Over-Expression of a Tobacco Nitrate Reductase Gene in Wheat (*Triticum aestivum* L.) Increases Seed Protein Content and Weight without Augmenting Nitrogen Supplying

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Abstract

Heavy nitrogen (N) application to gain higher yield of wheat (*Triticum aestivum* L.) resulted in increased production cost and environment pollution. How to diminish the N supply without losing yield and/or quality remains a challenge. To meet the challenge, we integrated and expressed a tobacco nitrate reductase gene (NR) in transgenic wheat. The 35S-NR gene was transferred into two winter cultivars, “Nongda146” and “Jimai6358”, by *Agrobacterium*mediation. Over-expression of the transgene remarkably enhanced T1 foliar NR activity and significantly augmented T2 seed protein content and 1000-grain weight in 63.8% and 68.1% of T1 offspring (total 67 individuals analyzed), respectively. Our results suggest that constitutive expression of foreign nitrate reductase gene(s) in wheat might improve nitrogen use efficiency and thus make it possible to increase seed protein content and weight without augmenting N supplying.

Introduction

Wheat (*Triticum aestivum* L.) is one of the most widely cultivated and most important food crops in the world, and its higher yield depends on heavy field-supply of nitrogen (N) fertilizer [1–3]. However, the N use efficiency of crops was low (approximately 33%) [4,5] and over 50% of the N applied was lost from the plant-soil system [6], leading to environmental damage and negative impacts on human health [7–10]. That was particularly pronounced in the areas along the Yellow River, Huai River and Hai River (called “Huanghuaihai Area”) in central China [11] where is one of the major areas of wheat production but with saline and alkaline sandy soils and relatively lower yield.

Nitrate (NO$_3^-$) is the main N source for crops under normal field conditions [9,12,13] and its availability strongly affects crop productivity and food quality [14], especially in wheat [15–17]. The nitrate uptake in plant is well known to be first reduced to nitrite and then to ammonium via the Glutamate synthesis cycle (GOGAT cycle) in two successive steps catalyzed by nitrate reductase (EC 1.6.6.1, NR) and nitrite reductase (EC 1.7.7.1, NIR) in cytosol and chloroplast, respectively [10]. Thus, the NR is considered a key enzyme in the overall process of nitrate assimilation [19], and how to increase NR content and/or activity, therefore, becomes one of the major challenges for increasing N use efficiency in crops including wheat. Using biotechnology to introduce and over-express exogenous tobacco NR gene was tested for lowering nitrate content in the leaf and edible organs of dicotyledonous crops [20–27], but no information about the effect on seed protein content and grain weight was released. To the best of our knowledge, integration and over-expression of foreign NR gene have not been tested in wheat although its foliar NR activity was demonstrated significantly correlated with yield [17,28], flour quality [16] and grain protein content [15,17]. The purpose of the present work was to test whether or not introduction and expression of a foreign NR gene in wheat could increase N use efficiency and hence improve quality and/or yield without augmenting N supply, or could maintain quality and/or yield with a diminished use of N fertilizer. Our results demonstrated that over-expression of a CaMV 35S-driven NR gene in two cultivated winter wheat cultivars remarkably enhanced foliar NR activity and significantly increased seed protein content and grain weight under normal soil N conditions.

Materials and Methods

Explants and *Agrobacterium tumefaciens*-mediated transformation

Two winter wheat (*Triticum aestivum* L.) cultivars, “Nongda146” (ND146) and “Jimai6358” (JM 6358) which are widely cultivated in the “Huanghuaihai Area”, China, were used throughout this study. Their immature embryos were isolated from the young caryopses 12–14 days after anthesis, and induced to produce embryogenic callus as previously described [29]. The calli were pretreated for 8–12 h on an osmotic medium with 0.4 M mannitol before *Agrobacterium tumefaciens* (strain LBA4404) inoculation. The
LBA4404 harbored a binary vector pBCLSL16 [21] which was kindly provided by Drs. Cabouche and Meyer (INRA, France). The vector carried a kanamycin-resistant gene (NptII) and tobacco nitrate reductase cDNA (nia) which was functionally fused to CaMV 35S promoter and terminator. The inoculation and coculture of the pretreated calli with Agrobacterium were performed as previously reported [29].

Selection and regeneration of G418-resistant wheat plants

After co-culture, the calli were subcultured, G418-resistance selected and shoot-regenerated, and the regenerated shoots rooted as previously described [29] except that G418 (Geneticin, an aminoglycoside antibiotic similar in structure to gentamicin B1; 25 mg/L) instead of PPT was used as the selective agent. The plantlets were vernalized for 2 weeks at 4°C, and then transplanted in pots in greenhouse and self-fertilized to produce T1 seeds. During greenhouse stage, one young leaf from each independent T0 transformant and WT was sampled for PCR verification.

Screening and cultivation of kanamycin-resistant T1 plants

Screening of kanamycin-resistant (Kan-R) T1 plants was conducted according to Xi and co-workers [30] and Zhang et al. [31] with slight modification. Briefly, the Kan tolerant threshold of WT (ND146 and JM6358) was first determined. The seeds were germinated in a set of Kan concentration (0, 40, 60, 80, 100, 120, 160 or 200 mg/L) at room temperature, and the seedlings were transferred into vermiculite-containing Petri dishes, irrigated with corresponding concentration of Kan and vernalized seedlings were selected as WT except with Kan at the threshold concentration. The green seedling was considered Kan-R.

The Kan-R T1 plants were further verified by PCR, and then transplanted in flowerpots (14×16.5 cm) together with untransformed control (WT) in greenhouse, one plant per pot. All pots contained equal quantity of the nutrient soil (1 vermiculite: 3 garden nutrient soil) and were randomly placed in an experimental plot with normal field managements.

PCR analysis

Total genomic DNA was isolated from fresh leaves using CTAB method developed by Doyle [32] with modifications described by Barro et al. [33]. PCR primers for amplification of a 735 bp fragment from npt HI-nos-ter were 5'-CTGGGCCACACAGACCAAT-3' (forward) and 5'-GAACGATCTCAGAAGAACGC-3' (reverse). The PCR reaction mixture of 20 µl was consisted of 2 µl of LaTaq PCR buffer, 1 µl genomic DNA (100 ng/µL), 2 µl dNTP (2.5 mM), 0.5 µl each primer (10 mM), 0.5 µl LaTaq DNA polymerase (5 U) (Tiangen, Tianjin, China) and 13.5 µl sterile distilled water. The PCR was run at the condition: 95°C for 5 min, followed by 33 cycles of 94°C for 30 s, 60°C for 40 s, 72°C for 40 s and 72°C for 10 min. PCR products were visualized by electrophoresis in 0.8% (w/v) agarose gel containing ethidium bromide.

Southern blot analysis

Southern blot analysis of PCR products was used to verify PCR-positive T0 transformants, and both PCR and Southern blot to identify T1 progeny.

For Southern blotting of PCR products, the PCR was run as described above with the genomic DNA from PCR-positive T0 transformants as template. PCR products were separated by electrophoresis in 0.8% (w/v) agarose gel. For Southern identification of T1 progeny, about 30 µg of genomic DNA from T1 individuals or the control (WT) were digested at 37°C for 12 h with NdeI that has no recognized site in the T-DNA region of pBCLSL16. The digested DNA was fractionated in 0.8% (w/v) agarose gel by electrophoresis run at 22 V for approximately 8 h. The PCR DNA and fractionated DNA were then transferred onto positively charged Hybond™-N+ nylon membrane (Amersham Pharmacia Biotech) by capillarity and fixed by UV cross-linking. The membranes were hybridized using the probe of npt HI nos fragments that were labeled with digoxigenin using the random primer labeling kit (DIG DNA Labeling and Detection Kit). Prehybridization, hybridization and detection of the probe were carried out using a non-radioactive, DIG Luminescent Detection Kit for Nucleic Acids (Roche Diagnostics) according to the manufacturer’s instructions.

Determination of nitrate reductase activity

The nitrate reductase activity (NRA) was measured in vivo according to Freschi et al. [34] with slight modification. Real and potential NRAs were those measured without and with KNO3 induction, respectively. The fresh flag leaf at grain filling period was collected between 9:00 and 10:00 a.m. from greenhouse-grown wheat, and cut into equal two parts along the main vein. One part was used for measurement of real NRA and another part, for potential NRA. To measure the potential NRA, the sample was first induced in 50 mM KNO3 for 12 h at 25°C under light of 3000 lux, and then vacuum-infiltrated. For vacuum-infiltration, leaf samples (0.2 g fresh weight) with or without KNO3 induction were cut into pieces (0.5–1 cm2), immersed in an incubation buffer (5 ml phosphate buffer (pH 7.5) + 5 ml 0.2 M KNO3 solution); vacuum-infiltrated 3–4 times, each for 20 min, and then incubated in darkness for 30 min at 30°C. After infiltration, the nitrate reduction was carried out at room temperature for 30 min in a reaction mixture containing 1 ml of sample infiltrate, 1 ml of 1% (w/v) sodium citrate in 36% HCl and 1 ml of 0.2% (w/v) 1-naphthylamine. The nitrite (NO2-) formed was detected spectrophotometrically at 540 nm, and the NRA was expressed in mg of nitrite (NIR) produced per hour and per gram of fresh leaf. The experiment was triplicated.

Measurement of nitrate contents

The foliar nitrate content was determined according to Cataldo et al. [35] slightly modified. Leaf segments were dried at 85°C until constant weight. The dried material (25 mg) was ground to powder and then incubated in 10 ml of distilled water for 2.5 h. Aliquots of 0.1 ml were mixed thoroughly with 0.4 ml of 5% (w/v) salicylic acid in concentrated H2SO4. After 20 min incubation at room temperature, 9.5 ml of 2 M NaOH were added. The samples were cooled to room temperature and nitrate concentration determined spectrophotometrically by measuring the absorbance at 410 nm.

Protein and 1000-grain weight analysis of T2 seeds

At harvest, the seeds from 57 T1 individuals with good seed-setting rate were chosen for determination of 1000-grain weight
and protein content. To determine 1000-grain weight, 15 seeds per individual plant were picked up randomly and weighted. For detecting protein content, the seeds were first dried at 40°C to constant weight, and then milled and sieved (100 mesh). The protein content of the flour was blindly measured by a commercial company using Kjeldahl method with a continuous flow analyzer (Auto Analyzer 3 Bran Luebbe, Germany) on three replicates, and calculated by using a conversion factor of 5.7.

**Statistical analysis**

Data of NRA, nitrate content, protein content and grain weight were analyzed by analysis of variance (ANOVA) followed by Duncan’s multiple test and T-test with SPSS 17.0 software (SPSS Inc, Chicago, IL, USA).

**Results**

Transformation and regeneration of transgenic wheat

Under promoting conditions of callus induction, 91.8% and 96% of immature embryos from JM6358 (2450 embryos cultured) and ND146 (3645 embryos cultured) developed embryogenic and non-embryogenic calli (Fig. 1a & 1b), respectively. After co-culture with the *Agrobacterium* and selected on G418-containing medium, 51.2% (1024/2000) and 86.2% (2843/3300) of embryogenic calli from JM6358 and ND146 formed resistant callus, whereas the calli from WT (not infected with the bacterium) became browning. On the regeneration medium containing G418, the WT calli ceased growing and did not differentiate (Fig. 1c), but the resistant calli regenerated green shoots (Fig. 1d) at the frequency of 42.1% (510/1210) and 34% (96/282) for JM6358 and ND146 rooted (Fig. 1e & 1f), but no one from WT. The plantlets grew well and were fertile after transplanting in pots in greenhouse (Fig. 1g & 1h).

**PCR and Southern blot identification of T0 transformants**

Among independent G418-resistant T0 transformants, 8 and 53 individuals from JM6358 and ND146 had one expected band of about 740 bp in the PCR product (Parts shown in Figs. 2a & 2b). This gave a transformation efficiency of 0.4% (8/2000) and 1.6% (53/3300), respectively, based on the number of PCR-positive plants/number of the embryogenic calli trans-infected. When Southern blotted, all PCR-positive products and the vector plasmid had a clear hybridized band, whereas no such a band appeared from untransformed control plant (Fig. 2c & 2d).

**Kanamycin screening of T1 offspring and PCR verification of the screening**

In the tested concentrations of Kan solution (0, 40, 60, 80, 100, 120, 160, or 200 mg/L), 95% of the WT seeds germinated, but more than 90% of the seedlings were albino when Kan concentration reached at 80 mg/L or more (Fig. 3a & 3c). In 80 mg/L of Kan solution, 1.4%–89.2% T1 seedlings from 9 independent T0 lines of NR-ND146 (73-212 plants) and 4.9%–49.4% from 7 T0 lines of NR-JM6358 (61–87 plants) remained green (Fig. 3b & 3d).

The T1 green seedlings were further verified by PCR. Overall 71.6% and 70.6% T1 green seedlings of NR-ND146 (225 plants) and NR-JM6358 (85 plants) were PCR-positive (PCR+), respectively (Table 1), but none of the albino seedlings from two families were PCR+ (Data not shown). As presented in Table 1, in NR-ND146 family 7 out of 9 lines had a ratio of 1 : 1 of the Kan-R : PCR+ individual, whereas in the family NR-JM6358, this ratio was only noted in 1 of 7 lines.

**Southern blot analysis of T1 offspring**

The presence of the transgene in PCR+ T1 progeny was further verified by Southern blot analysis. In 8 PCR+ individuals randomly picked (4 from NR-ND146 and 4 from NR-JM6358), the hybridizing band was clearly present, and the band number varied from 1 to 5, with fewer bands in the individuals from NR-ND146 family (lanes 1-4) than in those from NR-JM6358 (lanes 5-8) (Fig. 4).

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**Figure 1. Agrobacterium-mediated transformation and regeneration of transgenic plants from immature embryo-derived callus of common wheat (Triticum aestivum L.).**

a: Embryogenic calli (→) formed from immature embryos. b: Non-embryogenic calli (→) formed from immature embryos. c: Untransformed embryogenic calli (Control) on the regeneration medium supplemented with 25 mg/L G418. d: Shoot regeneration from Agrobacterium-infected embryogenic calli on the regeneration medium supplemented with 25 mg/L G418. e & f: Rooting of regenerated shoots on the rooting medium supplemented with 25 mg/L G418. g: G418-resistant plant in pot. h: Fertile G418-resistant plants in pot.

doi:10.1371/journal.pone.0074678.g001
The foliar NRA was significantly enhanced by 50 mM KNO₃ induction, and this increment took place both in WT and T1 progeny (Fig. 5). Compared with WT, the T1 offspring of NR-ND146 had a significant higher NRA in 5 of 7 individuals tested (146-50-5, 146-90-87, 146-90-110, 146-90-189 and 146-95-4), no matter with or without KNO₃ inducement (Fig. 5a). However, in NR-JM6358 descendants, all tested T1 individuals from 5 T0 lines displayed remarkably stronger NRA than WT when induced with KNO₃.

Table 1. Kanamycin screening of T₁ transgenic wheat and PCR verification of the screening.

| T₀ line  | No. of T₁ seeds | No. of Kan-R T₁ plants | No. of PCR+ T₁ plants | Kan-R: PCR+ |
|----------|-----------------|------------------------|-----------------------|-------------|
| NR-ND146-11 | 69              | 2                      | 2                     | 1:1         |
| NR-ND146-27 | 36              | 1                      | 1                     | 1:1         |
| NR-ND146-49 | 88              | 5                      | 5                     | 1:1         |
| NR-ND146-50 | 94              | 7                      | 7                     | 1:1         |
| NR-ND146-90 | 212             | 189                    | 128                   | 1.48:1      |
| NR-ND146-93 | 103             | 7                      | 5                     | 1:1         |
| NR-ND146-95 | 105             | 9                      | 8                     | 1.13:1      |
| NR-ND146-104 | 73              | 1                      | 1                     | 1:1         |
| NR-ND146-137 | 20              | 4                      | 4                     | 1:1         |
| NR-ND146 Total | 225             | 161                    |                       | 1:1         |
| NR-JM6358-1 | 57              | 5                      | 2                     | 2.5:1       |
| NR-JM6358-5 | 69              | 5                      | 3                     | 1.67:1      |
| NR-JM6358-11 | 87              | 43                     | 32                    | 1.34:1      |
| NR-JM6358-14 | 61              | 9                      | 4                     | 2.25:1      |
| NR-JM6358-16 | 61              | 3                      | 1                     | 3:1         |
| NR-JM6358-17 | 82              | 10                     | 8                     | 1.25:1      |
| NR-JM6358-18 | 80              | 10                     | 10                    | 1:1         |
| NR-JM6358 Total | 85              | 60                     |                       | 1.42:1      |

Kan-R: Kan-Resistant; PCR+: PCR positive. doi:10.1371/journal.pone.0074678.t001

Real and potential NR activities of T₁ progeny

The foliar NRA was significantly enhanced by 50 mM KNO₃ induction, and this increment took place both in WT and T₁ progeny (Fig. 5). Compared with WT, the T₁ offspring of NR-ND146 had a significant higher NRA in 5 of 7 individuals tested (146-50-5, 146-90-87, 146-90-110, 146-90-189 and 146-95-4), no matter with or without KNO₃ inducement (Fig. 5a). However, in NR-JM6358 descendants, all tested T₁ individuals from 5 T₀ lines displayed remarkably stronger NRA than WT when induced with KNO₃.
Among 24 leaf-intact T1 plants of NR-ND146 family, 70.8% increased their seed protein content in comparison with WT (protein content: 19.06%), with an increment range of more than 30% in 33.3% individuals and 20%-30% in 25% individuals. The highest seeds protein content reached at 31.49% (plant 146-93-3) which is 1.65 times of WT.

In NR-JM6358 family, 4 out of 26 leaf-intact T1 plants had the protein content higher than the WT (21.7%) by over 5%, 8 T1 individuals by 2%-5%, but about one half of individuals even declined their seed protein content, more or less (Table 2).

Discussion
Transformation and regeneration of cultivated winter wheat and rapid screening of T1 transformants
Although the first report on successful transformation and regeneration of wheat mediated by Agrobacterium tumefaciens was reported in 1997 by Cheng et al. [36], most reported transformation events were still limited to some "model" spring-type cultivars such as "Bobwhite" and "Chinese Spring" [37,38]. We successfully transferred a tobacco nitrate reductase gene (Nia2) into two commercially cultivated winter wheat cultivars, "ND146" and "JM6358" with Agrobacterium-mediation and obtained numbers of fertile transgenic plants (Figs. 1 & 2) following our protocol established [39] and improved [29,40,41]. We realized a transformation efficiency of 1.68% in "ND146" and 0.40% in "JM6358" based on the number of PCR-positive plants/number of calli inoculated.

After successful transformation and regeneration, we turned our attention to how to select transformants rapidly, efficiently and cost-effectively. In wheat as in other crops, using hygromycin resistance gene was considered an effective selection system that allowed few escape plants to survive [42]. However, taking consideration of the existing biosafety/regulatory rules about genetically modified crops (GMC) and possible commercial cultivation of the transgenic wheat, we used Kan-R gene (npt II) in place of hygromycin-R one as the selection gene. We used G418 in the place of Kan as the selectable agent at different in vitro stages of the transformation due to wheat's native resistance to Kan. Our results demonstrated that G418 at 25 mg/L was efficient for selecting npt II-transgenic calli, shoots and plantlets at corresponding stages of the transformation (Fig. 1c to 1h). Even so, we were aware that the G418 was much expensive than Kan, and its amount requested for "field" selection of T1 and then-after generations.

In order to select transformants more efficiently and cost-effectively, we turned our attention to how to select transformants rapidly, efficiently and cost-effectively. In wheat as in other crops, using hygromycin resistance gene was considered an effective selection system that allowed few escape plants to survive [42]. However, taking consideration of the existing biosafety/regulatory rules about genetically modified crops (GMC) and possible commercial cultivation of the transgenic wheat, we used Kan-R gene (npt II) in place of hygromycin-R one as the selection gene. We used G418 in the place of Kan as the selectable agent at different in vitro stages of the transformation due to wheat's native resistance to Kan. Our results demonstrated that G418 at 25 mg/L was efficient for selecting npt II-transgenic calli, shoots and plantlets at corresponding stages of the transformation (Fig. 1c to 1h). Even so, we were aware that the G418 was much expensive than Kan, and its amount requested for "field" selection of T1 and then-after generations.

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Nitrate reductase activity and nitrate content in the flag leaf of T1 progeny

In untransformed wheat, the nitrate reductase activities (NRA) of the leaf tissues [15,16], basipetal part of the youngest ligule emergent leaf [28], third leaf [44], flag leaf [17] and even shoots [45] were found to be correlated more or less with yield and/or grain (flour) quality. We used the flag leaf for determining NRA and nitrate content of T1 progeny, because its NRA was significantly correlated with both yield and grain protein content in winter wheat [17]. Over-expression of 35S-NR gene remarkably enhanced foliar NRA in more than 70% of T1 descendants analyzed in NR-ND146 and NR-JM6358 families, and a maximum increment level reached at 3.46 times and 4.08 times of the WT, respectively (Fig. 5). This kind of NRA-increment was also reported in 35S-NR Gene Increases Wheat Seed Protein and Weight
NR-transgenic dicotyledonous crops, such as in tobacco [20], Arabidopsis [46], lettuce [21], Chinese cabbage (Brassica campestris L. ssp. pekinensis) and pakchoi (B. campestris L. ssp. chinensis) [22].

Our data showed that without NO$_3^-$ inducement, the NRA of T$_1$ progeny was T$_0$ parent line-dependent, and the different T$_1$ individuals from one single T$_0$ line had also remarkably varied NRA (Fig. 5). Under NO$_3^-$ inducement, both WT and T$_1$ plants enhanced their leaf NRA, but the increment was much more pronounced in T$_1$ plants than in WT (Fig. 5), with a maximal 3.9-fold and 6.2-fold increment in the T$_1$ offspring of the family NR-ND146 (Fig. 5a) and NR-JM6358 (Fig. 5b), respectively. This implied that both endogenous and transgenic NR genes were nitrate-inducible, at least, in wheat, although the transgene NR was driven by constitutive promoter 35S.

Over-expression of 35S-NR gene significantly declined leaf nitrate content in 53.3% (8/15) to 76.5% (13/17) of T$_1$ individuals of NR-ND146 and NR-JM6358 families, respectively, with a maximal decrement of 78.6% (plant 6358-14-4) to 85.9% (plant 146-95-4) (Fig. 6). Such decrement of foliar nitrate content was also observed in numbers of NR-transgenic dicotyledonous crops: such as in tobacco [20,47–49], lettuce [21], potato [23–25], Chinese cabbage and pakchoi [22,27]. It was well known that the NR, as a rate-limiting enzyme, catalyzed reduction of NO$_3^-$ into NO$_2^-$, and thus logically over-expression of NR could decrease nitrate content in plant. Hu et al. [50] even speculated that the
higher NRA was the more nitrate would be reduced. However, in our NR-transgenic wheat under the greenhouse growth conditions, the increment of foliar NRA was sometimes correlated with the nitrate decrement in the leaves of T1 offspring of both NR-ND146 and NR-JM6358 families (Fig. S1). Sun et al. [51] reported that Arabidopsis plants transformed with a Chinese cabbage NR gene exhibited an enhanced level of both NO₃⁻ and NRA in leaves under NO₃⁻ inducement. Hoff et al. [52] also observed that Arabidopsis mutants affecting Nia2 and barley Nar1 mutants expressing only 10% of the WT NRA did not alter nitrate content and biomass under the greenhouse growth conditions. What is the reason remained to be investigated. In our transgenic

### Table 2. Protein content and 1000-grain weight of T₂ seeds.

| NR-ND146 |  | NR-JM6358 |
|----------|---|---|
| **Leaf-sampled** | **Leaf-sampled** |  |
| **T₁ plants** | **1000-grain weight (g)** | **Seed protein content (%)** | **T₁ plants** | **1000-grain weight (g)** | **Seed protein content (%)** |
| ND146 (WT) | 28.67±0.58 | 18.80±0.17 | JM6358 (WT) | 31.33±1.53 | 20.31±0.06 |
| 146-50-5 | 42.00±1.00*** | 25.12±0.12*** | 6358-5-4 | 53.33±0.58*** | 22.69±0.04*** |
| 146-90-110 | 37.67±1.53*** | 29.12±0.07*** | 6358-11-20 | 32.67±0.58 | 20.24±0.16 |
| 146-137-3 | not determined | 23.05±0.22*** | 6358-14-2 | 31.67±1.16 | 24.62±0.10*** |
| **Leaf-intact** |  |  | 6358-18-8 | 36.33±0.58*** | 23.42±0.08*** |
| ND146 (WT) | 33.67±2.08 | 19.08±0.01 | JM6358 (WT) | 33.33±0.58 | 21.70±0.02 |
| 146-11-1 | 34.33±2.08 | 27.42±0.16*** | 6358-1-2 | 36.00±1.00* | 17.23±0.03*** |
| 146-11-2 | 36.67±1.53 | 25.65±0.09*** | 6358-1-3 | 32.67±1.16 | 17.53±0.15*** |
| 146-27-1 | 45.00±3.61*** | 23.37±0.09*** | 6358-1-4 | 23.33±1.16*** | 21.32±0.06*** |
| 146-49-3 | 48.33±0.58*** | 20.50±0.07*** | 6358-5-2 | 50.00±3.61*** | 22.88±0.01*** |
| 146-49-4 | 32.67±2.08 | 27.75±0.05*** | 6358-5-5 | 38.00±1.00*** | 22.55±0.19*** |
| 146-49-5 | 49.67±6.03*** | 18.73±0.07*** | 6358-11-8 | 36.33±1.53* | 19.02±0.02*** |
| 146-50-1 | 45.33±2.08*** | 23.83±0.21*** | 6358-11-12 | 35.67±0.58* | 26.50±0.17*** |
| 146-50-2 | 40.00±1.00*** | 23.82±0.11*** | 6358-11-19 | 35.63±0.58*** | 22.48±0.02*** |
| 146-50-3 | 45.00±1.00*** | 24.21±0.121*** | 6358-11-24 | 34.67±0.58 | 21.49±0.06* |
| 146-90-5 | 43.00±2.00*** | 14.351±0.14*** | 6358-11-29 | 34.33±1.16 | 21.94±0.06** |
| 146-90-10 | 38.33±1.16** | 14.13±0.01*** | 6358-14-1 | 32.67±2.08 | 23.43±0.04*** |
| 146-90-27 | 50.00±1.00*** | 22.58±0.02*** | 6358-14-3 | 33.00±1.73 | 23.62±0.12*** |
| 146-90-109 | 43.67±1.16*** | 25.11±0.03*** | 6358-14-9 | 47.33±0.58*** | 22.22±0.02*** |
| 146-90-169 | 26.33±0.58*** | 22.48±0.05*** | 6358-16-1 | 32.33±0.58 | 22.78±0.08*** |
| 146-93-2 | 37.00±1.00* | 25.86±0.05*** | 6358-16-2 | 33.67±1.16 | 21.03±0.10*** |
| 146-93-3 | 33.67±1.53 | 31.49±0.11*** | 6358-16-3 | 38.33±1.53*** | 19.53±0.16** |
| 146-93-5 | 37.67±1.53* | 16.11±0.13*** | 6358-17-2 | 36.67±0.58*** | 20.36±0.11*** |
| 146-93-6 | 42.67±2.08*** | 23.70±0.06*** | 6358-17-4 | 43.00±2.00*** | 22.76±0.04*** |
| 146-95-1 | 38.67±1.53*** | 28.08±0.02*** | 6358-17-6 | 43.33±1.53*** | 20.13±0.11*** |
| 146-95-3 | 24.33±0.58*** | 29.17±0.04*** | 6358-17-7 | 38.00±1.00*** | 19.58±0.04*** |
| 146-95-6 | 34.00±1.00 | 14.83±0.14*** | 6358-17-9 | 38.00±1.00*** | 19.69±0.06*** |
| 146-95-7 | 43.67±1.16*** | 14.72±0.02*** | 6358-18-1 | 36.67±1.53*** | 22.48±0.10*** |
| 146-104-1 | 41.00±1.00*** | 24.10±0.06*** | 6358-18-2 | 39.67±2.08*** | 18.97±0.06*** |
| 146-137-1 | 40.33±2.31*** | 16.27±0.08*** | 6358-18-5 | 40.00±1.00*** | 20.17±0.20*** |
|  |  |  | 6358-18-6 | 34.33±0.58 | 22.78±0.08*** |
|  |  |  | 6358-18-9 | 46.33±1.53*** | 22.96±0.16*** |

T₁ plants of 35S-NR-transgenic wheat and wild-type (WT) were randomly grown in greenhouse under conventional conditions. Values represent mean ± S.D. of three replicates. Difference significant at P < 0.05 (*), < 0.01 (**) or < 0.001 (***).
wheat, the accumulated nitrate in the leaf would be used later for grain development.

Seed weight, protein content and their relationship with foliar nitrate reductase activity

Our data demonstrated that 70.8% (17/24) NR-ND146 and 50% (13/26) NR-JM6358 T1 descendants had significant higher protein content than WT, and a more than 30% increment was detected in 33% of T1 offspring in NR-ND146 family (Table 2). For a limited number of leaf-sampled T1 plants, the seed protein content looked like have a tendency of positive correlation with foliar NRA in both families (Fig. S2). In non-transformed spring wheat [15] and winter one [17], the foliar NRA was observed positively correlated with seed protein content. Kumar [17] thought that grain protein accumulation depended on the accumulation and partitioning of the reduced N accumulated during the vegetative stage and on the relative contributions of nitrate assimilation and N redistribution during grain development. When N-deficient wheat plants, lower shoot NRA resulted in decrement of reduced N accumulation daily in the shoots [45]. The plants grown in nitrate-rich conditions not only enhanced the activities of NR, ribulose bisphosphate carboxylase-oxygenase (RuBPCO) and glutamine synthetase etc. in growing and full expanded leaves, but also slowed the decrease of those activities in older leaves and delayed leaf senescence [44]. An increase in the supply of glutamine could enhance the rate of protein deposition in the wheat grain [53]. In 35S-NR-transgenic tobacco plants with higher foliar extractable NRA, Ferrario-Mery et al. [54] observed the increased glutamine level in the leaves. We suggested that the increased foliar NRA in 35S-NR-transgenic wheat might speed up nitrate assimilation and facilitate the N-flux to and/or N redistribution in seeds during grain development, and hence increased grain protein content.

We noted that in T1 plant with intact leaf, 70.8% and 65.4% of them remarkably augmented their grain weight in NR-ND146 and NR-JM6358 families, respectively (Table 2). In order to know whether the increase in grain weight has some relationship with foliar NRA, we analyzed T1 plants whose flag leaves were sampled for NRA and NO3− determination. Our data showed that the correlation between grain weight and flag leaf NRA was varied with transgenic wheat families: R2 = 1 in NR-JM6358, and R2 = 0.4569 in NR-ND146 (Fig. S3). In untransformed wheat, the NRA in the basipetal part of the youngest ligule emergent leaf [28], flag leaf [17] and leaf tissues at boot stage of maturity [15] correlated well with yield. Kumar [17] observed that on induction of NRA by nitrate supply at post anthesis stage, the flag leaf retained the ability to synthesize RuBPCO. We suggested that constant expression of the NR gene in 35S-NR-transgenic wheat might help consistent synthesis of RuBPCO, and thus confer to the leaves longer and higher capacity of photosynthesis, and hence increase grain weight.

Our data indicated also that in 35S-NR-transgenic wheat, there was not obvious relationship between grain weight and seed protein content (Fig. 7), and in some T1 individuals, the increment of grain weight was accompanied by the increase of seed protein content (Table 2). Jenner et al. [55] showed that the duration and rate of both starch and protein deposition in the endosperm of wheat were all independent events, controlled by separate mechanisms. Under N-rich growing conditions, both the duration and rate of starch deposition during grain filling were determined primarily by factors that worked close to or within the grain itself, whereas those of protein deposition were decided predominantly by factors of supply outside the grain. In studying the relationships between carbon and nitrogen metabolism in the leaves of NR-transgenic tobacco that expressed either a 5-fold increase or a 20-fold decrease in NRA, Foyer and colleagues [56] concluded that large decreases in NRA had profound repercussions for photosynthesis and carbon partitioning within the leaf, but the increases in NRA had negligible effects. In Arabidopsis, over-expression of NR led to 200% increase of seedlings protein content without any gain in the fresh and dry weights [46]. We are aware that more works are needed to address the mechanism of the cell- and organ-specific expression and metabolic regulation of NR gene and other genes involved in the nitrogen assimilatory pathway and to investigate the role of the enzymes in regulating flux through the nitrogen assimilation pathways, as indicated by Cullimore and Bennett [57].

We noted also, there was obvious variability in both seed protein content and grain weight among independent transformants and their progeny (Table 2). Random insertion of the transgene in the genome of T0 transformants and random recombination of the transgene in producing the progeny might be one of the explanations, because the insertion might change the expression of adjacent genes [58].

In conclusion, over-expression of 35S-NR gene in winter wheat significantly increased grain weight and seed protein content. This might be realized by an increased foliar NRA. The enhanced NRA might speed up nitrate assimilation and facilitate N-flux to and/or N redistribution in seeds during grain development in one hand, and make the leaf to have longer and higher capacity of photosynthesis, in other hands. Our results would provide an alternative way to breeding new wheat cultivars of higher protein content and higher nitrogen use efficiency, which makes it possible to reduce the need for excessive input of N fertilizers and improve or stabilize quality.

Supporting Information

Figure S1 Relationship between foliar NRA and nitrate content of T1 transformants of wheat. a: Leaf-sampled T1 offspring of NR-ND146. b: Leaf-sampled T1 offspring of NR-JM6358. (TIF)

Figure S2 Relationship between T1 foliar NRA and T2 seed protein content of transgenic wheat. a: Leaf-sampled T1 offspring of NR-ND146. b: Leaf-sampled T1 offspring of NR-JM6358. (TIF)

Figure S3 Relationship between T1 foliar NRA and T2 seed weight of transgenic wheat. a: Leaf-sampled T1 offspring of NR-ND146. b: Leaf-sampled T1 offspring of NR-JM6358. (TIF)

Acknowledgments

The authors thank Dr. XY Chen (Institute of Grain and Oil Crops, Hebei Academy of Agricultural and Forestry Sciences) for Jimai6358 (JM 6358) seeds, and XL Teng, LJ Yuan and L Han for assistance in isolation of wheat immature embryos.

Author Contributions

Conceived and designed the experiments: XGX XQZ. Performed the experiments: XQZ XLN. Analyzed the data: XQZ XGX. Contributed reagents/materials/analysis tools: XLN XQZ. Wrote the paper: XQZ XGX.
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Figure 7. Relationship between T2 seed weight and seed protein content of T1 wheat progeny with intact leaves. a: NR-ND146. b: NR-JM6358.
doi:10.1371/journal.pone.0074678.g007

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