13 and 90-03-1-5), four different nontransgenic barley lines of T1's isogenic controls, and the original transformation target cultivar (Golden Promise, noted as WT), totally 13 genotypes of barley, were employed. For our aim, *i.e.* to distinguish the unintended effects of genetic modification from the foreign gene insertion to those due to tissue culture variation, we averaged data from four different transgenic barley lines of T1 and its' isogenic controls as groups of T1 ($\mathrm{Tol}_{\mathrm{T1}}$) and its' control ($\mathrm{Tol}_{\mathrm{NT1}}$), separately. Parallelly, data from four different transgenic barley lines of T2 were averaged as group of T2 ($\mathrm{Tol}_{\mathrm{T2}}$).

Seeds of each line were initially sown in 5 cm diameter pots. After approximately 30 days, the germinated plants were potted into 23 cm diameter round pots for further development. In order to obtain plants influenced by the same environmental effects, the plants were grown side-by-side in a greenhouse of Experimental Station of Northeastern University, Shenyang (123°4'E, 41°8'N), Liaoning, at 25 ± 5°C, with an illumination period of 16 h light/8 h dark. Water stress was minimized with timely irrigation and insecticides were applied as needed during the season.

The plant transformed status for the introduced genes was verified by PCR analysis (Fig. 1*B*). Genomic DNA was extracted using the procedure of Edwards *et al.* (1991) and the presence of the *luc* gene was determined by the amplification of a 1.158 kb fragment of the gene using primers 5'-GCCGGTGTTGGGCGCGTT-3' and 5'-GCGGGAAGTTCACCGGCG-3' described by Harwood *et al*. (2000).

Photosynthetic gas-exchange properties and a leaf reflectance were completed approximately at the same

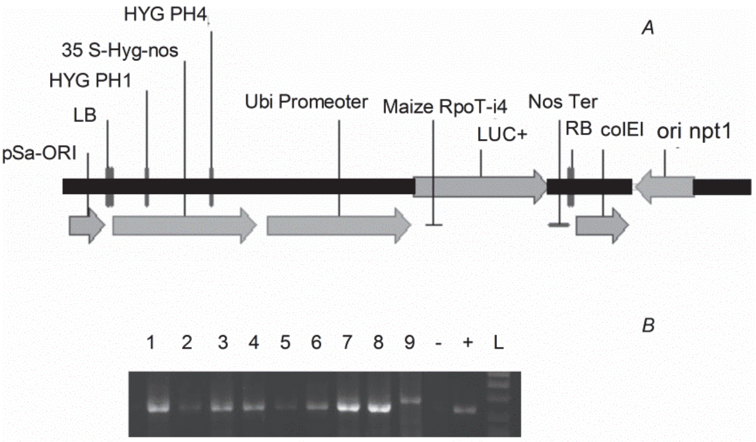

Fig. 1. Linear map of the construction of pBRACT 216 (*A*) and representative *luc* gene PCR analysis of T1 barley plants (*B*). Tracks labeled L contain a 2,000 bp ladder, - – water control, + – positive controls with 1 ng of plasmid pBRACT 216 and tracks 1 – 9 are DNA samples from T1 plants.

time and on the same, second young but fully mature leaf from the top on the main stem of each barley plant at a heading stage.

Photosynthetic gas-exchange measurements: P_N, g_s, C_i, E, and T_{leaf} of every single leaves were measured between 09:00 and 11:00 h on cloudless day with a portable photosynthesis system *LI-6400XT* (*LI-COR*, Lincoln, NE, USA) equipped with a standard 2 × 3 cm leaf cuvette, with an integral temperature and humidity sensor. Throughout the whole measurement, the light intensity of PAR was 1,100–1,500 μmol m^{-2} s^{-1}, the atmospheric CO_2 concentration (C_a) was 395 ± 5 μmol mol^{-1}, the air temperature was 25 ± 5°C, and the relative air humidity was 50–65%. At the beginning of the measuring day, the infrared gas analyzers of the *LI-6400XT* were calibrated according to manufacturer's instructions. Shading of the measured leaves was prevented as much as possible. Once the photosynthetic rate and stomatal conductance reached a steady state, measurements of photosynthetic parameters were taken at 1-min intervals. When 6 consecutive readings showed no substantive differences, these measurements were averaged to produce a single data point. Data stored by the *LI-6400XT* were automatically corrected by a leaf area corresponding to the leaf portion enclosed within the leaf chamber. LUE was calculated as P_N/PAR (Long *et al.* 1993). WUE was calculated as P_N/E (Nijs *et al.* 1997). L_s was calculated as $1 - C_i/C_a$ (Downton *et al.* 1988). Parameters from 10 leaves of the above mentioned insertion levels were determined in different plants of each line at heading stage.

Reflectance measurements: All spectral measurements were made with a field portable spectrometer *Unispec* (*PP Systems*, Haverhill, MA, USA), which consisted of a leaf clamp and bifurcated fiber-optic cable attached to a halogen light source. The *Unispec* has a nominal spectral range from 302 to 1,148 nm with approximately 3 nm nominal bandwidth. Thus, for each measurement, the spectrometer program automatically collects 256 data points covering the entire spectral range. The instrument was allowed to warm up for 20–30 min before use. Individual leaves were held in a black plastic polyvinylchloride leaf clip at a 60°angle relative to the foreoptic. The spectrometer used a halogen light source for leaf reflectance measurements. To account for instrument noise effects, dark scans preceded all measurements. The leaf reflectance was calculated by dividing a leaf radiance by the irradiance of a 99%-reflective white reference disk. The reflectance from this disk was measured before each measurement, as a white standard. The reflectance spectrum for each scan was calculated as R_λ = leaf radiance at wavelength λ /reflectance standard radiance at wavelength λ, and was averaged across the twenty separate scans made on each line. To characterize complex spectra and make possible comparisons between groups, we transformed the average raw reflectance spectrum using published indices, which have been recommended as excellent indicators of foliar biochemical components. A linear interpolation routine was used to estimate values at 1-nm intervals prior to calculation of indices.

Red edge λ (λ_{RE}) is associated with chlorophyll concentration (Curran *et al.* 1990), which is the wavelength (λ) of the maximum slope of the reflectance spectrum at wavelengths between 690 and 740 nm, measured in nm. λ_{RE} was calculated as $(R_n - R_{n-1})/(\lambda_n - \lambda_{n-1})$ over this range (Gitelson and Merzlyak 1996, Richardson and Berlyn 2002). The structure-independent pigment index (SIPI), which is correlated with the ratio of carotenoid to chlorophyll, was calculated as $(R_{800} - R_{445})/(R_{800} - R_{680})$. The SIPI is derived from a reflectance in the blue waveband (445 nm), in which both carotenoids and chlorophylls absorb, and the red waveband (680 nm), in which only chlorophylls absorb. A near-infrared wavelength (800 nm) is also incorporated; it mitigates the effects of within-leaf structural properties on the reflectance (Peñuelas *et al.* 1995, Moran *et al.* 2000). The ratio of the reflectance in the red and green regions of the spectrum (R_{RED}/R_{GREEN}) was effective for the estimation of the ratio of anthocyanin to chlorophyll (Gamon and Surfus 1999), it was calculated as $\sum_{i=600}^{i=699} \mathrm{R_i} / \sum_{i=500}^{i=599} \mathrm{R_i}$. The photochemical reflectance index (PRI) was calculated as $(R_{531} - R_{570})/(R_{531} + R_{570})$ (Gamon *et al.* 1997). By referencing R_{531} against R_{570}, this index partly normalizes for other factors besides the xanthophyll cycle that can affect the spectral reflectance in this spectral region, including pigment content and chloroplast movement; both of them can affect R_{531}. The PRI has been shown to be correlated with both the epoxidation state of xanthophyll cycle pigments and photosynthetic radiation-use efficiency (Gamon *et al.* 1997, Richardson and Berlyn 2002). The near-infrared reflectance at 800 nm ($NIRR_{800}$) was calculated by dividing the values for light energy reflected off the leaf by those for the white standard. This wavelength was selected to discriminate the effects of leaf structure which is known to affect photosynthesis *vs.* chemical or water content on the leaf reflectance (Slaton *et al.* 2001). The leaves samples were the same as those in the measurement of photosynthetic gas exchange.

Plant growth traits measurements: The total leaf area per plant (LA) was measured using a digital area meter *CI-202* (*CID,* Camas, WA, USA) on each plant after gas exchange and reflectance measurements. The plant height (PH) was determined following LA determination. Immediately after total LA and PH measurements were executed, each plant was destructively sampled for measurements of dry mass (DM). Harvest samples for DM measurement were weighed after an incubation at 70°C overnight or until no further decrease in the mass could be detected.

Statistical analysis: One-way analysis of variance (*ANOVA*) was used to test for overall differences among the treatments for all measured variables (*SPSS for Windows 7.5.1*, *SPSS Inc.*, Chicago). If *ANOVA* showed an overall significant effect, individual pairs of treatment means were compared using *Student-Newman-Keuls* analysis. Differences were considered significant at $P<0.05$.

Results

Photosynthetic gas-exchange properties: At single leaf level, all the presented gas-exchange parameters did not show significant differences between WT and Tol_{NT1} barley plants, and the same trend was found between Tol_{NT1} and Tol_{T1} (Fig. 2). However, the significantly higher P_N of Tol_{T2} was observed compared with Tol_{T1} (Fig. 2*A*). Accompanied by this, a significant increase in LUE of Tol_{T2} was also found compared with Tol_{T1} (Fig. 2*E*). The difference of *E* between Tol_{T2} and Tol_{T1} was too small to affect markedly the WUE (Fig. 2*D*,*F*). Additionally, there was a significant difference in C_i between Tol_{T2} and Tol_{NT1} (Fig. 2*C*). Compared with Tol_{NT1}, the higher P_N and C_i with the lower L_s implied that there was not stomatal limitation of photosynthesis in Tol_{T2} (Fig. 2*A*,*C*,*G*).

Reflectance spectra: The mean values of the leaf reflectance in different portions of the spectra showed that all barley leaves exhibited a strong absorption in the red part of the spectrum (620–670 nm), where the reflectance was lower than 11–12% (Table 1). The low reflectance in the red part of the spectrum (R_R) was strongly related to a strong light absorption from photosynthetic pigments. However, in the near-infrared part of the spectrum, about 50% of the light was reflected. Both in the visible part of the spectrum (from 400 to 700 nm, R_V) and in the far red region (beyond 700 nm, R_{FR}), where changes are associated with alterations of leaf anatomy or water content, the leaves of Tol_{T1} and Tol_{T2} had significantly different mean values of the reflectance (Table 1). As a consequence, they also showed a similar change of R_R and of R/FR ratio. However, leaves of Tol_{T1} and Tol_{NT1} had similar mean values of the reflectance both in the different partitions and of R/FR ratio. Except for R_R, WT and Tol_{NT1} had similar R_V, R_{FR} and R/FR ratio.

To illustrate fine differences among spectra of leaves from different barley groups, the ratio spectra were calculated by dividing one barley reflectance spectrum from 4 groups by another barley spectrum from the remaining groups, respectively (Fig. 3). The ratio spectra varied obviously between each pair, except for the pair of WT/Tol_{T2}. Most of the ratio spectra indicated that the reflectance differences in pairs were very obvious in the violet and blue region, between 400 nm and 500 nm, where energy per photon is at maximum, and then reached the highest level in the red region of the spectrum around the chlorophyll peaks (~680 nm). Meanwhile, the reflectance differences in pairs were much lower but moderately increasing within the near infra-red region (beyond 700 nm) and nearly identical in the green (500–580 nm) and yellow (580–600 nm) spectrum, near 550 nm especially. Across the entire calculated spectrum, the pair of WT/Tol_{NT1} showed similar change with Tol_{NT1}/Tol_{T1} and WT/Tol_{T1}. In particular, between 400 nm and 500 nm, WT reflected 7%, and around 680 nm 10% more than Tol_{NT1}. But in the reverse pattern, Tol_{T1} had lesser reflectance than Tol_{T2} in 13% in the range of 400–500 nm and 17% around 680 nm, respectively. The pair of Tol_{NT1}/Tol_{T2} showed a similar pattern as Tol_{T1}/Tol_{T2}.

Differences in the reflectance among the leaves from different groups could be most clearly seen using five common indices, λ_{RE}, SIPI, R_{RED}/R_{GREEN}, PRI and $NIRR_{800}$ (Table 2). Significant decreases in λ_{RE} (from 703.5 ± 0.1 nm to 702.4 ± 1.6 nm) and R_{RED}/R_{GREEN} (from 0.691 ± 0.033 to 0.660 ± 0.022) among WT, Tol_{T1}

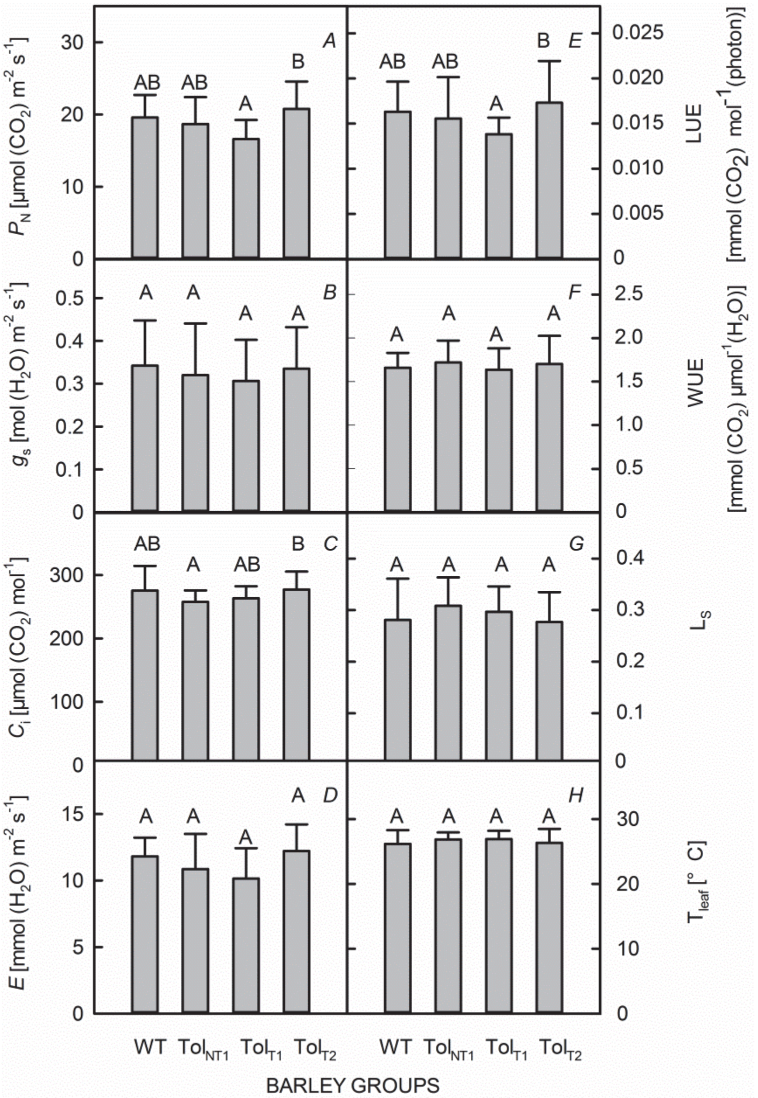

Fig. 2. Gas-exchange properties of transgenic barley groups (Tol$_{T1}$, Tol$_{T2}$) and their nontransgenic counterparts (WT, Tol$_{NT1}$). The values are the means from 10 to 40 individuals ± SD. *Different letters* above columns indicate significant differences, whereas *the same letters* show no significant difference (P<0.05). C_i – intercellular CO$_2$ concentration; E – transpiration rate; g_s – stomatal conductance; L$_s$ – stomatal limitation; LUE – light-use efficiency; P_N – net photosynthetic rate; T$_{leaf}$ – leaf temperature; WUE – water-use efficiency.

Table 1. Mean values of the leaf reflectance in the visible (R$_V$), far red (R$_{FR}$), and red (R$_R$) portion of the spectra, and the ratio of reflectance in the red and far red spectra (R/FR) of transgenic barley groups (Tol$_{T1}$, Tol$_{T2}$) and their nontransgenic counterparts (WT, Tol$_{NT1}$). The values are the means from 10 to 40 individuals ± SD. *Different letters* in the same column indicate significant differences (P<0.05).

Plants	R$_V$ (400–700 nm)	R$_{FR}$ (beyond 700 nm)	R$_R$ (620–700 nm)	R/FR
WT	0.137 ± 0.002^b	0.512 ± 0.008^b	0.124 ± 0.004^b	0.242 ± 0.004^b
Tol$_{NT1}$	0.131 ± 0.011^{ab}	0.491 ± 0.014^{ab}	0.115 ± 0.012^a	0.234 ± 0.018^{ab}
Tol$_{T1}$	0.125 ± 0.003^a	0.486 ± 0.002^a	0.107 ± 0.003^a	0.221 ± 0.006^a
Tol$_{T2}$	0.136 ± 0.002^b	0.506 ± 0.002^b	0.121 ± 0.003^b	0.239 ± 0.005^b

and Tol$_{NT1}$, indicated obvious decreases in chlorophyll and anthocyanin concentrations of Tol$_{NT1}$ leaves comparing with WT and Tol$_{T1}$, respectively. There was no significant difference among WT, Tol$_{T1}$ and Tol$_{NT1}$ in all other three indices. There was no apparent difference in chlorophyll concentration between Tol$_{T1}$ and Tol$_{T2}$ showed by λ_{RE}. However, SIPI, R$_{RED}$/R$_{GREEN}$, and PRI of Tol$_{T2}$ leaf increased significantly comparing with Tol$_{T1}$ indicating that carotenoid, anthocyanin and xanthophyll cycle pigments increased significantly. In this study, the increase in SIPI with stable λ_{RE} of Tol$_{T2}$ appeared to result primarily from changes in the pool sizes of carotenoid pigments rather than from chlorophylls. SIPI increased with increasing PRI and the observed change in xanthophyll cycle pigment inter-conversions was larger than the change in SIPI. It indicated that xanthophyll

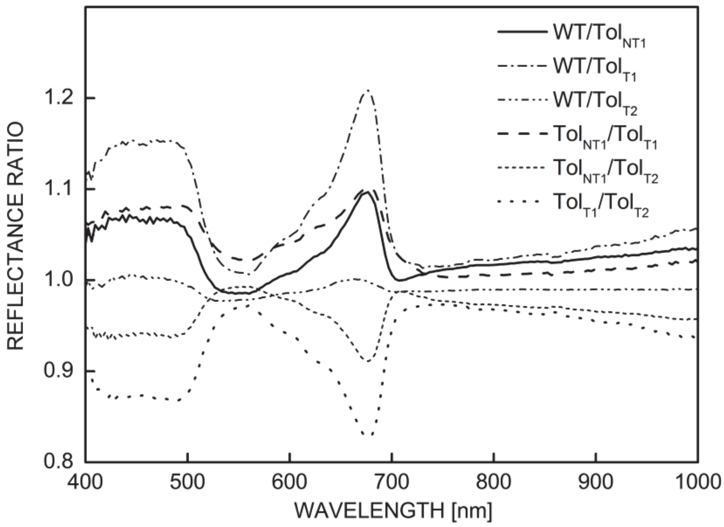

Fig. 3. Leaf reflectance ratios of transgenic barley groups (Tol$_{T1}$, Tol$_{T2}$) and their nontransgenic counterparts (WT, Tol$_{NT1}$). *n* = 80.

Table 2. Reflectance indices of transgenic barley groups (Tol$_{T1}$, Tol$_{T2}$) and their nontransgenic counterparts (WT, Tol$_{NT1}$). NIRR$_{800}$ – the near-infrared reflectance at 800 nm; PRI – the photochemical reflectance index; R$_{Red}$/R$_{Green}$ – the ratio of reflectance in the red and green spectra; SIPI – the structure-independent pigment index; λ_{RE} – red edge λ. The values are the means from 10 to 40 individuals ± SD. Different letters in same column indicate significant differences ($P<0.05$).

Plants	λ_{RE}	SIPI	R$_{RED}$/R$_{GREEN}$	PRI	NIRR$_{800}$
WT	703.5 ± 0.1^b	1.003 ± 0.015^{ab}	0.691 ± 0.033^c	0.017 ± 0.002^{ab}	0.521 ± 0.021^{ab}
Tol$_{NT1}$	702.4 ± 1.6^a	0.999 ± 0.009^a	0.660 ± 0.022^b	0.019 ± 0.005^{ab}	0.512 ± 0.024^a
Tol$_{T1}$	703.4 ± 0.5^b	0.995 ± 0.008^a	0.642 ± 0.019^a	0.016 ± 0.005^a	0.510 ± 0.020^a
Tol$_{T2}$	703.3 ± 0.9^b	1.006 ± 0.011^b	0.682 ± 0.021^c	0.019 ± 0.004^b	0.526 ± 0.015^b

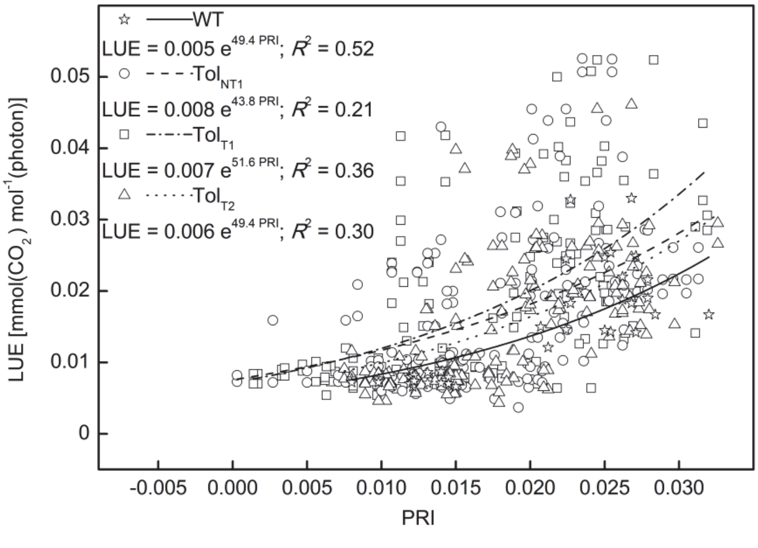

Fig. 4. Relationship between PRI and LUE for transgenic barley groups (Tol$_{T1}$, Tol$_{T2}$) and their nontransgenic counterparts (WT, Tol$_{NT1}$). *n* = 80. LUE – light-use efficiency; PRI – the photochemical reflectance index.

cycle pigment inter-conversions might account only for a small part of the observed variation in SIPI with the percentage of this variation in SIPI due to changes in total pool sizes of carotenoid pigments rather than xanthophyll cycle pigments. The higher values of PRI also suggest that the photosynthetic efficiency of Tol$_{T2}$ leaves was significantly increased relative to Tol$_{T1}$ leaves. Near infra-red reflectance at 800 nm also varied significantly from 0.510 ± 0.020 to 0.526 ± 0.015 between Tol$_{T1}$ and Tol$_{T2}$ showing the same trends as SIPI, R$_{RED}$/R$_{GREEN}$, and PRI and indicating probable changes of leaf structure between these barley plants.

Relationship between LUE and PRI: We analyzed the relationship between LUE and PRI at a leaf level to evaluate if it was common to different barley groups. The relationships between log LUE and PRI were significant for WT (R^2 = 0.52), Tol$_{NT1}$ (R^2 = 0.21), Tol$_{T1}$ (R^2 = 0.36) and $\mathrm{Tol}_{\mathrm{T2}}$ ($R^2 = 0.30$). The slopes and intercepts between different barley groups did not differ obviously (Fig. 4). The relative stable relationship between LUE and PRI among different barley groups was also revealed by the result that change of LUE based on gas-exchange measurement was consistent with PRI in reflectance measurement, especially for $\mathrm{Tol}_{\mathrm{T2}}$ barley plants,.

Plant growth traits: The $\mathrm{Tol}_{\mathrm{NT1}}$ barley plants had a greater DM, higher PH and larger LA than the $\mathrm{Tol}_{\mathrm{T1}}$ plants, but these differences were not statistically significant (Fig. 5). $\mathrm{Tol}_{\mathrm{T2}}$ transgenic barley plants exhibited obviously an increase in the biomass production compared with $\mathrm{Tol}_{\mathrm{T1}}$ plants, which was also reflected by a significant increase in LA and slightly increased PH. On leaf area basis, the difference between WT and $\mathrm{Tol}_{\mathrm{NT1}}$ barley plants was even more pronounced (Fig. 5*B*). In general, gas-exchange and reflectance measurement revealed changes of LUE and PRI, which were in agreement with the biomass production (Figs. 2,5; Table 2).

Discussion

Comparison of plant growth/morphology features, composition characteristics and the response to the environment between biotech corps and their conventional counterparts is a core component of the comparative safety assessment process for biotechnology-derived crops (Millstone *et al.* 1999, Kier and Petrick 2008). Since the ideal reference is an important factor to be taken into account, when evaluating the unintended effects of genetic modification, we started comparing the photosynthesis-related parameters of transgenic barley $\mathrm{Tol}_{\mathrm{T1}}$ and its isogenic nontransgenic control $\mathrm{Tol}_{\mathrm{NT1}}$. This enabled us to eliminate any change related to somaclonal variation regenerating from tissue culture and to determine the effects strictly associated with the genome alteration as a consequence of *Agrobacterium*-mediated transformation. And then, any difference detectable in the photosynthesis-related parameters of plants between $\mathrm{Tol}_{\mathrm{NT1}}$ and WT can be linked to the somaclonal variation. Finally, it was also possible to evaluate how transgenic plants responded in different ways to the same environmental conditions by comparing the photosynthesis-related parameters between transgenic barley $\mathrm{Tol}_{\mathrm{T1}}$ generation and its $\mathrm{Tol}_{\mathrm{T2}}$ progenies grown side-by-side (Zolla *et al.* 2008). The results of our study concluded that the most significant changes of the gas exchange (P_{N} and LUE; Fig. 2*A*,*B*), the leaf reflectance ($\mathrm{R_V}$, $\mathrm{R_R}$, $\mathrm{R_{FR}}$, R/FR, SIPI, $\mathrm{R_{RED}/R_{GREEN}}$, PRI and $\mathrm{NIRR_{800}}$; Tables 1, 2) and the plant growth (LA; Fig. 5*A*) were found between $\mathrm{Tol}_{\mathrm{T1}}$ and $\mathrm{Tol}_{\mathrm{T2}}$, indicating that those two subsequent generations responded in a different way to the same environmental conditions. One possible reason for the differences could be due to the expression instability of the transgene through T1 and T2 generations; the highest level of transgene expression was found in T1 plant, while it showed an important reduction in expression level in the T2 generation (Bourdon *et al.* 2002). It was also mentioned by Butaye *et al.* (2005) that transgenic plants, which exhibit a desired level of transgene expression, might alter its characteristic in subsequent generations. We found only a few significant alterations comparing $\mathrm{Tol}_{\mathrm{NT1}}$ and WT, showing that only a minority of leaf reflectance properties ($\mathrm{R_R}$, λ_{RE} and $\mathrm{R_{RED}/R_{GREEN}}$; Tables 1, 2) and plant growth traits (LA; Fig. 5*B*) of transgenic plants as affected by a tissue culture. Indeed, somaclonal variation resulting from changes in a chromosome number, a chromosome breakage and rearrangement, point mutations, changes in DNA methylation, and an activation of transposons were reported in many studies of plant genetic manipulation (Cassells and Curry 2001, Papazova *et al.* 2006, Bednarek *et al.* 2007). Compared with WT, some unintended changes of the leaf reflectance and plant growth traits found in $\mathrm{Tol}_{\mathrm{T1}}$ were significant, but disappeared in $\mathrm{Tol}_{\mathrm{T2}}$, implying the variation could be an unstable epigenetic variation, which resulted usually from changes in DNA methylation (Kaeppler *et al.* 2000). In addition, the comparison between $\mathrm{Tol}_{\mathrm{NT1}}$ and $\mathrm{Tol}_{\mathrm{T1}}$ showed that except for the leaf reflectance (λ_{RE} and $\mathrm{R_{RED}/R_{GREEN}}$; Table 2), the gas exchange and growth of barley plants seemed not to be

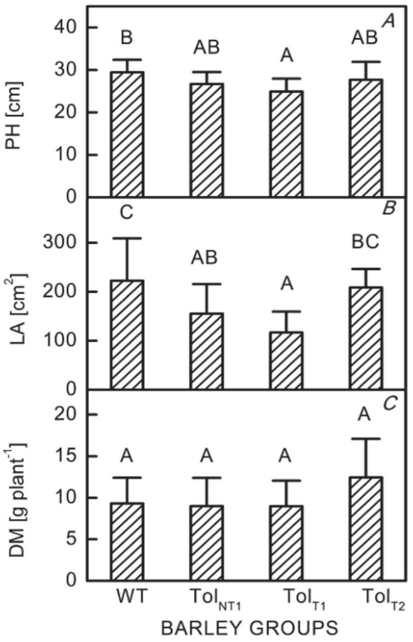

Fig. 5. Plant growth traits of different transgenic barley groups ($\mathrm{Tol}_{\mathrm{T1}}$, $\mathrm{Tol}_{\mathrm{T2}}$) and their nontransgenic counterparts (WT, $\mathrm{Tol}_{\mathrm{NT1}}$). The values are the means from 5 individuals ± SD. *Different letters* above columns indicate significant differences, whereas the same letters show no significant difference ($P<0.05$). DM – dry mass; LA – total leaf area per plant; PH – plant height.

disturbed significantly by the inserted DNA. The results would be more thoroughly understand by sequencing the genomic DNA flanking the insertion sites. The characterizations of the genetic modification at molecular level, including introduced DNA sequences and an insertion site, are important to identify any unintended effects. In particular, the junction sequence of the inserted recombinant DNA and the plant genome by sequence alignment with published sequence data may provide clues on the site of recombinant DNA insertion in the plant genome, *i.e.* they may help to predict, whether any functional or regulatory genes have been disrupted and whether any new open reading frames have been created, resulting in the possible synthesis of novel chimeric proteins (Königa *et al.* 2004, Cullen *et al.* 2011, Harper *et al.* 2012). Especially in barley, the data from transgene flanking regions indicated that transgene insertions were preferentially located in gene-rich areas of the genome, *i.e.* some significant alignments for the transgene flanking regions matched to a cytochrome B gene, a catalase gene, lipase-like protein gene, and a high-molecular-mass glutenin gene (Salvo-Garrido *et al.* 2004). A similar result has been obtained in transgenic *Bt* rice, where the analysis of flanking sequence indicated that there is a strong trend for T-DNA to carry the *Bt* gene sequence into transcriptionally active regions of the rice genome (Zaidi *et al.* 2007). However, due to the limited knowledge of a gene function and limited genomic databases for the crop species, flanking sequence information would not necessarily provide unequivocal evidence for searching for possible unintended effects of the transgene insertion (Cullen *et al.* 2011, Harper *et al.* 2012).

The relationship between the photosynthetic performance and the leaf reflectance are tightly coupled *via* several mechanisms (Carter 1991, Pieruschkaa *et al.* 2010, Zu *et al.* 2010). First of all, a large number of spectral indices, based on certain reflectance bands, have been developed for the estimation of a leaf pigment content (Richardson *et al.* 2002, Sims and Gamon 2002). PRI associated with the zeaxanthin-antheraxanthin-violaxanthin interconversion and the related thylakoid membrane energization was significantly correlated with the maximum quantum yield of PSII estimated by fluorescence techniques and the photosynthetic radiation-use efficiency based on an instantaneous gas exchange (Peñuelas *et al.* 1995, Gamon *et al.* 1997, 2001). However, the radiation that is dissipated by the xanthophyll cycle is not available for photosynthesis, and hence LUE is high when PRI is high (Demmig-Adams and Adams 2006). This again supported the finding of the present study that Tol_{T2} leaves had both higher PRI and LUE than Tol_{T1} leaves (Fig. 2, Table 2). Furthermore, the generally consistent correlation between LUE and PRI (Fig. 4) indicated that these two indices may be applied not only for nontransgenic barley plants, but also for transgenic ones, and they were all good estimates shared with each other. However, in contrast to significant changes in LUE, PRI or other gas-exchange and reflectance parameters, the biomass production of Tol_{T2} based on the whole-plant photosynthetic capacity altered slightly compared with Tol_{T1} (Fig. 5*C*). One possible reason for this could be the measurements based on a single leaf that neglect some important features such as a distance and a position of leaves to each other, while the observed biomass production might be the result of long-term adaptations to the complicated unintended alteration (Terashima and Saeki 1985, Stirling *et al.* 1993, Rosati and DeJong 2003). In combination with PH and LA, the data obtained from the gas-exchange and leaf reflectance measurements were generally in a good agreement with plant growth measurements.

Both the mean values of the leaf reflectance in different portions of the spectra and the reflectance ratios showed that Tol_{T1} had less reflectance than $\text{Tol}_{T2,}$ Tol_{NT1} and WT (Table 1, Fig. 3). However, Tol_{T1} also possessed the lowest P_N, LUE, SIPI, R_{RED}/R_{GREEN}, PRI, LA, PH and DM among different barley groups (Figs. 2, 5; Table 2). One possible reason was that besides the light level, the light quality (red/far red ratio or blue irradiance) was also well correlated with photosynthetic acclimation (Rosati *et al.* 1999, Frak *et al.* 2002). In detail, the R/FR ratio of irradiance may influence photosynthetic characteristics at molecular, biochemical, and physiological levels, such as thylakoid morphology, chlorophyll *a/b* ratio and the partitioning of leaf nitrogen into carboxylation and electron transport (Chow *et al.* 1990, Frak *et al.* 2002). On the other hand, the leaf reflectance in the visible region could provide the information on photosynthetic pigments, whereas the reflectance in the far red region, *i.e.* NIRR_{800}, could diagnose the leaf structure and anatomy (Carter 1991, Slaton *et al.* 2001, Sims and Gamon 2002). Coincided with this speculation, the NIRR_{800} of Tol_{T1} showed a significant difference compared with Tol_{T2} (Table 2). In our recent study, it was revealed that the changed NIRR_{800} could be the alteration in a composition and/or metabolism of structure-forming substances like lignin or alteration in anatomical characteristics, *i.e.* sclerenchymal tissue (Sun *et al.* 2012). In addition, the reflectance-based ratio spectra were sensitive to identify the differences between two barley groups (Fig. 3). This is of fundamental importance, as the comparison of genetically modified plants with their counterparts represents the most basic concept of 'substantial equivalence' (Millstone *et al.* 1999, Kabouw *et al.* 2012).

Furthermore, this study provides a plausible complex explanation for the unintended effects of genetic transformation on photosynthetic properties in barley. The higher photosynthetic activity of Tol_{T2} indicated by increased P_N and LUE based on gas-exchange measurement was most likely due to the possible alteration in photosynthetic pigment and the leaf structure linked to altered SIPI, R_{RED}/R_{GREEN}, NIRR_{800} and the ratio spectra based on the leaf reflectance measurement compared with

$\mathrm{Tol}_{\mathrm{T1}}$. Then, the higher light-use efficiency of $\mathrm{Tol}_{\mathrm{T2}}$, revealed by increased LUE based on gas-exchange measurement and confirmed by higher PRI based on leaf reflectance measurement, was basically in accordance with the plant growth and biomass production based on plant growth parameters in comparison with $\mathrm{Tol}_{\mathrm{T1}}$. Thus, the interactions among photosynthetic gas-exchange behavior, leaf reflectance properties, and plant growth traits were tightly coupled.

Selection marker genes and reporter genes are required virtually in all plant transformation approaches, but they are not generally required for the expression of the trait gene in the transgenic plants. The safety of selection marker genes and reporter genes are another potential problem in genetically engineered plants (Ouakfaoui and Miki 2005, Shrawat and Lörz 2006). Luciferase can be used as a noninvasive reporter in plants to accurately mark changes in a transgene expression. It should be mentioned that its selection through LUC assay is conditional on the presence of external substrates. Although Baruah-Wolff *et al.* (1999) reported that embryogenic callus proliferation and plant regeneration were unaffected by luciferin treatment and luminograph screening during rice transformation study, Jordá and Vera (2000) found that luciferin, the substrate for the reporter luciferase gene, was able to activate an expression of pathogenesis-related genes. The gene encoding hygromycin phosphotransferase (*hpt*) is one of the most widely used selection markers in a cereal transformation. The use of kanamycin as selective agents was also reported as leading probably to an inhibition of the growth and development of transformed tissues (Miki and McHugh 2004). Duan *et al.* (2006) also reported that some physiological characteristics of transgenic *bar* rice, such as the photosynthetic rate, changed significantly after spraying by Liberty®, a glufosinate-ammonium nonselective herbicide. These facts urged caution against the unintended effects of *hpt* and *luc* genes that still need further study.

It was concluded that the photosynthesis-related properties of transgenic barleys with the gene-transfer vector containing the *hpt* and *luc* genes responded to the same environment in a more different way in two subsequent generations. This response, based on gas exchange, leaf reflectance, and plant growth measurements, was rather less dependent on the processes of the gene insertion by *Agrobacterium* and associated to a tissue culture. However, more related studies are needed to follow the basic principle of case-by-case in safety assessing of the genetic transformation.

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