Identification and characterization of improved nitrogen efficiency in interspecific hybridized new-type Brassica napus

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INTRODUCTION

Nitrogen (N) is a primary constituent of nucleotides and proteins that are essential for life (Ladha and Reddy, 2003; Guo et al., 2008). The application of chemical N fertilizers resulted in a great increase in global food production in the past. In China, a 3-fold increase in fertilizer-N application in agriculture has contributed to a near 70% increase in grain production since 1980 (Guo et al., 2010). However, it has been estimated that plants can generally consume less than half of the fertilizer-N applied in soils (Good et al., 2004). The excessive N fertilizers in soils lead to increasingly severe adverse effects to the environment, such as greenhouse gas emission, soil acidification or water eutrophication (Guo et al., 2010; Liu et al., 2013). Furthermore, N is one of the most expensive nutrients to supply, and the commercial N fertilizers represent a major cost in crop production. The cultivation of N-efficient genotypes with better N utilization efficiency (NutE) would enable a reduction in the N fertilization level without drawbacks in yield. NutE has been defined for crops as the grain yield per unit of available N in soils, and/or for other plants used for biomass production as the fresh matter or dry matter produced per N content (Chardon et al., 2010). Comprehensive genetic variation in traits that contribute to NutE were observed among different species as well as in different varieties of the same species (Svečnjak and Rengel, 2006; Dawson et al., 2008; Ikram et al., 2012). It would be of great importance to identify cultivars with significant difference in NutE among plant species and/or genotypes within one species, and for a long-term goal to elucidate the physiological and molecular mechanisms on high N efficiency.

The major form of inorganic N is nitrate in aerobic soils, and ammonium in flooded wetland or acidic soils. The utilization of nitrate and ammonium by plants involves several steps including uptake, assimilation, translocation, recycling and remobilization (Xu et al., 2012). The first step is the active transport across the plasma membrane of root epidermal and cortical cells (Garnett et al., 2009). Molecular data have established that multiple gene family members encoding putative transporter proteins are present for both nitrate and ammonium transport systems (Ludewig et al., 2007; Dechorgnat et al., 2011). In arabidopsis, four nitrate transporters, i.e. NRT1-1 (CHL1), NRT1-2,
NRT2.1 and NRT2.2, take up nitrate (Tsay et al., 2011). At least four ammonium transporters (AMT1-1, AMT1-2, AMT1-3 and AMT1-5) can take up ammonium from soil (Yuan et al., 2007). These genes play important roles in N uptake, plant growth and development under N stress conditions (Garnett et al., 2009). Moreover, the expression of nitrate and ammonium transporters is regulated by different N forms and concentrations (Sonoda et al., 2003). After being taken up by the roots, different forms of N are assimilated. The nitrate is reduced to ammonium by nitrate reductase (NR) and nitrite reductase (NiR), and ammonium, taken directly from the soil or converted from nitrate, is then incorporated into amino acids via the glutamine synthetase (GS)/glutamine-2-oxoglutarate aminotransferase (GOGAT) pathway (Bernard and Habash, 2009; Xu et al., 2012). Expression of several genes related to N metabolism is regulated by nitrate and N metabolites, as well as N deficiency (Takahashi et al., 2001; Cai et al., 2009).

Brassica napus is widely used as food oil for humans and as animal feed worldwide. A disadvantage of oilseed rape is its relatively high N fertilizer demand and the resulting N surpluses in the environment (Rathke et al., 2006). One way to reduce N surpluses is to develop N-efficient cultivars, as genotypic variation was observed for B. napus under contrasting N supplies (Svečnjak and Rengel, 2006; Balint et al., 2008; Kessel et al., 2012). Different traits were used in the selection of N-efficient oilseed rape cultivars, and N-efficient oilseed rape cultivars were characterized by high N uptake efficiency (NupE; Berry et al., 2010; Schulte auf’m Erley et al., 2011). Furthermore, genotypic variation in nitrogen remobilization efficiency contributes to N efficiency of oilseed rape cultivars (Ulras et al., 2013). However, it is not well documented for B. napus in the regulation of physiological processes and in the expression of key genes involved in N transport and assimilation under N starvation conditions, which are essential for developing rapeseed cultivars with enhanced NutE in low-N-availability soils. Moreover, allopolyploid B. napus (genome AACc, 2n = 38) was derived from a natural cross between B. rapa (genome AA, 2n = 20) and B. oleracea (genome CC, 2n = 18) (Nagaharu, 1935). The short domestication history (about 400 years) and traditional breeding schedule of B. napus has led to a narrow genetic range in the population (Gómez-Campo and Prakash, 1999). To widen the genetic diversity of B. napus (A′A′C′C′), new-type B. napus (A′A′C′C′) was developed by interspecific crosses from B. rapa (A′A′), traditional B. napus (A′A′C′C′), and B. carinata (B′B′C′C′) (Xiao et al., 2010). The new-type B. napus harboured exotic genomic components from B. rapa and B. carinata and showed rich phenotypic variation with plenty of valuable traits including seed oil content, yield and yield-related traits, and flowering time (Zou et al., 2011; Fu et al., 2012). This population of new-type B. napus is of great use for an efficient breeding programme for increasing target traits such as nutrient efficiency. Our previous research showed that there were abundant genetic variability and wide genotype differences in N efficiency in the new-type B. napus (unpubl. res.).

The aim of this study was: (1) to identify the variation in N efficiency within a large number of new-type B. napus genotypes at the seedling stage; and (2) to characterize some crucial physiological and molecular mechanisms that were involved in response to N limitation in B. napus.

MATERIALS AND METHODS

Germlasm collection and experimental design for nitrogen efficiency analysis

A population of 150 new-type Brassica napus (A′A′C′C′) inbred lines was derived from interspecific crosses of traditional B. napus (A′A′C′C′), B. rapa (A′A′) and B. carinata (B′B′C′C′), and was used for phenotypic investigation. Ten traditional B. napus lines were employed as a control. The field trial was conducted in paddy soil in Qichun county, Hubei Province in the 2009–2010 crop season. Two N treatments were used: low N treatment with an application of N 75 kg ha−1, and normal N treatment with an application of N 150 kg ha−1. The amount of P, K and B fertilizers applied for each treatment was calculated according to the following nutrient rates: P2O5 90 kg ha−1, K2O 120 kg ha−1, borax 15 kg ha−1. Seed yield and other yield-related traits were investigated for all the materials at the maturing stage. Then, 20 new-type B. napus inbred lines with relatively high N efficiency at the maturing stage were selected for microspore culture. Finally, a total of 46 double haploid (DH) lines were obtained from seven of the 20 new-type B. napus inbred lines, i.e. M9C102-1 (D1), M9C114-1 (D2), M9C035-1 (D3), M9C144-1 (D4), M9C135-1 (D5), M9C144-2 (D6) and M9C039-1 (D8). The 46 new-type B. napus DH lines together with two traditional B. napus genotypes Ningyou 7 (NY7) and Qingyou 10 (QY10) were grown in three independent hydroponic culture experiments to investigate phenotypic variation including biomass production, root morphology, N concentration and accumulation at the seedling stage. Plants were grown in an illuminated culture room at a cycle of 16 h/24°C day and 8 h/22°C night and a light intensity of 300–320 μmol proton m−2 s−1 with a relative humidity of 65–80%. The composition of the full-strength nutrient solution was: 0.25 mM CaCl2·2H2O, 2.0 mM KCl, 0.28 mM Na2HPO4·12H2O, 0.64 mM NaH2PO4·H2O, 2.0 mM MgSO4·7H2O, 46.0 μM H3BO3, 9.0 μM MnCl2·4H2O, 0.3 μM CuSO4·5H2O, 0.8 μM ZnSO4·7H2O, 0.1 μM (NH4)6Mo7O24·4H2O and 500 μM Fe-EDTA. Two N levels, high N (HN; 3.0 mM NH4NO3) and low N (LN; 0.15 mM NH4NO3) were designed with four replicates. The pH of the nutrient solution was controlled at 5.8–6.0 adjusted by NaOH or HCl. The solution was replaced every 5 d with quarter- and half-strength solution for the first and second time, and then with full-strength solution. Uniform seeds were germinated on moistened gauze that was fixed to a tray filled with deionized water, and were grown for 6 d at 22–24°C in the illuminated culture room until the cotyledons were fully developed. Then the uniform seedlings were transferred carefully to HN (3.0 mM NH4NO3) and LN (0.15 mM NH4NO3) solutions, respectively. Plants were harvested after being grown for 24 d, and the phenotypes including dry weight, N concentration in the leaf, and root and root morphology were investigated.

Plant materials and hydroponic experiment for characterizing nitrogen efficiency mechanisms

Two DH lines of new-type B. napus, N-efficient genotype D4–15 and N-inefficient genotype D1–1, and two traditional B. napus cultivars NY7 and QY10 were used in the hydroponic experiments. The growth conditions were the same as the above. After germinating for 6 d, uniform seedlings of the four genotypes were grown in HN solution with 3.0 mM NH4NO3.
for 15 d, and then were transferred to LN solution with 0-15 mm NH4NO3 for 8 d. Then the plants were harvested to analyse root morphology, dry weight, N content, GS and NR activities, and gene expression levels. C2H2N4 (Shanghai Chemical Co., www.reagent.com.cn) was added at 35-34 mg L−1 to inhibit nitrification in the culture solution. Each treatment comprised three replicates, and the experiments were repeated twice.

Root morphology investigation

After plants were harvested, shoots and roots were separated and washed with deionized water. Root morphological parameters including total root length, root volume and root surface area were quantified with root image analysis software WINRHIZO Epson Perfection V700 Photo (JZZIA, Seiko Epson Corp. Japan).

Measurement of dry weight and nitrogen concentration

Shoots and roots were dried at 60 °C to a constant mass, and then the dry weights were recorded. The dried samples were ground into fine powder, and were then digested with H2SO4–H2O2. The concentration of N was determined using a flow injection analysis instrument (FLAstar 5000 analyzer; FOSS, Hilleroed, Denmark). Dry weight (d. wt), N concentration (NC), N accumulation (NA = d. wt × NC), NupE (shoot NA/total NA) and NutE (d. wt/NA = 1/NC) of each sample were calculated.

Determination of tissue NR and GS activities

When the fresh plants were harvested, the samples were immediately frozen in liquid N and then stored at −80 °C. The NR activity was assayed according to the method of Silveira et al. (2001). The NR activity was expressed as µg N dioxygen (NO2−) h−1 g−1 fresh weight (f. wt). The GS activity was determined in a reaction mixture containing imidazole buffer (Taira et al., 2004). The frozen samples (approx. 1-0 g) were ground in an ice-cold mortar and homogenized with 3 mL of 0.05 mol L−1 HONH3Cl) and 0.7 mL of 0.1 mol L−1 ATP. The composition of nutrients except N was as follows (mg kg−1 soil): P, 100; K, 166; Mg, 50; Ca, 140; B, 0.2; Mo, 0.1; and Zn, 0.1. After the two lines D4-15 and D1-1 were first grown in LN solution with 0-15 mm NH4NO3 for 8 d, they were transferred to stable isotope 15N solution with 3.0 mm 15NH4NO3 or NH42NO3 for 3, 11 and 24 h. Plants were sampled and divided into shoots and roots (the roots were washed in ddH2O), and then were placed in an oven at 105 °C for 30 min and dried to a constant weight at 60 °C. All the samples were ground into fine powder. The 15N concentration (15NH4 and 15NO3) of the samples was measured using 15N mass spectrometry (EA-Delta VMS, Thermo Scientific, USA).

Pot culture

The two genotypes D4-15 and D1-1 were grown in soil in pots supplied with two N treatments under greenhouse conditions. The basic properties of the soil were as follow: pH 6.2 (soil:water ratio of 1:2.5), organic matter 5.9 g kg−1, alkaline hydrolysis N 32.3 mg kg−1, available phosphorus 10.3 mg kg−1, available potassium 46.3 mg kg−1. The two N treatments were: ample N (0-2 g N kg−1 soil) and low N (0-02 g N kg−1 soil), with four replicates for each treatment. Each pot contained 7 kg of soil. The composition of nutrients except N was as follows (mg kg−1 soil): P: 100; K: 166; Mg: 50; Ca: 140; B: 0-2; Mo: 0-1; and Zn, 0-1. All pots were watered with 1-5 L of distilled water and incubated for 2 weeks in a greenhouse to reach nutrient balance in the soil before planting. Plants were harvested at the maturing stage, and seed yield and yield components were investigated.

Analysis of introgressed exotic genomic components in new-type B. napus

Genomic DNA was extracted from young leaves of the two new-type B. napus genotypes and their parents. Simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) markers were used to analyse the introgressed exotic genomic components. The SSR markers were selected and experiments were performed according to Xiao et al. (2010). For AFLP analysis, the genomic DNA was digested with two restriction enzymes, EcoRI and MseI. Adaptor ligation and the two successive PCRs for AFLP analysis were performed according to the method described by Vos et al. (1995). The ratio of introgressed genomic components of B. rapa and B. carinata in new-type B. napus was described by the index of sub-genomic
components (ISG) for \( A^\prime, C^\prime \) or \( A^\prime + C^\prime \), which was calculated according to the approach described by Xiao et al. (2010).

Statistical analysis

The data were subjected to analysis of variance, post-hoc comparisons with Duncan’s multiple range tests at \( P < 0.05 \), principal components analysis and clustering analysis using SPSS 17-0 (SPSS Inc., Chicago, IL, USA). Ascendant hierarchical clustering was performed with Ward’s method, and the dissimilarity cut-off was chosen to define four distinct groups.

RESULTS

Identification of nitrogen-efficient germplasm in new-type \textit{B. napus}

Seed yield and relative seed yield of 20 new-type \textit{B. napus} inbred lines and ten traditional \textit{B. napus} lines are shown in Supplementary Data Table S2. The results showed that both traditional and new-type \textit{B. napus} had large variation on seed yield under the LN and HN condition. However, new-type \textit{B. napus} had a higher relative seed yield, ranging from 0.41 to 0.99, than traditional \textit{B. napus} (from 0.40 to 0.78), in particular for M9C104-1, M9C144-1 and M9C144-2. These three new-type \textit{B. napus} lines had higher relative seed yield (>0.8) regarding of their low seed yield at HN condition, showing the characterization of \textit{B. rapa} (\( A^\prime A^\prime \)) and \textit{B. carinata} (\( B^\prime B^\prime C^\prime C^\prime \)) as having high tolerance to infertile soil and low seed yield. By microspore culture, a total of 46 new-type \textit{B. napus} DH lines were successfully obtained from seven of the 20 new-type \textit{B. napus} inbred lines. These DH lines and two traditional \textit{B. napus} cultivars NY7 and QY10 were then grown at high- and low-N levels by hydroponic culture. The phenotypes of the 48 samples are shown in Supplementary Data Table S3. Among them, large genetic variation was observed, and the coefficient of variation ranged from 7.33 to 48.88 %. By principal component analysis, the 19 N-efficient-related traits were divided into four principal components, and the eigenvectors of shoot dry weight (0.350), root dry weight (0.312) and shoot N accumulation (0.300) under the LN condition were higher than other indexes (Supplementary Data Table S4). Thus, shoot and root dry weight under the LN condition could be considered as the first key indicator, and shoot N accumulation under the LN condition as the secondary key indicator in identifying N-efficient genotypes. The analysis and comparisons of key indicators facilitated the clustering of 48 genotypes into four different classes (Supplementary Data Fig. S1). Class 1 and 2 include lines with high N efficiency, and Class 3 and 4 include lines with low N efficiency. Clustering results showed that D4-15 has the highest total score with 21.99, while D1-1 had the lowest total score with −12.41, suggesting that D4-15 could be considered as a N-efficient genotype and D1-1 as a N-inefficient genotype. The traditional \textit{B. napus} NY7 was N inefficient and QY10 was N efficient (Supplementary Data Fig. S1). Interestingly, D4-15 and D1-1 were derived by microspore culture from M9C144-1 and M9C102-1 (Supplementary Data Table S2), respectively.

Dry weight, root morphology and NutE of the four genotypes

Dry weight, root morphology and NutE of D4-15, D1-1, QY10 and NY7 were analysed in hydroponics experiment under LN and HN conditions (Fig. 1; Table 1). For all the genotypes, shoot dry weight was decreased while root dry weight was increased under the LN level compared with the HN level (Fig. 1). The shoot and root dry weights of D4-15 under both N levels were significantly higher than those of QY10, D1-1 and NY7, as were the relative shoot and root dry weight (Fig. 1). The traditional \textit{B. napus} QY10 which was N efficient showed higher shoot and root dry weight compared with D1-1 and NY7 under the LN supply (Fig. 1). Regarding N uptake, a higher concentration and accumulation were observed in both the shoot and root of the four genotypes under the HN condition than under the LN condition. The shoot and root N concentrations of D1-1 and NY7 were significantly higher than those of D4-15 and QY10 under the LN condition, but D4-15 can
accumulate more N in the shoot and root under both N levels than QY10, D1-1 and NY7 (Table 1). The NupE of the four genotypes was decreased under the LN condition, and D4-15 showed the highest value among them (Table 1). For NutE, there was no significant difference among the four genotypes under the HN condition, but it was significantly higher for D4-15 than QY10, D1-1 and NY7 under the LN condition. Compared with the HN condition, a significant increase of NutE was observed for all the genotypes under the LN condition, and D4-15 had the highest increment (Table 1). All four genotypes had a more developed root system under the LN condition than the HN condition (Table 1), suggesting that root growth was induced at the seedling stage by LN stress. Compared with QY10, D1-1 and NY7, the total root length, root volume and root surface area of D4-15 were significantly higher, suggesting an important role for a developed root system in N efficiency for D4-15.

Genotypic differences in nitrogen ($^{15}$NH$_4{^+}$ and $^{15}$NO$_3$-) uptake

Because few growth differences were observed between the B. napus genotype NY7 and the new-type B. napus N-inefficient genotype D1-1 under both N levels (Table 1; Fig. 1), only D4-15 and D1-1 were used for the following experiments. Using stable isotope $^{15}$N ($^{15}$NH$_4{^+}$ and $^{15}$NO$_3^-$) labelling, we analysed genotypic differences in N uptake (Fig. 2). Significantly higher N accumulation was observed in D4-15 than in D1-1 in shoot $^{15}$NH$_4{^+}$ at all the time points ($P < 0.01$) (Fig. 2A, C). Moreover, significantly higher uptake in root was detected for D4-15 at 3 h for $^{15}$NH$_4{^+}$ and at 11 h for $^{15}$NO$_3^-$ than for D1-1 ($P < 0.05$) (Fig. 2B, D).

Activities of NR and GS of the two genotypes

In order to unravel the N metabolic difference in the two new-type B. napus genotypes, the activities of NR and GS in leaves and roots of D4-15 and D1-1 were determined at the seedlings stage under the LN and HN conditions (Fig. 3). The activities of GS in D4-15 leaves were significantly higher compared with D1-1 leaves under both N conditions, but no differences were detected in roots of D4-15 and D1-1 (Fig. 3C, D). As regards the activities of NR, different results were observed. The activity of NR in roots of D4-15 was significantly higher than that in D1-1 under both levels of N supply. In leaves, the activities of NR were significantly higher for D4-15 than for D1-1 under LN stress, but no differences were observed under ample N supply between the two genotypes.

Expression of nitrogen transporter genes

Subsequently, using real-time RT–PCR, we analysed the expression levels of BnAMT1;1 and BnNRT1;1, BnNRT2;2, BnNRT2;5, BnNRT2;6 and BnNRT2;7 in leaves and roots of D4-15 and D1-1 under the two N conditions (Fig. 4). The expression levels of these genes were higher under the LN condition than under the HN condition in both the leaves and roots of the two genotypes, except for BnAMT1;1 and BnNRT2;2. For all the genes in roots, the expression levels were significantly higher in D4-15 than in D1-1 under the N stress condition. However, ambiguous results were observed for the expression levels of all the transporter genes in leaves.

Table 1. Nitrogen accumulation, nitrogen uptake efficiency (NupE), nitrogen utilization efficiency (NutE) and root morphology of D4-15, D1-1, QY10 and NY7 under high-N and low-N conditions (different superscript letters indicate significant difference at $P < 0.05$)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N concentration (%)</th>
<th>N accumulation (mg per plant)</th>
<th>Root morphology</th>
<th>Surface area (cm$^2$ per plant)</th>
<th>Total length (cm per plant)</th>
<th>Root volume (cm$^3$ per plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot</td>
<td>Root</td>
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<td></td>
<td>(mg d. wt per N$^{15}$)</td>
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<td>(mg d. wt per N$^{15}$)</td>
<td>(mg d. wt per N$^{15}$)</td>
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<td>(mg d. wt per N$^{15}$)</td>
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<tr>
<td>High N</td>
<td></td>
<td></td>
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<tr>
<td>D4-15</td>
<td>5.55$^a$</td>
<td>4.59$^a$</td>
<td>17.39$a$</td>
<td>0.84$a$</td>
<td>13.59$a$</td>
<td>1.08$a$</td>
</tr>
<tr>
<td>D1-1</td>
<td>5.69$^a$</td>
<td>4.66$^a$</td>
<td>10.52$^a$</td>
<td>0.97$^a$</td>
<td>10.76$^a$</td>
<td>0.93$^a$</td>
</tr>
<tr>
<td>QY10</td>
<td>5.89$^a$</td>
<td>4.63$^a$</td>
<td>14.31$^b$</td>
<td>0.94$^a$</td>
<td>15.88$^a$</td>
<td>0.98$^a$</td>
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<tr>
<td>NY7</td>
<td>5.70$^a$</td>
<td>4.67$^a$</td>
<td>9.95$^a$</td>
<td>0.94$^a$</td>
<td>10.78$^a$</td>
<td>1.02$^a$</td>
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<tr>
<td>Low N</td>
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<tr>
<td>D4-15</td>
<td>2.75$^d$</td>
<td>2.21$^d$</td>
<td>5.92$^d$</td>
<td>0.85$^d$</td>
<td>6.77$^d$</td>
<td>3.37$^d$</td>
</tr>
<tr>
<td>D1-1</td>
<td>3.26$^b$</td>
<td>2.86$^b$</td>
<td>2.99$^c$</td>
<td>0.85$^d$</td>
<td>3.73$^d$</td>
<td>3.64$^d$</td>
</tr>
<tr>
<td>QY10</td>
<td>2.59$^b$</td>
<td>2.48$^b$</td>
<td>4.67$^c$</td>
<td>0.82$^d$</td>
<td>3.47$^d$</td>
<td>3.62$^d$</td>
</tr>
<tr>
<td>NY7</td>
<td>2.93$^b$</td>
<td>2.78$^b$</td>
<td>3.79$^c$</td>
<td>0.82$^d$</td>
<td>3.27$^d$</td>
<td>3.60$^d$</td>
</tr>
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</table>
of the two genotypes under low N supply. Interestingly, the expression levels of both BnNRT2;5 and BnNRT2;7 were greatly induced by LN stress and were significantly higher than those of any other N transporter genes under the LN environment (Fig. 4G, H, K, L), indicating their key roles in N uptake under the LN condition.

Expression of GS and NR family genes

Using real-time RT-PCR, the expression levels of GS gene family members (BnGln1;1, BnGln1;2, BnGln1;3, BnGln1;4, BnGln1;5 and BnGln2) and NR gene family members (BnNR1 and BnNR2) in leaves and roots of the two genotypes were also detected under both levels of N supply (Figs 5 and 6). The expression levels of most of the GS gene family members in leaves and roots of the two genotypes were downregulated by LN stress, but different expression patterns were observed between D4-15 and D1-1. The expression levels of BnGln1;1, BnGln1;2 and BnGln1;5 were significantly higher in both leaves and roots of D1-1 than in those of D4-15 under the LN condition, but no significant differences were observed for the expression of BnGln1;3 and BnGln2 in either leaves or roots of the two genotypes under the same N condition (Fig. 5). Interestingly, the expression of Gln1;1 and Gln1;4 was induced by LN stress in leaves of D4-15, suggesting their important roles in N assimilation in the N-efficient line D4-15 under the LN condition. The expression levels of BnNR1 in both leaves and roots of D4-15 and D1-1 were downregulated by LN stress, but BnNR2 was downregulated in leaves but upregulated in roots by LN stress (Fig. 6). Furthermore, the expression levels of BnNR1 in both leaves and roots of D4-15 were significantly lower than those in D1-1 under the two N conditions. However, the expression levels of BnNR2 in both leaves and roots of D4-15 were significantly higher than those in D1-1 under the LN condition. In particular, the expression of BnNR2 in roots under the LN condition showed nearly a 4-fold increase in D4-15 compared with D1-1 (Fig. 6D).

The responses of D4-15 and D1-1 to N starvation at the maturing stage

The results in hydroponics are often not reproducible in soil. Thus, in order to test further the responses of D4-15 and D1-1 to N starvation at the maturing stage, a pot experiment was conducted. Seed yield and yield components of the two genotypes
were investigated (Table 2). The results showed that the N-efficient line D4-15 produced significantly more yield per plant than the N-inefficient line D1-1 under LN and LN conditions, and the relative seed yield, which was defined as the ratio of seed yield under the LN level to that under the HN level, was significantly higher for D4-15 (0.52) than for D1-1 (0.29). A significant decrease was observed for pod number per plant of the two lines under the LN condition compared with the HN condition. However, D4-15 could produce nearly twice as many pods per plant as D1-1 under the LN condition. The seed numbers per pod and 1000-seed weight of D4-15 were significantly higher than those of D1-1 under both N levels.

Evaluation of introgression of exotic genomic components in new-type B. napus

The ratio of introgressed exotic genomic components in new-type B. napus was evaluated using SSR and AFLP molecular markers (Table 3). About 1140 polymorphic bands were produced from the two new-type lines and their parents. The ISG (A') in D4-15 and D1-1 were 32-74 and 17-65 %, respectively, and the ISG (C') in D4-15 and D1-1 were 32-23 and 14-58 %, respectively. In total, the ISG (A' + C') in D4-15 was 64-97 %, which was >2-fold higher than in D1-1 (32-23 %). This result revealed that the genome of the new-type B. napus changed considerably after the introgression of exotic genome components from B. rapa and/or B. carinata, and more exotic genome components were introgressed into the genome of D4-15 than D1-1.

DISCUSSION

Large phenotypic variation exist in new-type B. napus

There is much genetic variation in traits that contribute to NutE (Svečniak and Rengel, 2006; Balint et al., 2008; Dawson et al., 2008; Kessel et al., 2012). Identification of N-efficient genotypes from a natural population is an important first step to improve N efficiency of crops and reduce environmental pollution (Hakeem et al., 2011). Previous studies have extensively characterized various crop performances under high- and low-N environments, such as arabidopsis, rice, maize and B. napus (Ikram et al., 2012; Kessel et al., 2012; Wei et al., 2012; Abdel-Ghani et al., 2013).

In the current research, the performances of new-type B. napus and traditional B. napus were analysed under high- and low-N conditions at both the seedling stage and maturing stage. The results showed that N availability greatly influenced the variation of all the measured traits and most of the computed traits, and large phenotypic variation for N efficiency was observed in new-type B. napus (Supplementary Data Tables S2 and S3). NutE is defined differently, depending on whether vegetative biomass productivity or grain production are important traits to be considered (Dawson et al., 2008). In this study, principal component analysis and clustering analysis using SPSS 17.0 were performed to determine key indicators under high- and low-N conditions (Supplementary Table S4, Fig. S1). Finally, two extreme genotypes (D4-15 and D1-1) were noted and were used for further study.

Developed root systems and efficient transport systems contribute greatly to plant growth under low nitrogen environments

Nitrogen uptake depends on the extent and effectiveness of the root system. Differences in N uptake may have been caused by differences in root growth. For example, Kamh et al. (2005) showed that the variety with a high yield under an LN environment had greater root growth following stem extension in a comparison of two B. napus varieties. Similarly, Ye et al. (2010) analysed two B. napus genotypes differing in N use in nutrient solution and reported a greater root volume and root active absorbing area in the N-efficient genotype. The same results were observed in the present study. The N-efficient line D4-15 had a more developed root system compared with NY7 and D1-1 under both N levels (Table 1). In terms of N uptake among the four genotypes, the results showed that D4-15 could accumulate more N in both shoots and roots than NY7 and D1-1 under the LN condition (Table 1). The result was confirmed in the 15N experiment by adding two different forms of 15N (15NH4+ and 15NO3−) to the nutrient solution (Fig. 2). A larger root system and higher NupE of D4-15 facilitated the accumulation of more N in the shoot than in D1-1, and a larger shoot of D4-15 might result in a change in sink strength which then would drive N uptake.

It is reported that nitrate and ammonium transporters play important roles in N acquisition by regulating root growth and development under N starvation (Remans et al., 2006; Engineer and Kranz, 2007; Shi et al., 2010). For example, Remans et al. (2006) found that NRT2.1 had a key dual function in coordinating root development with external NO3− availability.

In this study, we examined the expression of six nitrate and ammonium transporters under N stress conditions, and found that the
expression levels of all of these genes were significantly higher in roots of the N-efficient genotype D4-15 than in roots of the N-inefficient genotype D1-1 under the LN condition (Fig. 4), and some genes were greatly induced by LN stress, such as BnNRT2;5 and BnNRT2;7 (Fig. 4G, H, K, L). Our results suggest that efficient transport systems and developed root systems play important roles in B. napus N acquisition under N starvation. However, the mechanism by which gene expression regulates shoot and root growth in B. napus needs further study, and more evidence of its molecular biology needs to be uncovered.

The importance of NR and GS in nitrogen assimilation and the regulation mechanisms of gene expression at the seedling stage of new-type B. napus

Different forms of N are assimilated after being taken up by plant roots (Xu et al., 2012). Reports show that NR and GS play important roles in N assimilation for plants grown under LN conditions (Shi et al., 2010; Hakeem et al., 2011). Using two B. napus genotypes differing in N efficiency in nutrient solution, Ye et al. (2010) found that higher NR and GS activity were detected in the more N-efficient genotype. Similar results were observed in the present study. The activities of NR were higher in roots and leaves of the N-efficient line D4-15 compared with the N-inefficient line D1-1 under the LN condition (Fig. 3). It is reported that NR could play an indirect role in the absorption of NO₃⁻, regulating the levels of NO₃⁻ and amino acids in root cells (Hakeem et al., 2011). In our experiment, the pattern of NO₃⁻ uptake by the root and the NO₃⁻ concentration and accumulation in roots and shoots were coincident with the pattern of NR activities (Fig. 2; Table 1). The activities of GS were significantly higher in D4-15 leaves than in D1-1 leaves, but no significant differences were observed in GS activities between D4-15 and D1-1 roots (Fig. 3). The reasons might be that a more efficient transport system of D4-15 resulted in more N accumulated in leaves (Fig. 4G), which led to higher GS activities for assimilation in leaves of D4-15. Our results show that the N-efficient genotype D4-15 had a higher nitrate reduction in leaves and roots and
higher ammonium assimilation in leaves than the N-inefficient genotype D1-1 under LN supply, suggesting that NR and GS may play vital roles in the N efficiency of new-type *B. napus* under LN conditions.

As NO$_3^-$ is assimilated via conversion to NO$_2^-$, then of NH$_4^+$ into amino acids, the internal pools of amino acids within plants may indicate the N status by providing a signal that is somehow sensed and can feed back to regulate N uptake and assimilation by the plant (Miller *et al.*, 2008). The feedback regulation can occur by changing the expression of transporters, and may also involve the post-translational modification of protein levels (Bernard and Habash, 2009). For example, Finnemann and Schjoerring (2000) found that GS1 was regulated post-translationally by reversible phosphatases. In this research, although the activities of GS and NR were significantly higher in leaves of D4-15 than in D1-1 under LN supply (Fig. 3), the gene expression levels were different (Figs 5 and 6). The reason might be that D4-15 had higher N accumulation and higher activities of NR and GS in *vivo* which resulted in a larger internal pool of downstream N metabolites. Thus, certain N-sensing systems were started, and the feedback regulation may occur by post-translationally modifying NR and GS protein levels.

**Application potential of interspecific hybridized new-type Brassica napus in breeding cultivars with enhanced NutE and seed yield**

The role of cultivated *B. napus* as a commercial oil crop in Asia, Europe, North America and Australia has progressively
High-N: plants were grown in 3.0 mM NH4NO3 solution for 23 d. Low-N: BnNR2

Increased due to better production potential and seed quality improvement. However, the short domestication history and intensive breeding has resulted in a narrow genetic base of B. napus (Gómez-Campo and Prakash, 1999), which was not beneficial in breeding B. napus cultivars with higher seed yield and oil content, and even better tolerance to biotic and abiotic stresses. It is reported that the introgression of A’ and/or C’ genomic components from B. rapa and B. carinata into new-type B. napus (A’A’C’C’) can lead to considerable differences in the gene expression profiles (Chen et al., 2008). In this research, large genetic variation was observed among agronomic traits contributing to N efficiency of new-type B. napus (Supplementary Tables S2 and S3). Further research on D4-15, QY10, NY7 and D1-1 demonstrated that the N-efficient new-type B. napus line showed higher values than the N-inefficient line and the traditional B. napus genotypes on several phenotypic traits under both LN and HN conditions by hydroponic culture, suggesting that the N-efficient new-type B. napus line had a better overall growth potential under both N levels at the seedling stage (Tables 1 and 2). However, at the maturing stage, there was no significant difference in seed yield between D4-15 derived from M9C144-1 by microspore culture and QY10 under low N conditions, but D4-15 had a higher relative seed yield (0.87) than QY10 (0.71), indicating that D4-15 had higher N efficiency (tolerance to LN stress) than QY10 which would be of importance in breeding N-efficient B. napus cultivars in the field (Supplementary Data Table S2). By evaluation of introgression of exotic genomic components in D4-15 and D1-1 with SSR and AFLP molecular markers, we found that more exotic genome components were introgressed into the genome of D4-15 (64-97%) than D1-1 (32-23%) (Table 3). This may be one of the key factors that contribute to the better performance of D4-15 than D1-1 at both the seedling stage and maturing stage under an LN environment. However, more research is needed to confirm whether the differentially expressed N-related genes were genetically localized in the genomic region containing the exotic genome components that were introgressed. Moreover, depending on the morphological and physiological features, it would be very interesting to use D4-15 together with D1-1 or NY7 as parent lines of populations to perform quantitative trait locus mapping of traits related to NutE in B. napus in the future.

**SUPPLEMENTARY DATA**

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: systemic classification of 46 new-type Brassica napus and two traditional B. napus cultivars. Table S1: primer sequences used for real-time RT–PCR. Table S2: seed yield and relative seed yield of 20 new-type Brassica napus inbred lines and ten traditional Brassica napus lines. Table S3: phenotypic analysis of 46 new-type Brassica napus genotypes and two traditional B. napus genotypes in the hydroponic experiment. Table S4: principal components analysis of 46 new-type Brassica napus genotypes with 19 nitrogen-efficient indicators in the hydroponic experiment.

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