Glucose-6-Phosphate Dehydrogenase Is Involved in the Tolerance of Soybean Seedlings to Low Nitrogen Stress

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Abstract: Nitrogen (N) deficiency affects plant growth and crop yield. In this study, we investigated the role of glucose-6-phosphate dehydrogenase (G6PDH) in response to N availability in three soybean cultivars, JINDOU 19 (JD19), LONGHUANG 3 (LH3), and LONGDOU 2 (LD2), that have different tolerances to low-N stress. The results showed that the leaf area and primary root length of JD19 and LH3 were greater than that of LD2 under low-N stress, suggesting that the growth of JD19 and LH3 was impaired less than LD2, and thus are more tolerant to low-N stress than LD2 is. Interestingly, the G6PDH expression showed different degrees of change in these soybean cultivars under low-N conditions, and the G6PDH activity in JD19 and LH3 was higher than that in LD2. When G6PDH was inhibited by glucosamine (GlcN), the contents of malondialdehyde (MDA) and reduced glutathione (GSH) in JD19 and LH3 were increased more than that in LD2. In contrast, the activity of the plasma membrane (PM), NADPH oxidase, and nitrite reductase (NiR), respectively [3]. However, the accumulation of ammonium (NH4+) and nitrate (NO3−) by nitrate reductase (NR) and nitrite reductase (NiR), respectively [3]. However, the accumulation of ammonium can cause damage to plant cells. Thus, it is rapidly assimilated into amino acids, catalyzed primarily by glutamine synthetase (GS) and glutamate synthase (GOGAT) [4]. Soybeans (Glycine max L.) are a major crop that provide a large amount of protein and vegetable oil for humans. Due to soybean seeds providing protein, there is a great demand for N during

Keywords: low nitrogen; glucose-6-phosphate dehydrogenase; antioxidant; nitrogen metabolism; soybean

1. Introduction

Nitrogen (N) is the most important nutrient and a core component of a variety of cellular metabolites, including nucleic acids, amino acids, and proteins, in virtually all living organisms [1]. Plants acquire inorganic nitrogen through the soil, where ammonium (NH4+) and nitrate (NO3−) are the universal forms [2]. After entering the plant cell, NO3− is successively reduced to nitrite (NO2−) and ammonium (NH4+) by nitrate reductase (NR) and nitrite reductase (NiR), respectively [3]. However, the accumulation of ammonium can cause damage to plant cells. Thus, it is rapidly assimilated into amino acids, catalyzed primarily by glutamine synthetase (GS) and glutamate synthase (GOGAT) [4].
the growth and development stage [5]. Soybeans generally obtain N through biological nitrogen fixation (BNF) and absorption from soil [6]. It was reported that 50–60% of soybean N demand is obtained through BNF, while the rest comes from soil absorption [6]. However, under natural conditions, plants often face N-deficient environments, which results in the decline of the yield [7].

Abiotic stresses, including low-N stress, will enhance the production of hydrogen peroxide (H$_2$O$_2$) and other reactive oxygen species (ROS), and the overproduction of ROS leads to damage in plants [8,9]. Therefore, plants have evolved an underlying strategy to counteract adverse environmental conditions, especially oxidative stresses. NADPH is a critical regulator of the cellular redox balance in protecting plant growth against oxidative stresses [10,11]. On the one hand, NADPH is the major metabolic product of several physiological reaction processes. On the other hand, NADPH is an essential substrate for reductive biosynthetic reactions in cell growth and proliferation in plants [12]. Meanwhile, NADPH is recognized as reducing the potential for the production of reduced glutathione (GSH) [13]. GSH is a main substance in the process of anti-oxidation that includes a range of antioxidant enzymes: α-tocopherol, glutathione, and ascorbate. The network is responsible for defense against ROS [14]. Furthermore, GSH plays a key role in ROS detoxification in early plant development. In Arabidopsis, the loss of function of AtGSH1 leads to a phenotype of recessive embryo-lethality [15]. Some studies indicate that GSH and phytochelatins (PCs) play a central role in plant tolerance to heavy metals [16,17]. Therefore, maintaining the cellular GSH balance is crucial for enhancing the resistance to oxidative stresses in plants. In addition, in response to various environmental stresses, plants also accumulate soluble sugars [18], abscisic acid, and proline [19] to diminish stress damages. Proline participates in the cellular redox modulation and soluble sugars, especially glucose, fructose, and sucrose, play an important role in plant metabolism and structure at the whole-organism level [20].

Glucose-6-phosphate dehydrogenase (G6PDH) is an important enzyme in the pentose phosphate pathway (OPPP), and G6PDH regulates the NADPH content by modulating the glucose metabolism through OPPP [11]. In contrast, the G6PDH activity is competitively inhibited by NADPH [21]. In higher plants, G6PDH is located in the peroxisomes, cytosol, and plastids [22–24]. In Arabidopsis, there are two cytosolic (Cyt) isoforms and four plastidic (Pla) isoforms. Among them, G6PD1, G6PD2, G6PD3, and G6PD4 encode Pla-G6PDH, while G6PD5 and G6PD6 encode Cyt-G6PDH [25]. Previous studies have demonstrated that G6PDH participates in responses to salt, drought, aluminum, and UV-B stresses [26–30]. For example, Cyt-G6PDH reduces the oxidative damage caused by salt stress through increasing the activity of antioxidant enzymes in Arabidopsis [31]. In soybeans, G6PDH maintains redox homeostasis by regulating ABA and H$_2$O$_2$ levels under drought stress [11]. Furthermore, increased G6PDH expression enhances the GSH levels, thus mitigating the damage of oxidative stress [32]. Additionally, it was shown that the plasma membrane (PM) NADPH oxidase requires NADPH to produce superoxide, which subsequently leads to the generation of H$_2$O$_2$ [13]. Overproduction of ROS in cytosol will trigger redox signaling and activate the OPPP [33,34].

Previous studies mostly focused on the relationship between G6PDH and salt or drought stress. Few studies addressed the role of G6PDH in soybean plants under low-N stress. Here, three soybean cultivars, JD19, LH3, and LD2, exhibiting different tolerances to low-N stress, were used as the materials for studying the relationship between G6PDH and low-N stress.
2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Seeds of three soybean cultivars, JD19, LH3, and LD2, were provided by Prof. Guohong Zhang (Gansu Academy of Agricultural Sciences, Lanzhou, Gansu Province, China). JD19 and LH3 were bred in 2003 and 2018, respectively, with large seeds and lodging resistance. LD2 was bred in 2005, with 4–5 branches and small seeds. The seeds were surface sterilized for 5 min with 1% sodium hypochlorite first, and then for 20 min in 2% H$_2$O$_2$. After washing 5 times with sterile water, these seeds were germinated on aseptic sponge and gauze for 2 days in the dark at 28 °C. Subsequently, the germinated seeds were transferred to 300 mL hydroponic pots and grown in 1/4 Hoagland medium (pH 6.0) with different NO$_3^-$ level for 10 d. Ca(NO$_3$)$_2$·4H$_2$O was used as the nitrate source. In this study, the N levels were 3.75 mM (3.75 N, control level N) and 0.01 mM (0.01 N, low N). At 0.01 N, the reduced nitrate was replaced by chloride. Glucosamine (GlcN) was used as a competitive inhibitor of G6PDH activity [35]. For chemical treatment, soybean seedlings were cultured in 1/4 Hoagland medium with 3.75 N or 0.01 N for 10 d and then transferred to fresh medium with 10 mM GlcN for 48 h. The nutrient solutions were replenished every day. Plants were grown in greenhouse (16 h/8 h light/dark cycle and 300 µmol m$^{-2}$ s$^{-1}$ photon flux density) at 25 °C. At the end of the treatment, the leaves and roots were used for analysis immediately.

2.2. Phenotypic Analysis

After 10 d of treatment, the primary root length and leaf area of the seedlings were photographed with a Nikon digital camera and analyzed with Image J software (version 1.43). Each sample was comprised of a minimum of 15 seedlings, with three replicate samples per treatment.

2.3. Measurements of MDA, H$_2$O$_2$ and Proline, Soluble Sugar Content

MDA content was determined according to the method of Hodges et al. [36]. Samples were extracted at 4 °C in 10% trichloroacetic acid. After centrifugation for 10 min at 4000× g, the supernatant was incubated for 1 h with the same amount of 0.5% thiobarbituric acid at 95 °C. The absorbance was measured at 440, 535, and 600 nm after centrifugation at 10,000× g for 10 min. H$_2$O$_2$ content was measured using the method of Yang et al. [31]. Proline and soluble sugar content were determined according to the methods described by Bates et al. [37] and Jin et al. [38], respectively. In each independent assay, 0.5 g of roots and 0.5 g of leaves were used in each single repeat.

2.4. Determination of GSH, NADP$^+$ and NADPH Content

GSH content was analyzed according to the method of Wang et al. [30]. Samples were homogenized in 7% sulfosalicylic acid at 4 °C and centrifuged at 10,000× g for 10 min. The supernatant was used to determine the glutathione content. The absorbance of the reaction mixture was measured at 412 nm. The corresponding GSH content was quantified from the standard curve according to the above method. NADP$^+$ and NADPH were extracted in 3.8 mL of 0.2 M HCl and 0.2 M NaOH, respectively. The homogenates were centrifuged at 12,000× g for 10 min at 4 °C, and the supernatants were used to measure the NADP$^+$ and NADPH content, respectively. The content of NADP$^+$ or NADPH was determined spectrophotometrically according to the method described by Wang et al. [39].

2.5. Determination of G6PDH Activity

The total G6PDH (EC 1.1.1.49) activity was determined according to Zhao et al. [28]. 0.5 g of roots and 0.5 g of leaves were homogenized with extract buffer (100 mM Heps-Tris (pH 7.8), 10 mM EDTA (pH 7.5) and 10 mM MgCl$_2$) and then centrifuged at 12,000× g for 20 min at 4 °C. Subsequently, the reaction buffer (100 mM Heps-Tris (pH 7.8), 10 mM MgCl$_2$) pre-heated at 37 °C, the supernatant, 15 mM NADPNa$_2$, and 15 mM D-glucose-6-phosphate disodium salt were added in order. The Cyt-G6PDH activity was determined...
with the same reaction buffer, adding an extra 62.5 mM DTT to inhibit Pla-G6PDH activity. The absorbance change rate of the reaction mixture at 340 nm was measured and recorded within 4 min. The activity of Pla-G6PD equals the total G6PD activity minus the Cyt-G6PD activity [30].

2.6. Plasma Membrane (PM) NADPH Oxidase Activity Assay

PM was isolated according to the method of Qiu and Su [40]. The PM NADPH oxidase activity was determined according to Duan et al. [41]. The protein content was determined according to Bradford [42]. Bovine serum albumin (BSA) was used as the standard.

2.7. Determination of NO$_3^-$ Content and N Metabolism-Related Enzyme Activities

The NO$_3^-$ content was determined according to the method of Wang et al. [43]. 0.5 g of roots and 0.5 g of leaves were ground with 5 mL H$_2$O to extract NO$_3^-$, and the NO$_3^-$ content was measured spectrophotometrically following the procedure of Wang et al. [43]. NR in soybean roots and leaves was extracted in an enzyme extract buffer [100 mM Hepes-KOH (pH 7.5), 7 mM cysteine, 3% PVPP, and 1 mM EDTA]. The NR activity was determined by using the method of Du et al. [44]. NiR was extracted in an enzyme extract buffer [50 mM potassium phosphate (pH 8.8), 1 mM EDTA, 3% (w/v) bovine serum albumin, and 25 mM cysteine]. The NiR activity was measured spectrophotometrically following the procedure of Wang et al. [43]. GS was extracted with the enzyme extract buffer [50 mM Tris-HCl (pH 8.0), 400 mM sucrose, 2 mM DTT, and 2 mM MgSO$_4$], and the GS activity was determined according to the method of Wang et al. [43].

2.8. Total RNA Isolation and Quantitative RT-PCR

The total RNA was extracted from the leaves and roots of 10-day-old soybean seedlings using the Plant RNA Extraction Kit (Thermo Fisher, Shanghai, China). The cDNA was synthesized from total RNA (1 µg) using the PrimeScript RT Reagent Kit with gDNA Eraser (Thermo Fisher) and was used as the template for qRT-PCR amplification. The CFX96 Real Time System (Bio-Rad, Hercules, CA, USA) with an SYBR Premix Ex Taq II Kit (Thermo Fisher) was used for PCR reactions. GmACTIN2 was used as the internal control. Fold changes ($2^{-\Delta\Delta Ct}$) were expressed relative to the control. The primer sequences used in this study were listed in Table S1.

2.9. Statistical Analysis

Each assay was carried out with three independent biological replicates. Data analyses were performed with SPSS 20.0. The values were expressed as mean ± SE ($p < 0.05$). Statistical analyses were done with one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test ($p < 0.05$). Principal component analysis (PCA) was performed using Statistic 6.0 (Statsoft, Tulsa, OK, USA).

3. Results

3.1. Low-N Stress Affects Soybean Growth

Plants often change root systems, leaf production, and chlorophyll contents to minimize adverse effects under low-N stress [39]. In our study, the leaf area and the primary root length were decreased dramatically in all three soybean cultivars under 0.01 N conditions (Figure 1). In JD19, LH3, and LD2, the leaf area was decreased by 17.7%, 23.4%, and 44.4%, respectively, compared to the control (Figure 1a,b). The primary root length under 0.01 N was decreased by 21.0% in both JD19 and LH3, and by 61.0% in LD2 (Figure 1c,d). These results indicated that LD2 is more sensitive to low-N stress than JD19 and LH3.
3.2. Low-N Stress Increases MDA and H$_2$O$_2$ Content but Decreases Proline and Soluble Sugar Content in Three Soybean Cultivars

MDA is considered a credible indicator of oxidative stress [8]. To investigate the oxidative damage and ROS accumulation caused by low-N stress, we measured MDA and H$_2$O$_2$ contents. Results showed that with N deprivation, MDA and H$_2$O$_2$ contents were increased in three soybean cultivars (Figure S1a–d). However, the MDA and H$_2$O$_2$ contents were increased more in LD2 than in JD19 and LH3. Thus, the more severe oxidative damage might be one of the prime reasons for low tolerance to low-N stress in LD2.

Plants accumulate proline and soluble sugars to deal with environmental stresses [45]. We observed that the contents of proline and soluble sugars were increased under low-N stress in both the leaves and the roots (Figure S1e–h). Overall, the proline and soluble sugar contents in the roots were significantly lower than that in the leaves in all three cultivars. Consistently, JD19 and LH3 accumulated more soluble sugars and proline than LD2 in both shoots and roots. The above results further demonstrate that JD19 and LH3 have higher tolerance to low-N than LD2.

3.3. Low-N Stress Affects G6PDH Activity and G6PDH Transcript Levels in Three Soybean Cultivars

In order to explore whether G6PDH is involved in the response to low-N stress, we measured the activity of G6PDH in three soybean cultivars under low-N stress. Figure 2 showed that the activity of Tot-G6PDH in the roots of JD19 and LH3 was increased by 51.0% and 49.7%, respectively, while it was only increased by 20.1% in LD2 roots under low-N stress (Figure 2b). Similarly, Pla-G6PDH activity was markedly enhanced in the three cultivars, especially in JD19 and LH3 (Figure 2f). However, no significant difference in Cyt-G6PDH activity was found when the three soybean cultivars were exposed to low-N conditions (Figure 2d). Similar patterns were observed in the leaves (Figure 2a,c,e). We further analyzed the role of the G6PDH gene family involved in low-N stress. BLAST (Basic Local Alignment Search Tool) searches against the soybean genome database identified eight G6PDH homologs in the soybean genome (Figure S2). The phylogenetic analysis revealed that the G6PDH proteins between Arabidopsis and soybeans share the highest similarity.
Five G6PDH proteins in soybeans, named GmG6PD1, GmG6PD2, GmG6PD3, GmG6PD4, and GmG6PD5, belong to Pla-G6PDH, while GmG6PD6, GmG6PD7, and GmG6PD8 are grouped together with Cyt-G6PDH proteins (Figure S2). qRT-PCR results showed that the expression of GmG6PDH in the three soybean cultivars was induced to varying degrees in response to low-N stress, especially the Pla-G6PDH genes (Figure 2g,h). However, the expression patterns of different G6PDH genes in response to low-N stress among the three soybean cultivars were different. Specifically, the transcript levels of G6PD1, G6PD2, and G6PD3 in JD19 leaves were significantly increased, while the levels of G6PD2, G6PD3, and G6PD5 were significantly increased in LH3 leaves (Figure 2g). The transcript level of G6PD5 was also increased in LD2 leaves (Figure 2g). Meanwhile, the expression levels of G6PD2 and G6PD3 in JD19 roots and G6PD1, G6PD2, G6PD3, and G6PD4 in LH3 roots were significantly enhanced under low-N stress. Moreover, the transcript level of G6PD3 was also induced in LD2 roots (Figure 2h). Altogether, these results suggested that Pla-G6PDH play an important role in soybean response to low-N stress.

![Figure 2](image-url)

**Figure 2.** Effects of G6PDH activity and relative expression levels in three soybean cultivars under low-N stress. (a) Tot–G6PDH activity, (c) Cyt–G6PDH activity, (e) Pla–G6PDH activity in the leaves (b) Tot-G6PDH activity, (d) Cyt-G6PDH activity, (f) Pla-G6PDH activity in roots. Data are the means ± SE (n = 3, p < 0.05). (g) relative expression levels of G6PDH in the leaves (h) relative expression levels of G6PDH in the roots. Data are means ± SE (n = 3). The letters show significant differences under N conditions.
3.4. Low-N stress Increases ROS Levels after Dysfunction of G6PDH in Three Soybean Cultivars

To further elucidate the role of G6PDH in soybean tolerance to low-N, exogenous GlcN (an inhibitor of G6PDH) was used to inhibit the G6PDH activity. As shown in Figure 3a,b, 10 mM GlcN strongly eliminated the low-N-induced G6PDH activity in leaves and roots. Expectedly, MDA and H$_2$O$_2$ contents were markedly increased in the three soybean cultivars under the 0.01 N + GlcN treatment (Figure 3c–f). These results further confirmed that G6PDH plays a positive role in soybean tolerance to low-N stress.

![Figure 3](image-url)

**Figure 3.** Effects of glucosamine (GlcN, a competitive inhibitor of G6PDH) on G6PDH activity, MDA, and H$_2$O$_2$ content in three soybean cultivars. (a) G6PDH activity, (c) MDA content, (e) H$_2$O$_2$ content in leaves, (b) G6PDH activity, (d) MDA content, (f) H$_2$O$_2$ content in roots. 10 mM GlcN was added into 1/4 Hoagland medium (pH 6.0) with 3.75 N or 0.01 N for 48 h. Data are means ± SE ($n = 3$, $p < 0.05$). The letters show significant differences under N conditions.

3.5. Changes of the Redox Status under Low-N Stress

G6PDH catalyzes NADP$^+$ to produce NADPH, which is the substrate of NADPH oxidase [11]. Additionally, the G6PDH activity is inhibited when the NADPH/NADP$^+$ ratio is increased [21]. As shown in Figure 4, 0.01 N treatment significantly increased the NADPH content, but decreased the NADP$^+$ content, resulting in a high NADPH/NADP$^+$ ratio in LD2 leaves and roots. However, low-N stress did not significantly affect the levels of NADPH and NADP$^+$ in the leaves of JD19 and LH3, thus the NADPH/NADP$^+$ ratio had no significant changes. The higher NADPH content and NADPH/NADP$^+$ ratio in turn inhibited the G6PDH activity in LD2. These results suggest that JD19 and LH3 dissipate excessive reducing equivalents more efficiently than LD2 under low-N stress.
3.6. Effects of Low-N Stress on GSH Content and Plasma Membrane NADPH Oxidase Activity

NADPH is the only reducing force for the generation of reduced glutathione (GSH), which plays an important role in the process of removing H$_2$O$_2$ [46,47]. To test whether the different tolerances among three soybean cultivars is caused by different GSH levels under low-N stress, the GSH content was measured. As shown in Figure 5a, under 0.01N treatment, the GSH content was increased more in JD19 and LH3 leaves (about 40.0% of the control) than in LD2 leaves (about 22.0% of the control). Similarly, under 0.01 N treatments, the GSH content was increased by 141.8% and 104.9% in JD19 and LH3 roots, respectively (Figure 5b). However, it was only increased by 48.3% in LD2 roots.

The PM NADPH oxidase, which uses G6PDH-generated NADPH as the substrate, is mainly responsible for H$_2$O$_2$ generation [11,48]. Figure 5c,d showed that the NADPH oxidase activity was increased in both the leaves and the roots under low-N stress. In the leaves, the PM NADPH oxidase activity was increased by 26.1% and 25.3% in JD19 and LH3, respectively. However, in LD2 leaves, it was increased by 43.8% (Figure 5c). The PM NADPH oxidase activity in the roots showed a similar pattern as in the leaves, increased by 41.5%, 40.0%, and 50.4% in JD19, LH3, and LD2, respectively (Figure 5d).
Figure 5. Effects of GSH content and PM NADPH oxidase activity in three soybean cultivars under low-N stress. (a) GSH content (c) PM NADPH oxidase activity in the leaves (b) GSH content (d) PM NADPH oxidase activity in the roots. Data are means ± SE (n = 3, p < 0.05). The letters show significant differences under N conditions.

3.7. Principal Component Analysis

The purpose of principal component analysis was to clarify the relationship between the investigated parameters. In the leaves, some variables showed distinct separation. Factor 1 explained 64.44% of the total variance, of which PNO (PM NADPH oxidase) contributed the most variation (approximately 16.1%), while H2O2 and G6PDH accounted for approximately 15.7% and 14.5% of the variation, respectively (Figure S3a, Table S2). NADP+ (35.9%) and NADPH (33.9%) were loaded on factor 2, which explained 27.6% of the variation. The next was MDA, accounting for 10.4% of the variation (Table S2). According to the average value of all detected parameters in the leaves among the three soybean cultivars, the plot case factor coordinates for each nitrogen concentration were analyzed. PCA plot showed a significant difference between 3.75 and 0.01 mM N concentrations in three soybean cultivars (Figure S3b). Factor 1 and factor 2 explained 69.5% and 30.3% of the total variance, respectively. The 0.01 mM N concentration showed the highest contribution (20.1%) to factor 1 in LH3, followed by JD19 (18.8%). Additionally, the 0.01 mM N concentration was significantly loaded (71.9%) on factor 2 in LD2 (Figure S3b). Furthermore, our results showed that the three soybean cultivars had no difference in the leaves under 3.75 mM N concentration. The tolerance of the leaves had no difference between JD19 and LH3 seedlings under 0.01 mM N concentration; however, the tolerance of the leaves showed significant difference between LD2 and JD19/LH3 at 0.01 mM N concentration (Figure S3b).

In the roots, some variables also showed obvious separation. Factor 1 explained 77.3% of the total variance, of which NADP+ contributed the highest variation (12.7%), while H2O2 and G6PDH contributed about 12.5% and 12.2%, respectively, followed by proline (11.9%) and GSH (11.2%) (Figure S3c, Table S2). NADPH (22.3%) and MDA (18.0%) were loaded on factor 2, which explained 15.9% of the variation. The next was soluble sugar and GSH, accounting for 12.3% and 9.7%, respectively (Figure S3c, Table S2). Similarly, based on the mean values of all examined parameters in the roots among the three soybean cultivars, the plot case factor coordinates of each nitrogen concentration were analyzed. PCA plot revealed good distinction of the 3.75 and 0.01 mM N concentrations from the three soybean cultivars (Figure S3d). Factor 1 and factor 2 explained 93.4% and 6.2% of the total variance, respectively. The 0.01 mM N concentration showed the highest contribution (33.8%) to
factor 1 in JD19, followed by LH3 (26.4%). Additionally, the 0.01 mM N concentration was significantly loaded (83.2%) on factor 2 in LD2 (Figure S3d). Furthermore, our results showed that the three soybean cultivars had no significant difference in the roots under 3.75 mM N concentration, however, the tolerance in roots showed significant difference between LD2 and JD19/LH3 at 0.01 mM N concentration (Figure S3d).

3.8. Low-N Stress Affects Nitrate Assimilation after Dysfunction of G6PDH

In order to examine the effect of G6PDH on nitrate assimilation, NO$_3^-$ content and the activities of nitrate assimilation-related enzymes were determined. As shown in Figure 6a, low-N stress significantly decreased the NO$_3^-$ content by 72.8% and 72.7% in JD19 and LH3 leaves, respectively, compared to the contents at 3.75 N concentrations. The NO$_3^-$ content was decreased by nearly 79.4% in LD2 leaves. When G6PDH was inhibited by GlcN under low-N treatment, the NO$_3^-$ content in JD19, LH3, and LD2 leaves was markedly decreased to 33.2%, 33.5%, and 28.3% of the low-N treatment alone, respectively. Similar results were observed in the roots (Figure 6b). For NR, NiR, and GS activities, they were significantly reduced in all cultivars under low-N stress (Figure 6c–h). Compared with low-N treatment alone, NR, NiR, and GS activities were further decreased to various degrees under 0.01N+GlcN treatment. These results suggested that the nitrate assimilation in the three cultivars is impaired when G6PDH is not functional.

![Figure 6. Effects of GlcN on NO$_3^-$ content and NR, NiR, and GS activity in three soybean cultivars under low-N stress. (a) NO$_3^-$ content, (c) NR activity, (e) NiR activity, (g) GS activity in leaves, (b) NO$_3^-$ content, (d) NR activity, (f) NiR activity, (h) GS activity in roots. 10 mM GlcN was added into 1/4 Hoagland medium (pH 6.0) with 3.75 N or 0.01 N for 48 h. Data are means ± SE (n = 3, p < 0.05). The letters show significant differences under N conditions.](image-url)
4. Discussion

G6PDH is the first and rate-limiting enzyme of the oxidative pentose phosphate pathway (OPPP), which is a basic component of cellular metabolism [49]. Previous studies revealed that the activity of G6PDH increases the establishment of tolerance to environmental stresses, such as salinity and drought, in several different plant species [28,31,50–52]. In the present study, we aimed to investigate whether G6PDH is involved in the response to low-N stress in soybean seedlings and their possible physiological mechanisms.

Our results showed that the two cultivars JD19 and LH3 differ significantly from the third cultivar LD2 in terms of physiological responses to low-N stress. After 10 days of growth, the leaf area and the primary root length were reduced to different degrees with reduced N concentration in all three cultivars. The decrease in LD2 was more pronounced than that in JD19 and LH3 (Figure 1). Low-N stress often leads to increased oxidative stress in plants [11]. In our study, H$_2$O$_2$ and MDA contents were higher in LD2 than in JD19 and LH3 (Figure S1). These results suggest that LD2 suffers more damages than JD19 and LH3 do under low-N stress. Moreover, JD19 and LH3 accumulate more beneficial compounds, such as proline and soluble sugars, than LD2 to reduce stress damage (Figure S1).

G6PDH maintains the redox homeostasis of plant cells, and this is one of the main reasons for enhanced environmental stresses [31,52]. The differentially increased H$_2$O$_2$ and MDA contents under low-N stress indicated that soybean seedlings suffered from different degrees of oxidative damages (Figure S1). However, JD19 and LH3 had higher G6PDH activities compared with LD2 under low-N stress (Figure 2). This might be one of the reasons for tolerance to low-N in JD19 and LH3. Similar to our results, the drought-tolerant soybean cultivar has enhanced G6PDH activity and lower H$_2$O$_2$ levels than the drought-sensitive cultivar upon drought stress [52]. Furthermore, exogenous H$_2$O$_2$ treatment also increases the total G6PDH activity [11]. In addition, when the low-N-induced G6PDH activity was inhibited under 0.01 N + GlcN treatment, high levels of MDA and H$_2$O$_2$ were detected in the soybean seedlings (Figure 3). Based on these results, we concluded that G6PDH is not only involved in the response to low-N stress, but also has a positive regulatory effect on ROS levels in soybean resistance to low-N stress.

GSH is considered a non-enzymatic scavenger. It is generated from its oxidized form (GSSG) through the glutathione cycle with NADPH as the reductant [10]. G6PDH is responsible for NADPH generation [32]. Therefore, enhancing G6PDH expression could increase the GSH content, which further enhances the antioxidant capacity [32]. In our study, the GSH content was consistent with the Tot-G6PDH activity in three soybean cultivars under low-N stress (Figure 5a,b). In Arabidopsis, AtG6PD5 and AtG6PD6 utilize NADPH to regulate the GSH level, which decreases the ROS accumulation under salt stress [31]. Our data showed that the total NADPH content was slightly higher in LD2 than that in JD19 and LH3, but the NADP$^+$ content was lower in LD2 than that in JD19 and LH3, ultimately resulting in a higher NADPH/NADP$^+$ ratio in LD2 (Figure 4). On the one hand, NADPH is the only reductant for GSH production [13]. Lower GSH content in LD2 means less NADPH consumption under low-N conditions. On the other hand, NADPH and Fd (reduced) are mainly used for N and C assimilation [53]. Under low-N conditions, the reduction equivalence of N assimilation in chloroplasts will accumulate greatly, resulting in an excessive reduction in the photosynthetic electron transfer chain, photo-inhibition, and the accumulation of ROS [54–56]. It was reported that the NADPH content and the NADPH/NADP$^+$ ratio in sensitive barley Ganpi6 are increased more than that in tolerant barley Kunlun12 under low-N stress [11]. Furthermore, the higher NADPH/NADP$^+$ ratio would feedback to inhibit the G6PDH activity [57]. Recent findings reported that G6PDH is essential for maintaining the cellular NADPH/NADP$^+$ homeostasis and the expressions of stress-related genes in soybean tolerance to drought [30]. Overexpression of a soybean Cyt-G6PDH gene, GmG6PD7, increased the salinity tolerance of the transgenic Arabidopsis by regulating the GSH and NADPH level and activating ROS scavengers, which maintained the intracellular redox balance [58]. In the study, the response of three soybean cultivars to low-N stress was also involved in the modulation of NADPH/NADP$^+$
and GSH levels (Figures 4 and 5). These results suggest the key function of G6PDH in soybean tolerance to low N, and moreover, the physiological roles for G6PDH might be similar upon the different stresses in plants. In addition, the PM NADPH oxidase acts as a central enzyme to oxidize cytoplasmic NADPH, further transferring an electron to molecular O$_2$ to form O$_2^-$, which is eventually converted to H$_2$O$_2$ [59]. Our results indicate that the NADPH oxidase activity is also higher in LD2 than that in JD19 and LH3 (Figure S1b,d). This result was consistent with the H$_2$O$_2$ content in the three soybean cultivars (Figure S1b,d). Based on these results, we concluded that JD19 and LH3 utilize more NADPH to produce GSH, thus reducing the ROS level under low-N stress. Additionally, NO$_3^-$ reduction to NH$_4^+$ and glutamate formation both require reductants, which are provided by photosynthetic oxidation processes [60]. When G6PDH is dysfunctional, the content of NADPH is decreased, which hinders the process of N metabolism (Figure 6). However, in this study, it is limited to using only three genotypes, and further work is needed across a more diverse range of genotypes to follow through with conclusions on genotypic variation.

5. Conclusions

In conclusion, our results indicated that G6PDH plays an active role in the response to low-N stress in soybean seedlings. Low-N treatment induces G6PDH expression and enhances G6PDH activity. On the one hand, increased G6PDH activity provides more NADPH for GSH to remove excess ROS. On the other hand, G6PDH provides NADPH for NO$_3^-$ reduction and NH$_4^+$ assimilation to maintain N supply under low-N conditions. The homeostasis of the NADPH/NADP$^+$ ratio is essential for maintaining G6PDH activity. Moreover, NADPH oxidase is responsible for H$_2$O$_2$ accumulation. Increased proline and soluble sugars contribute to reducing oxidative damage caused by low-N stress.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/103390/agronomy11040637/s1, Figure S1: MDA, H$_2$O$_2$ and proline, soluble sugar content in leaves (a, b, e, f) and roots (c, d, g, h) of three soybean cultivars affected by low-N stress, Figure S2: Phylogenetic analysis of G6PDH family proteins in soybean and Arabidopsis using MEGA 6.0, Figure S3: Plot variable factor coordinates and plot case factor coordinates in leaves (a, b) and roots (c, d) of three soybean cultivars. (a, c) projection of all examined parameters, Table S1: Gene-specific primers used in qRT-PCR analyses of G6PDH in three soybean seedlings, Table S2: Contributions of variables in soybean leaves and roots by principal component analysis.

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