Screening of differentially expressed miRNAs and target genes in two potato varieties under nitrogen stress

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Abstract

Background: Nitrogen is an important element for potato growth and development, and improving nitrogen utilization efficiency is an effective way to reduce the amount of nitrogen fertilizer. Transgenic breeding is an effective means to solve the problem that traditional breeding is difficult to solve, it is of great significance to excavate genes that improve nitrogen utilization for breeding nitrogen-efficient potato. It was found that the key enzyme genes in potato nitrogen metabolism pathway were highly responsive to nitrogen stress, and the activity of the key enzyme gene *NiR* (nitrite reductase) was significantly regulated with the increase of nitrogen supply. With the continuous in-depth study of the functions of miRNAs, it has been found that key enzyme genes can regulate their spatiotemporal expression through specific miRNAs, thereby regulating plant phenotypic traits. For example, miR159 regulates *MYB*, miR169 regulates *NFYA*. miRNAs are endogenous non-coding RNAs with regulatory functions that recognize target genes through complementary base pairing. In recent years, some mechanisms have been elucidated through the correlation between the expression levels of potato miRNAs target genes and regulated genes under stress conditions, but the role of miRNAs in the inhibition/expression of key genes regulating nitrogen metabolism under N stress is still unclear.

Results: Through the extension of nitrogen-efficient potato, Yanshu4 ("Y") and nitrogen-inefficient potato Atlantic ("D"), the roots and leaves at the seedling and budding stages after different nitrogen fertilization treatments were collected. miRNAs sequencing, degradome analysis, qRT-PCR and luciferase activity measurement to analyze the expression changes of miRNAs under different N treatment conditions. The study found that with the increase of nitrogen application, NiR activity increased significantly from seedling stage to budding stage, and *NiR* responded significantly to different nitrogen application treatments. miRNAs sequencing predicted 48 families of conserved miRNAs, mainly involved in nitrogen metabolism, carbon metabolism and amino acid biosynthesis. The following differences of miRNAs were screened out (high expression and $P < 0.05$): *stu-miR396-5p*, *stu-miR408b-3p_R-1*, *stu-miR3627-3p*, *stu-miR482a-3p*, *stu-miR8036-3p*, *stu-miR482a-5p*, *stu-miR827-5p*, *stu-miR156a_L-1*, *stu-miR827-3p*, *stu-miR172b-5p*, *stu-miR6022-p3_7*, *stu-miR398a-5p*, *stu-miR166c-5p_L-3*. Degradome analysis showed that most miRNAs had many-to-many relationships with target genes. The main target genes involved in nitrogen metabolism include *NiR*, *NiR1*, *NRT2.5* and *NRT2.7*. qRT-PCR verification found that *stu-miR396-5p*, *stu-miR8036-3p* and *stu-miR482a-3p* were significant differences in leaves and roots between Yanshu4 and Atlantic at seedling and budding stages under the conditions of no nitrogen application and excess nitrogen application, which are induced in response to N stress. The correlation between differential *stu-miR396-5p* and its corresponding target gene *NiR* was further verified by luciferase activity, and it was found that there was a strong negative correlation, which was conducive to further functional research.

Conclusion: From seedling stage to budding stage, the activity of NiR was significantly positively correlated with nitrogen application. Differential miRNAs and target genes showed a many-to-many relationship. The expression of *stu-miR396-5p*, *stu-miR482a-3p* and *stu-miR8036-3p* in Yanshu4 and Atlantic, seedling and budding stage at roots and leaves were most different under two N stresses. Under

two N stresses, in Yanshu4, *stu-miR396-5p* was down-regulated in seedling leaves and shoot-stage roots, and up-regulated in seedling stage roots and shoot-stage leaves; *stu-miR482a-3p* was up-regulated in seedling stage and shoot stages; The expression of *stu-miR8036-3p* was up-regulated in leaves and roots at the seedling and budding stages, and down-regulated in roots under both nitrogen stresses. The N metabolism key enzyme gene *StNiR* and the *stu-miR396-5p* luciferase activity assay reporter detected that they have a strong regulatory relationship.

Introduction

Potato(*Solanum tuberosum* L.)is the fourth largest food crop in the world[1]. The application of nitrogen fertilizer is closely related to potato yield. In order to ensure high and stable yields, China's fertilizer input has accounted for 31% of the world's total in recent years, four times the world average[2]. Excessive nitrogen application will not only lead to the phenomenon of "high fertilizer inefficiency", but also bring negative impact on the environment and economy[3]. Improving the nitrogen use efficiency (NUE) of crops to obtain equal or higher yield with less nitrogen is considered an effective way to solve this problem.

Studies have shown that potato NUE can only be improved to a limited extent through selection of high-yielding varieties or soil management, and further improvements are more difficult[4]. Therefore, from the perspective of molecular biology, by regulating nitrogen stress in potato, the roles of differential miRNAs and corresponding target genes in nitrogen metabolism pathways were analyzed, and then potato NUE was improved to achieve high and stable potato yield. According to previous studies, under N stress, nitrate transporter protein (NRT), nitrate reductase (NR), glutamine synthase (GS), Glutamate dehydrogenase (GDH), and Nitrite reductase (NiR), all of which respond to different N treatments in two potato varieties[5]. And with the increase of nitrogen supply, the activity and expression of *StNiR* also showed an obvious upward trend[6]. miRNAs are endogenous non-coding RNAs with regulatory functions, about 18–25 nucleotides in length. The target gene is recognized by base complementary pairing, and the silencing complex degrades the target gene or inhibits the translation of the target gene according to the degree of complementarity[7], Thus participating in many growth and development processes such as cell signal transduction and development, biotic and abiotic stresses[8]. In barley and gramineous crops such as wheat and corn[9], miR396 is considered to be a metabolic sensor regulating plant nitrogen balance[10]. MiR169 and the target gene *NFYA* were shown to be significantly negatively regulated under nitrogen stress in maize[11]. miR174 and miR167 are involved in N signaling in root development[12], and miR156,miR166,and miR169 are up-regulated in plant growth under nitrogen deficiency[13]. Studies have shown that the regulation pattern of miR159-regulated target gene *MYB* and miR169-regulated target gene *NFYA* in potato drought stress has been confirmed[14]. Therefore, we speculate that the screened differential miRNAs and their target genes have regulatory relationships in nitrogen metabolism pathways, and then work together to regulate nitrogen metabolism .In conventional production, 'YanShu4' is a high absorption and high utilisation potato and 'Atlantic' is a low absorption and low utilisation potato[1]. Therefore, the two types of potatoes were treated with no nitrogen application and excessive

nitrogen application, and the roots and leaves of the two types of potatoes at the seedling and budding stages were subjected to miRNA sequencing and degradome sequencing. Combined with sequencing results and qRT-PCR, the differences in N stress miRNA screening were verified, and the expression relationship between differential miRNAs and corresponding target genes under N stress was clarified.

Results

miRNAs Sequencing and analysis

The RNA extracted from potato leaves and roots was sequenced, and the output raw sequencing data (Appendix A, Table 1) were counted to obtain the sequencing data Unique Sequence and the copy number corresponding to each Unique sequence. First remove the 3' linker sequence from the original sequencing data, and the length of the removed sequencing base is less than 18nt if there are 80% sequences in the sequence A or C or G or T, 3N (not necessarily continuous), only A and C without G and T, or Only G and T, no A and C, or continuous nucleotide dimers, trimers. At the same time, we will compare and filter the sequence and target genes (some species may not), RFam (including rRNA, tRNA, snRNA, snoRNA, etc.) and the Repbase database. The filtered data is called Valid Data, and the Valid Data is further processed miRNA alignment, identification, prediction and analysis. Clear adapter trimming, quality trimming, read label unification, and map to Rfam after filtering database and duplicate database and filtering read length, a clean read is obtained from the original read (size: 18-25nt).

Based on the analysis and statistics of the original sequencing data, the statistical analysis of the length distribution of the filtered effective data is performed. According to the typical characteristics of Dicer digestion, most of the data are distributed in 20-24nt. Figure 1 shows the length distribution of miRNAs clean reads obtained by miRNA Sequencing under N stress, showing that the largest miRNAs are 25 nucleotides(nt) in length, followed by 24 nucleotides.

Differential miRNAs expression analysis

Test was used to screen genes with significant differences and differentially expressed genes, with P < = 0.05 as the threshold. (See Appendix A, Table 2 for details) miRNAs Sequencing predicts 48329A conserved miRNAs in a family. According to miRNA sequencing and degradome analysis, it is found that the differentially expressed miRNAs screened out involve a large number of metabolic pathways and biological processes, many of which are miRNAs have a one-to-many relationship with target genes.

Differential miRNAs Analysis

The overall distribution of differentially expressed miRNAs can be known by drawing volcano plots. It can be seen from the figure that there are obvious up-down regulation and expression differences in the leaves of the two types of potatoes at the germination stage without nitrogen application. The comparison of two kinds of potatoes in seedling and budding stage leaves under excessive nitrogen application, the comparison of two kinds of potatoes in seedling and budding stage roots under

excessive nitrogen application, and the comparison of two kinds of potatoes in seedling and budding stage roots under no nitrogen application. See Fig. 1 in Appendix A.

Under the condition of excessive nitrogen application, the leaves of two kinds of potatoes were significantly up-regulated in the comparison of seedling and budding stage, and when nitrogen was not applied, the leaves of two kinds of potatoes were significantly down-regulated in the comparison of seedling and budding stage.

Statistical analysis of differential up- and down- miRNAs

Statistics of up- and down-regulated frequencies of differential genes can determine the number of differentially expressed miRNAs under different experimental conditions. Comparison of miRNAs in different groups showed that the number of up- and down- regulated miRNAs in nitrogen-fertilized leaves were 151 and 181, respectively. The numbers of up- and down-regulated miRNAs in the corresponding non-nitrogen- treated leaves of the two potatoes were 146 and 180. There were no up-regulated miRNAs in roots of seedling and budding stage of Yanshu4 under excessive nitrogen application treatment, seedling and budding stage of Atlantic under no nitrogen application treatment, budding stage of Yanshu4 under different nitrogen application treatments. At the same time, there were no up-regulated miRNAs in leaves of Atlantic budding stage under the non-nitrogen treatment and in roots of two kinds of potatoes in budding stage under the non-nitrogen treatment.

Differential miRNAs Cluster analysis

Cluster expression models provide better and more intuitive cluster graphs. KEGG enrichment analysis of the target genes corresponding to the differential miRNAs showed that *stu-miR396-5p*, *stu-miR482a-3p* and *stu-miR8036-3p* were expressed in two N stresses, two potatoes, two periods and two tissues; Under different nitrogen fertilization treatments, *stu-miR482a-5p* had higher expression in two periods and two sites in Atlantic. The expression of *stu-miR482a-5p* was higher in two periods and two parts of Atlantic under different N treatments.

The expression of *stu-miR166c-5p_L-3* and *stu-miR3627-3p* was lower in both N stresses, two potatoes, two periods and two tissues. *stu-miR156a_L-1* was less expressed in the seedling roots of two kinds of potatoes with non-nitrogen treatment, in Atlantic seedling roots under different N treatments, and in Atlantic roots at the budding and seedling stages with non-nitrogen treatment. The expression of *stu-miR172b-5p* in two tissues and two periods of Yanshu4 was lower under different nitrogen treatments.

Prediction and enrichment analysis of target genes corresponding to miRNAs

Target gene prediction of differential miRNAs

Target genes were predicted for those miRNAs with significant differences using psRobot (v1.2) [15]. Target penalty strategy based on plants predicts targets (the default threshold is Score ≤ 2.5). We

performed target gene prediction for differential miRNAs, as well as target gene information corresponding to miRNAs and annotation information of target genes GO and KEGG.

Differential miRNAs target gene enrichment analysis

Enrichment analysis mainly includes two parts, one is GO function annotation (Fig. 2), and the other is KEGG Pathway function annotation (Fig. 3).

Our analysis revealed that *stu-miR396-5p* is involved in metabolic pathways such as nitrogen metabolism, carbon metabolism, pyruvate metabolism, alanine, aspartate and glutamate metabolism, biological processes such as phytohormone biosynthesis, amino acid biosynthesis, zeatin biosynthesis, and molecular functions such as RNA degradation, RNA transport and endoplasmic reticulum protein processing, predicting that *stu-miR396-5p*, which has 65 target genes including *NiR*, *PERK2* and *HAK26*, *stu-miR8036-5p* is mainly involved in plant-pathogen interactions, cyanine metabolism, keratin, hypoglycine and wax biosynthesis. *stu-miR482a-5p* has 15 target genes were predicted, including *CNX2*, *Os04g0386900* and *PSS1*. which is mainly involved in folate biosynthesis, glycerophosphate metabolism, glucosinolate metabolism. Sequencing and analysis of *stu-miR156a_L-1* found that it is mainly involved in biological processes and metabolic pathways such as nitrogen metabolism, endoplasmic reticulum processing, RNA degradation, and ubiquitin-mediated protein degradation. The predicted target genes include *NRT2.5*, *SRF6* and *AGL15* and other genes 50 target genes.*stu-miR172b-5p* is mainly involved in starch and sucrose metabolism, arginine and proline metabolism and other pathways. The predicted target genes include 13 target genes including *MT2287*, *grpE* and *WRKY48*. The analysis of *stu-miR827-3p* found that it was mainly involved in biological processes such as aminoacyl biosynthesis and plant hormone signal transduction. The predicted target genes included 31 target genes including *SD31*, *EXD1* and *RS31*. The analysis of *stu-miR408b-3p_R-1* found that it was mainly involved in metabolic pathways and biological processes such as nitrogen metabolism, carbon metabolism, ascorbic acid and uronic acid metabolism. The predicted target genes included 9 *UCC1*, *LAC5*, *TPS32*, and *TPS31*. *stu-miR3627-3p* was found to be mainly involved in RNA transport, carbon metabolism and other pathways, and the predicted target genes were *HSP70-14*, *B3GALT5*, and *B3GALT7*.The *stu-miR482a-3p* analysis found that metabolic pathways were mainly involved in endocytosis, plant-pathogen interaction, and ubiquitin-mediated proteolytic metabolism. The predicted target genes included 14 target genes including *R1B-16*, *RPP13L3*, *FZR1*, and *At1g61180*.The *stu-miR827-5p* analysis found that it is mainly involved in amino sugar and nucleotide sugar metabolism, plant-pathogen interaction, glutathione metabolism and other pathways. The predicted target genes include *R1B-16*, *RPP13L3*, *FZR1*, *At1g61180*, etc. *stu-miR6022-p3_7* was found to be mainly involved in nitrogen metabolism, amino acid biosynthesis, thiamine metabolism and other metabolic pathways and biological processes, etc. The predicted target genes include *CYTB5-B*, *PSY1R*, *PAE12*, etc. The analysis of *stu-miR398a-5p* found that it mainly involved metabolic pathways such as peroxisome, homologous recombination and photosynthesis, and the predicted target genes included *PSBY*, *STY17*, *ATX1*, *Os12g0234800*. *stu-miR166c-5p_L-3* is mainly involved in metabolic pathways such as pyruvate metabolism, glycolysis/ gluconeogenesis, valine, leucine and isoleucine degradation, and predicted 26 metabolic pathways including *PEX11B*, *GT7*, and *LECRKS5*. target gene.

GO has three ontologies, which describe the molecular functions, cellular components, and biological processes involved in genes, respectively. (See Appendix A, Table 4 for details).

GO enrichment analysis was performed to count the top 20 annotations closely related to nitrogen metabolism, such as GO0003674 (molecular function), GO0003700 (DNA-binding transcription factor activity), GO0004674 (protein serine/threonine kinase activity), GO0005634 (nucleus), GO0006355 (DNA template, transcriptional regulation), etc. The results show that the most annotated are plasma membrane (GO0005886), defence response (GO0006952) and ATP binding (GO0005524). GO enrichment analysis of target genes corresponding to differential miRNAs showed that defense response was the most annotated among the 25 biological processes, followed by biological processes, the nucleus was the most annotated among the 15 cellular components, followed by the plasma membrane, and 10 The most annotated molecular function is ATP binding, followed by protein binding. The top 20 annotations closely related to nitrogen metabolism, such as KO00910 (nitrogen metabolism), KO01200 (carbon metabolism), KO04146 (peroxisome), KO00970 (amino acid biosynthesis) and KO04075 (phytohormone signalling), were enriched for KEGG analysis. KO04626) and shear bodies (KO03040) and phytohormone signalling (KO04075). KEGG enrichment analysis showed that target genes were mainly enriched in phytopathogenic interactions (KO04626) and phytohormone signaling (KO04075).

Validation of differential miRNAs by qRT-PCR

Differential expression of miRNAs under the same N treatment

The differential expression of *stu-miR396-5p* in LN_YLS and LN_DRA was significant, as shown in Fig. 4. The differential expression of *stu-miR408b-3p_R-1* was more significant. The differential expression of *stu-miR482a-3p* was significant under LN_YRS treatment and more significant in LN_YRA. *stu-miR166c-5p_L-3* was significantly differently expressed under LN_YRS treatment. The differential expression of *stu-miR8036-3p* was significant under LN_YLA treatment and more significant in LN_DLS. The differential expression of *stu-miR482a-5p* was significant under LN_DLA treatment and more significant in LN_DLS. The differential expression of *stu-miR827-3p* was more significant in LN_DLA treatment. *stu-miR827-5p*, *stu-miR172b-5p* and *stu-miR398a-5p* were more significantly expressed in LN_DLA and LN_DLS. The differential expression of *stu-miR3627-3p*, *stu-miR156a_L-1* and *stu-miR6022-p3_7* was not significant in the absence of nitrogen application.

The expression of differential miRNAs under the treatment of excess nitrogen application in Fig. 5 showed that *stu-miR396-5p* was significantly expressed under HN_YLS treatment and more significantly expressed in HN_YRS. The differential expression of stu-miR482a- 3p was significant under HN_YRA treatment and more significant in HN_YRS. The differential expression of *stu-miR8036-3p* was significant under HN_YLA treatment and more significant in HN_DLS. The differential expression of *stu-miR482a-5p* was significant under HN_DLA treatment and more significant in HN_DLS. The differential expression of *stu-miR156a_L-1* was more significant under HN_YLA treatment. *stu-miR827-3p* was significantly

expressed under HN_DLA treatment and more significantly expressed in HN_YRA. The differential expression of *stu-miR166c-5p_L-3* was more significant under HN_DRA treatment. The differential expression of *stu-miR408b-3p_R-1*, *stu-miR827-5p*, *stu-miR172b-5p*, *stu-miR6022-p3_7* and *stu-miR398a-5p* was more significant under HN_DLA treatment. None of the differential expression of *stu-miR3627-3p* was significant under excessive nitrogen application.

Expression of differential miRNAs under different N treatment

The differentially expressed miRNAs are shown in Fig. 6. In Atlantic, *stu-miR396-5p* was significantly differentially expressed under LN_DRA treatment and more significant in LN_DRS. The differential expression of *stu-miR482a-3p* was significant under LN_DRS and more significant in HN_DRS. The differential expression of *stu-miR8036-3p* was significant under HN_DLS treatment and more significant in HN_DLA. The differential expression of *stu-miR166c-5p_L-3* was more significant under HN_DRA treatment. *stu-miR482a-5p* and *stu-miR398a-5p* were significantly differently expressed under HN_DLA treatment and more significantly expressed in LN_DLA. The differential expression of *stu-miR827-5p* and *stu-miR827-3p* was more significant under LN_DLA treatment. The differential expression of *stu-miR408b-3p_R-1*, *stu-miR156a_L-1* and *stu-miR172b-5p* was more significant under HN_DLA treatment. The differential expression of *stu-miR3627-3p* and *stu-miR6022-p3_7* was not significant in the Atlantic.

The differential expression of *stu-miR396-5p* and *stu-miR156a_L-1* were significantly expressed under the treatment of LN_YLS and more significantly expressed in HN_YLS as seen from the expression of differential miRNAs in Fig. 7. The differential expression of *stu-miR408b-3p_R-1* and *stu-miR398a-5p* was more significant under the treatment of HN_YLS. The differential expression of *stu-miR3627-3p* was more significant under the treatment of LN_YLA. The differential expression of *stu-miR482a-3p* was significant under the treatment of HN_YRA and more significant in HN_YRS. The expression of *stu-miR8036-3p* and *stu-miR172b-5p* was significantly different in the treatment of HN_YLA and more significantly in LN_YLA. The differential expression of stu-miR827- 3p was significant under the treatment of LN_YRA and more significant in HN_YRA. The differential expression of *stu-miR6022-p3_7* was more significant under the treatment of HN_YRA. The differential expression of *stu-miR166c-5p_L-3* was more significant under the treatment of HN_YRS. The differential expression of *stu-miR482a-5p* and *stu-miR827-5p* was more significant under the treatment of LN_YLS.

Expression of *stu-miR396-5p* was down-regulated in seedling leaves and budding stage roots and up-regulated in seedling roots and budding stage leaves under both N stresses in Yanshu4. *stu-miR408b-3p_R-1* and *stu-miR482a-3p* were up-regulated in seedling leaves and in roots and leaves at budding stage. *stu-miR8036-3p* was expressed up-regulated in seedling leaves and roots and down-regulated in leaves and roots at budding stage. *stu-miR482a-5p* was up-regulated in seedling leaves and roots. *stu-miR827-5p* was down-regulated in expression in seedling leaves and up-regulated in seedling roots. *stu-miR156a_L-1* was down-regulated in seedling leaves and roots and in roots at budding stage. *stu-miR827-3p* was expressed down-regulated in seedling leaves and roots and up-regulated in seedling roots

at budding stage. *stu-miR172b-5p* was expressed up-regulated in seedling and budding stage roots and down-regulated in seedling leaves. up-regulated expression of *stu-miR6022-p3_7* in seedling roots. *stu-miR398a-5p* was expressed up-regulated in seedling and budding stage leaves and down-regulated in seedling and budding stage roots. *stu-miR166c-5p_L-3* was down- regulated in seedling leaves and roots and up-regulated in leaves and roots at budding stage.

The expression of differential miRNAs in the two potato leaves from Fig. 8 shows that *stu-miR396-5p* was significantly differently expressed in the treatment of a LN_YLS and more significantly in the HN_YLS. The differential expression of *stu-miR408b-3p_R-1* was significant under the treatment of HN_DLA and more significant in LN_DLA. *stu-miR3627-3p* was differently expressed in HN_DLA. *stu-miR482a-3p* was differently expressed in the treatment of HN_YLS and differently expressed in LN_YLS. The differential expression of *stu-miR8036-3p* was significant under the treatment of LN_YLA and more significant in HN_YLA. The differential expression of stu-miR482a- 5p was significant under the treatment of HN_DLA and more significant in HN_DLS and LN_DLA. The differential expression of *stu-miR827-3p* was significant under the treatment of LN_DLA and more significant in LN_DLS. The differential expression of *stu-miR827-3p* was significant under the treatment of LN_DLA and more significant in LN_DLS. The expression of *stu-miR172b-5p* and *stu-miR398a-5p* was significantly different under the treatment of HN_DLA and more significantly in LN_DLA. The differential expression of *stu-miR156a_L-1* and *stu-miR6022-p3_7* in the two potato leaves was not significant.

The expression of differential miRNAs in the two kinds of potatoes roots as seen in Fig. 9 showed that the differential expression of *stu-miR396-5p* was significant under the treatment of LN_DRA and more significant in LN_YRA. The differential expression of *stu-miR408b-3p_R-1* and *stu-miR156a_L-1* was more significant in HN_DRA. The differential expression of *stu-miR3627-3p* was more significant in LN_DRA. The differential expression of *stu-miR482a-3p* was significant under the treatment of HN_YRA and more significant in LN_YRS. The differential expression of *stu-miR8036-3p* was significant under the treatment of LN_YRS and more significant in HN_YRA. The differential expression of *stu-miR482a-5p* and *stu-miR398a-5p* was significant under the treatment of HN_DRS and more significant in HN_DRA. The differential expression of *stu-miR827-5p* was more significant in HN_DRS. The differential expression of *stu-miR827-3p* and *stu-miR172b-5p* was significant under the treatment of LN_DRS and more significant in HN_DRA. *stu-miR166c-5p_L-3* was significantly differently expressed in the treatment of LN_DRA and more differently expressed in LN_YRS. The differential expression of *stu-miR6022-p3_7* in the two potato roots was not significant.

The differential expression of the two potato seedling miRNAs in Fig. 10 shows that *stu-miR396-5p* was significantly expressed under the treatment of LN_YLS and more significantly in HN_YLS. The differential expression of *stu-miR408b-3p_R-1* was more significant in HN_DLS. The differential expression of *stu-miR3627-3p* was more significant in LN_DLS. The differential expression of *stu-miR482a-3p* was significant under the treatment of HN_YRS and more significant in HN_YLS. The differential expression of *stu-miR8036-3p* was significant under the treatment of HN_DLS and more significant in HN_YLS. The differential expression of *stu-miR482a-5p* and *stu-miR398a-5p* was significant under the treatment of

HN_DLS and more significant in LN_DLS. The differential expression of *stu-miR827-5p* and *stu-miR827-3p* was significant under the treatment of LN_DLS and more significant in HN_DLS. The differential expression of *stu-miR156a_L-1* was more significant in LN_YLS. The differential expression of *stu-miR172b-5p* and *stu-miR6022-p3_7* was more significant in HN_DLS. The differential expression of *stu-miR166c-5p_L-3* was more significant in HN_DRS.

The differential expression of *stu-miR396-5p* in LN_DRA and more significantly in LN_YRA can be seen from the expression of the two potato differential miRNAs at budding stage in Fig. 11. The differential expression of *stu-miR408b-3p_R-1* was more significant in HN_DLA. *stu-miR482a-3p* was significantly differently expressed in the treatment of HN_YRA and more significantly in LN_YRA. The differential expression of *stu-miR8036-3p* was significant under the treatment of HN_YLA and more significant in LN_YLA. *stu-miR482a-5p* and *stu-miR398a-5p* were significantly differently expressed under the treatment of HN_DLA and more significantly expressed in LN_DLA. *stu-miR827-5p* and *stu-miR827-3p* were differently expressed in LN_DLA. The differential expression of *stu-miR156a_L-1* was significant under the treatment of LN_YLA and more significant in HN_DLA. The differential expression of *stu-miR172b-5p* was significant under the treatment of HN_YLA and more significant in LN_DLA. *stu-miR166c-5p_L-3* was differently expressed in HN_DRA. The difference between *stu-miR3627-3p* and *stu-miR6022-p3_7* was not significant at budding stage in the two potatoes.

Analysis of traits, target genes and differential miRNAs

Analysis of the relationship between traits and target genes

As shown in Fig. 12, the NiR activities of Yanshu4 and Atlantic showed a significant upward trend with the increase of nitrogen application from seedling stage to germination stage, either excess nitrogen application or no nitrogen application. This indicated that the two kinds of potatoes NiR activities were positively correlated with the two nitrogen application, i.e. the two kinds of potatoes *NiR* genes were highly responsive to nitrogen stress.

The analysis of target genes and their corresponding differential miRNAs is shown in Fig. 13. In Yanshu4 and Atlantic, the relative expression of *NiR* from seedling stage to budding stage showed a significant upward trend from no nitrogen application to super nitrogen application. *stu-miR396-5p* decreased significantly from seedling stage to budding stage from no nitrogen application to super nitrogen application, especially in Yanshu4.

The relative expression of *NiR* in the two kinds of potatoes showed a significant upward trend from excessive nitrogen application to no nitrogen application. The relative expression of *stu-miR396-5p* in the two potato varieties showed a significant downward trend from excessive nitrogen application to no nitrogen application. The expression of *NiR* in two potatoes was positively correlated with nitrogen application, while the relative expression of *stu-miR396-5p* was negatively correlated with nitrogen application application.

The prediction of the binding site of *stu-miR396-5p* to *StNiR* was performed using the online URL and the predicted results are shown in Fig. 14. There may be a binding site between *stu-miR396-5p* and *StNiR*, and there may be a splicing relationship between the 8th base of *stu-miR396-5p* and the 8th base of *StNiR* from the 5' end of *stu-miR396-5p*.

As shown in Fig. 15, the binding ratio of NiR-luc to miR396-5p was 0.2715, the binding ratio of NiR-luc to 1300-35S-X was 1, the binding ratio of 1300-luc to NiR-luc was 0.9452, and the binding ratio of 1300-luc to NiR-luc was 0.9452. The binding ratio to 1300-35S-X was 0.9143, so it could be confirmed that miR396-5p binds to the NiR sequence and causes product degradation. Therefore, it can be confirmed that *stu-miR396-5p* does have a targeting relationship with *StNiR*.

Discussion

Expression analysis of differential miRNAs in two potat0 cultivars, root and leaf tissues, and two growth stages under two N stress conditions

A large number of studies have shown that different miRNAs are involved in different stress conditions and play an extremely important role in plant growth and development. We screened for differential miRNAs between Yanshu4 and Atlantic in roots and leaves at seedling and budding stages, and our analysis found that *stu-miR396-5p*, *stu-miR408b-3p_R-1*, *stu-miR3627-3p*, *stu-miR482a-3p*, *stu-miR8036-3p*, *stu-miR482a-5p*, *stu-miR827-5p*, *stu-miR156a_L-1*, *stu-miR827-3p*, *stu-miR172b-5p*, *stu-miR6022-p3_7*, *stu-miR398a-5p*, *stu-miR166c -5p_L-3* were significantly different.

Researchers found that miR396 is directly involved in the regulation of plant metabolism and plant growth and development[16], such as water stress, temperature stress, salt stress, oxidation process, fatty acid metabolism, root tip growth and development[17] and bacterial infections[18]. The study found that miR396-GRF plays an important role in regulating the response to different nitrogen forms. In lettuce,*LSA-miR396* affects the content of different nitrogen forms by regulating LsaGRFs during leaf growth[19]. We found that differential miR396-5p was highly sensitive to N stress, and *stu-miR396-5p* was down-regulated in both seedling leaves and roots and budding leaves and roots of large Atlantic, in seedling leaves and budding roots of Yanshu4 and in It was up-regulated in the roots of seedlings and leaves at the budding stage, which is consistent with the previous study. Fischer[20] studies on the regulation of miRNAs expression in plants revealed that miR396 was also found to be down-regulated under nitrogen-deficient conditions[21]. We found that *stu-miR398a-5p* was up-regulated in leaves at seedling and budding stage and down-regulated in roots at seedling and budding stage under two N stresses. In Atlantic, *stu-miR398a-5p* was up-regulated in leaves and roots at seedling stage and budding stage. The expression of stu-miR408b-3p_R-1 was up-regulated in leaves and roots at seedling stage and budding stage, which indicated that *stu-miR408b-3p_R*-1 was highly responsive to N stress. Several studies have found that MiR160 is highly responsive to N and S deficiency. Under N starvation conditions, the

expression of miR169, miR171, miR398, miR399, miR408, miR827, and miR857 was repressed, whereas the expression of miR160, miR826, miR842, and miR846 was induced. At the same time, it was found that miR156, miR172 and miR398 were responsive to low temperature stress. Based on the research of others, we further clarified the specific expression location and period, which provided some references for future related research. Fischer[20] discovered the regulatory mechanism of miRNA expression under nitrogen deficiency in plants, and found that the expression of miR156 was up-regulated under nitrogen deficiency. Studies have shown that miR156 plays an important role in many metabolic pathways in potato, such as miR156 inhibits the formation of potato tubers[22]. miR156e regulates potato tuber development by regulating the expression of its target gene StPTB6,other studies pointed out that the expression level of potato miR156 is also regulated by photoperiod, and miR156/157 targets *SPL* transcription factors A study on regulating the polarity of potato flower organs.[23]. We found that *stu-miR156a_L-1* was down-regulated in leaves and roots at seedling stage and roots at budding stage under two N stresses. Under two N stresses, the expression of *stu-miR156a_L-1* was down-regulated in seedling leaves and sprouting roots of Atlantic, and up-regulated in seedling roots and sprouting leaves, and the expression of *stu-miR156_L-1* in leaves of these two types of potatoes was over-fertilized with nitrogen. The expression level was significantly higher under N stress than in non-N treated leaves, suggesting that *stu-miR156_L-1* plays an important role in the process of N stress. Its corresponding target gene is the key enzyme gene *NRT2.5* in the nitrogen metabolism pathway. The early identification of the *NRT* family showed that when N is sufficient, family members play an active role in the nitrogen metabolism pathway, while nitrogen deficiency induces potato stress. Therefore, we speculate that *stu-miR156a_L-1* may have a regulatory relationship with its target gene *NRT2.5*, which regulates the nitrogen metabolism pathway of potato, but the specific regulatory mechanism needs to be further explored. Studies indicate that inhibition of miR482 has been shown to inhibit miR482 in potato, tomato and cotton. Effectively inhibit plant infection with pathogenic bacteria. Through the study of two kinds of potatoes, we found that under the two kinds of N stress, with the increase of nitrogen application, the expression levels of *stu-miR482a-5p* and *stu-miR482a-5p* showed an upward trend, indicating that miR482a-5p has a sensitive response to different N treatment. The expression of *stu-miR482a-3p* in Yanshu4 was up-regulated in leaves at seedling stage and roots and leaves at budding stage under two N stresses. The expression of *stu-miR482a-3p* was down-regulated in leaves and roots at seedling stage and up-regulated in leaves and roots at budding stage in the Atlantic under two N stresses. *stu-miR482a-5p* was up-regulated in leaves and roots at seedling and budding stages. We initially found that miR482a induced expression changes under N stress, but the specific function of miR482a needs further study. Studies have shown that in potato plants,miR172 is involved in flower and tuber induction signal transduction pathways and a clear link between solute transport and flowering and nodulation induction[24]. other study showed that tomato miR172 targets and regulates the APETALA2 transcription factor *SlAP2a* to regulate fruit ripening[25], Ferdous found that miR172,miR396a and miR396c regulate *P5CS* genes respectively under water stress in barley, thereby regulating proline accumulation and providing molecular evidence for the drought tolerance process in potato[26]. There are also research found that miR172 was significantly expressed in the developmental stage of potato tubers[27]. We found that the expression of *stu-miR172b-5p* in Atlantic roots was higher than that in leaves at the budding stage under excessive nitrogen

application, and the expression of *stu-miR172b-5p* was up-regulated in Atlantic seedling and budding leaves and roots, but down-regulated in roots at seedling stage. In Yanshu4, *stu-miR172b-5p* was up-regulated in roots at seedling and shoot stages, but down-regulated in leaves at seedling stage, and was highly sensitive to nitrogen stress. This is similar to the previous conclusions, but its deeper function needs further study. *stu-miR827* is mainly involved in biological processes such as ribosome formation, aminoacyl biosynthesis, and plant hormone signal transduction in eukaryotes. Studies have pointed out that miR827 plays a key role in the adaptation of barley to drought tolerance, and MiR827 has a strong induction effect on PI starvation through shoots and roots[28]. We found significant differences in the enlargement of *stu-miR827-3p* and *stu-miR827-5p* between the two types of potatoes under N treatment. In Yanshu4, *stu-miR827-5p* was down-regulated in leaves and up-regulated in roots. The expression of *stu-miR827-3p* was down-regulated in leaves and roots at seedling stage and in roots at budding stage, but up-regulated in roots at seedling stage. In Atlantic, *stu-miR827-5p* was up-regulated in roots and down-regulated in leaves at seedling and budding stages. The expression of *stu-miR827-3p* was down-regulated in leaves and up-regulated in roots at seedling and budding stages. However, we intend to screen out genes closely related to nitrogen metabolism, and their target genes do not include key enzymes in nitrogen metabolism pathways, so we can consider studying from different directions. miR408 is involved in plant growth and stress response, and miR408 can regulate plastid cyanin (PC) by down-regulating target proteins, thereby affecting photosynthesis and ultimately promoting grain yield, revealing that miR408 plays an important role in regulating plant growth and development and plant responses to various abiotic and biotic stresses[29]. Previous studies have shown that overexpression of miR408 can significantly enhance the drought tolerance of chickpeas, while recent studies have shown that functional deficits of miR408 can negatively regulate light-dependent seed germination[30]. We found that *stu-miR408b-3p_R-1* was up-regulated in leaves at seedling stage and roots and leaves at budding stage in Yanshu4 under two N stresses, and *stu-miR408b-3p_R-1* was up-regulated in leaves and roots at seedling stage and budding stage in Atlantic, which was highly responsive to N stress. We verified the expression of miR408 from a new abiotic stress perspective, providing a new direction for people to further understand this ancient and highly conserved miRNA. Little is known about miR8036. We found that *stu-miR8036-3p* was up-regulated in leaves and roots at seedling stage and budding stage in Atlantic under two N stresses. In Yanshu4, *stu-miR8036-3p* was up-regulated in leaves and roots at seedling stage and down-regulated in leaves and roots at budding stage. The specific regulatory mechanism is still unclear, and miRNA, as a relatively blank, needs further study. miR398 is considered to be a kind of miRNA directly related to plant stress regulation network, which regulates plant responses to oxidative stress, water deficit, salt stress, abscisic acid stress, ultraviolet stress, copper and phosphorus deficiency, high sugar and bacterial infection[11], and has great value for the study of biology and biological stress. Studies have shown that miR398 can enhance SOD activity in wheat roots by regulating the expression of *WRKY*, thus alleviating non-induced oxidative toxicity in wheat roots[31]. Recent studies have shown that miR398 can alleviate symptoms and accumulation of bamboo mosaic virus by regulating antioxidants[32]. We found that *stu-miR398a-5p* was up-regulated in leaves and roots at seedling stage and budding stage in Atlantic under two N stresses, and up-regulated in leaves at seedling stage and budding stage in Yanshu4, but down-regulated in roots at seedling stage and budding stage.

We studied the regulatory relationship of miR398 from the direction of N stress, which enriched the research content of miR398.

It was found that the relative abundance of Streptococcus tomato and Streptococcus Habrochetti subtypes changed significantly under low temperature stress, which indicated that miR6022 played an important role in cold stress response[33]. Other studies have shown that *sly-miR6022* can regulate tomato R gene *Cf-9* at post-transcriptional level in tomato[34]. Through the interweaving study of different regulatory networks in potato, it is found that miR6022 participates in regulating the regulatory networks of other miRNAs, revealing that miRNAs are balanced by mutual regulation in developmental signaling, disease symptom development and stress signaling[35]. We found that *stu-miR6022-p3_7* was up-regulated in seedling roots in Yanshu4 under two N stresses, and in seedling leaves and bud roots in Atlantic. Our research provides new research ideas on the basis of predecessors, and the specific regulation mechanism needs further study. Recent studies have found that *sly-miR166* and *SlyHB* modules are susceptible factors of ToLCNDV (New Delhi Tomato Leaf Curl Virus) in tomatoes, Moreover, *sly-miR166* and *SlyHB* were negatively regulated. Therefore, by regulating the expression of *sly-miR166*, *SlyHB* can be regulated, thereby regulating the pathogenesis of ToLCNDV[36]. Studies have found that tomato plants carrying the resistant alleles of *Slhb15a* and miRNA166 develop into normal ovules, and fruit setting can be promoted at extreme temperatures by mutual regulation of *Slhb15a* and miRNA166[37]. We found that *stu-miR166c-5p_L-3* was down-regulated in leaves and roots at seedling stage and up-regulated in leaves and roots at budding stage in Yanshu4 under two N stresses, and up-regulated in roots at budding stage and down-regulated in leaves at budding stage in Atlantic. The specific regulatory mechanism needs further research to fully exert its role in plant biotic and abiotic stress.

Analysis of miRNA sequencing expression under N stress

In recent years, with the continuous development of sequencing technology, its application is more and more extensive. Studies have shown that there are differences in aluminum tolerance of miRNAs between Ailanthus sinensis and Ailanthus macrophylla roots through Illumina sequencing technology: miR160 promotes the development of adventitious roots and lateral roots; miR3627 citric acid secretion increased; miR3627 and miR482 more flexibly control the alternative glycolytic pathway and TCA cycle; miR172s flexibly metabolically regulate miRNA metabolism. miR160 was found to affect root development in citrus, and *cas-miR5139* and *csi-miR1210*5 played important roles in citrus Al tolerance by regulating cell wall components[38]. Fische's study on the regulation of miRNAs expression in plants found that miR396 expression was down-regulated under nitrogen deficiency conditions[21]. Trindade and others have revealed that MiR396 response to water stress of alfalfa[39]. We found by miRNA sequencing that *stu-miR396-5p*, *stu-miR8036-3p* and *stu-miR482a-3p* were significantly different in root and leaf expression in Yanshu4 and Atlantic over- and under-fertilized seedlings and roots and leaves at the germination stage.

N under stress difference expression of miRNAs, target genes and traits

Previous research found that *StNiR* activity showed a significant upward trend with the increase of N application. This study found that the relative expression of *StNiR* showed a significant upward trend with the increase of N application, while the relative expression of *stu-miR396-5p* showed a significant downward trend with the increase of N application, especially in Yanshu4. Degradome analysis showed that the target gene corresponding to *stu-miR396-5p* was *NiR*. Our previous study found that the expression level of *StNiR* gene in Yanshu4 and Atlantic showed an upward trend under N stress, indicating that it showed a high response to N stress. The results are consistent with those of previous studies[40]. We also found that *stu-miR396-5p* in Yanshu4 and Atlantic showed a downward trend under N stress. Therefore *stu-miR396-5p* and *StNiR* showed a negative correlation under N stress, so we speculated that *StNiR* might play an important role in N metabolism pathway of potato.

Some studies have found that the *sit-miR396* target gene is *Type-IV*, and its main function is in catalysis, such as hydrolase, isomerase and peroxidase, etc. The functional enrichment study of *sit-miR396* shows that it can effectively regulate plant growth, but The role of *sit-miR396* and the specific mechanism of regulating root development in millet are still unclear[41]. And some studies have pointed out that the expression of conservative miR408a and miR408b can be changed under low temperature non-adaptation, while miR156 and miR169 differential expression only under low temperature adaptation conditions[42]. And some studies have pointed out that the transient overexpression of miR169o in rice protoplasts can rapidly detect the decrease in the activity of the verification target gene *OsNF-YA4*,which indicates that miR169o can regulate the expression level of the target gene *OsNF-YA4*[43]. Others have found that the S1COL4 transcription factor in tomato plays a negative role in fruit ripening by regulating the expression of *ASC* genes, thereby achieving the purpose of regulating the ethylene biosynthetic pathway[44]. Therefore, we designed a test scheme according to the following Fig. 16 to fully explore the relationship between *StNiR* and *stu-miR396-5p*-specific regulation in potato nitrogen metabolism pathway.

Conclusion

The miRNA sequencing predicted 329 conserved miRNAs from 48 families, and 13 miRNAs closely related to N metabolic pathway were screened. degradome analysis revealed that most miRNAs showed many-to-many relationship with target genes. GO and KEGG enrichment analyses were performed and revealed numerous biological processes and metabolic pathways involved in nitrogen metabolism, carbon metabolism and phytohormone biosynthesis. Screening miRNAs with nitrogen metabolism pathways and related pathways according to experimental ideas. qRT-PCR validation of the screened differential miRNAs showed that *stu-miR396-5p, stu-miR408b-3p_R-1*, *stu-miR3627-3p*, *stu-miR482a-3p*, *stu-miR8036-3p*, *stu-miR482a-5p*, *stu-miR827-5p*, *stu-miR156a_L-1*, *stu-miR827-3p*, *stu-miR172b-5p*, *stu-miR6022-p3_7*, *stu-miR398a-5p*, and *stu-miR166c-5p_L-3* were all found in Yanshu4 and Atlantic, seedling and budding stage, root and leaf with differential expression in response to N stress. The most significant differences of miRNAs were *stu-miR396-5p*, *stu-miR8036-3p* and *stu-miR482a-3p*. *stu-miR396-5p*, and they were down-regulated in seedling leaves and roots and in budding leaves and roots in Atlantic under

both N stresses; *stu-miR482a-3p* was down-regulated in seedling leaves and roots and up-regulated in budding leaves and roots; *stu-miR482a-3p* was up-regulated in seedling leaves and roots and in budding leaves. The expression of *stu-miR482a-3p* was down-regulated in seedling leaves and roots and up-regulated in leaves and roots at budding stage; *stu-miR8036-3p* was up-regulated in seedling leaves and roots and in leaves and roots at budding stage. Previously, it was found that NiR activity in leaves and roots of Yanshu4 and Atlantic showed positive correlations at seedling and budding stages under no and excessive N application, and both were highly responsive to N stress. The qRT-PCR validation revealed that *NiR* was positively correlated with the expression of both Yanyan4 and Atlantic in seedling and budding, root and leaf, and *stu-miR396-5p* was negatively correlated with the expression of both Yanyan4 and Atlantic in seedling and budding, root and leaf from no N application to excessive N application, and the difference in expression was highly significant. The splicing relationship between *StNiR* and *stu-miR396-5p* was found using online software prediction, and it was verified that the two were indeed cut relation by luciferase assay.

Materials And Methods

Plant material and treatments

The tetraploid cultivar potato N-efficient variety Yanshu4 (*Solanum tuberosum* L. var. *Yanshu4*) (Y) and nitrogen-inefficient varieties Atlantic (*Solanum tuberosum* L. var. *Atlantic*) (D) as test material. Courtesy of the Potato Innovation Team, Jilin Agricultural University. Seed potatoes were cut and treated for potting and set up for two N stresses, with the main source of N fertiliser being urea (containing 46% N) unapplied N:0kg/667m^2 and overapplied N: 25kg/667m^2. A uniform application of calcium superphosphate (containing P_2O_5 46%) at a rate of 18kg/667m^2 and potassium sulphate (containing K_2O 50%) at a rate of 36kg/667m^2 was made, all fertilisers were applied at once as a base fertiliser,10 pots were planted in each treatment and three biological replicates were set up. Roots and leaves were collected at seedling stage (S) and Budding stage (A), snap-frozen in liquid nitrogen, stored at -80℃and sent to Biotechnology Ltd. for miRNA sequencing and degradome analysis and qRT-PCR validation.

Construction of miRNA and degradation libraries

The miRNA library was constructed according to the standard construction steps provided by Illumina, including library preparation and sequencing experiments. miRNA Preparation of sequencing library TruSeq Small RNA Sample Prep Kits (Illumina, San DieGO, USA) Kit.

The end repair technology was used to construct the degradation group library, 5'adaptorConnection,3'Adaptor Connection, reverse transcription, PCR Short amplification, enzyme digestion,3 'Double chain DNA Joint connection and PCR long amplification[45]. The library construction was carried out on the basis of the Axtel1[46] degradome library construction, which was further optimised and simplified by the introduction of Beads screening. After library preparation, the constructed libraries were sequenced using the Illumina Hiseq2000/2500 at a single-end read length of 1 x 50 bp.

Analysis of miRNAs and prediction of target genes

Through a series of data processing, the original data obtained from sequencing can be used to comparable pairs of sequencing sequences for subsequent analysis. Re-aligning sequences with sequencing species cDNA database sequence alignment generates degradation group density file (degradome density file). Then through the shear site prediction software (GSTAr) Predicting and sequencing species miRNA sequence pairing of target gene sequences. Finally, the predicted miRNAs and their corresponding target genes were combined with the target genes in the generated degradomic data to find common target genes.

Degradation group was analyzed by Cleave Land Program[47], Application OliGO map the short reading frame calibrator finds out the target genes matching the degradation group sequence[48]. Standard sequences of value to the degradome sequences were compared in the database at NRPM (reads per million) to remove redundancy. The OliGO map was again applied to extract 13 sequences upstream and 13 sequences downstream of the paired site for each accurately paired degradome sequence to form a 26nt target gene, and the Needle program in the EMBOSS package was applied to derive all sequences that matched the sequences in the provided miRNA library, then Columns were scored against the plant miRNAs/target pairing criteria. The score should not exceed a user-set threshold and should retain nucleotides 10 at the 5' end of the degradome sequence paired with the miRNA.

miRNA identification and predicted expression profiles

The expression profile information of miRNAs was identified using a normalization method. First find a regular sequence in all samples, construct a reference data family, then do a logarithmic transformation log2(copy#) with base 2 on the copy number for all samples and the reference data family, then calculate the difference Δlog2(copy#) between log2(copy#) of the respective samples and the reference data family, select the |Δlog2(copy#)|<2 sequence, and finally the correction factor algorithm fi = 2Δyi for sample i; the number of copies in each sample is corrected by multiplying the original number of copies by the algorithm correction factor fi.

Identification and functional annotation of target genes

Predicted target genes were mapped to the potato genome GDDH13 Version 1.1(https://iris.angers. inra. fr/GDDH13/the-apple-genome-downloads. html) using HISAT2 (Johns Hopkins University Center for Computational Biology, Baltimore, MD, USA) differently expressed genes with |log2(fold change)| ≥ 1 and statistically significant (P < 0.05) were selected using the R package BallGOwn.

Based on the GO database (http://www.gene ontology.org/) and the KEGG database (http://www.genome.jp/KEGG/). The enrichment analysis consists of two main parts, one for GO functional annotation and one for KEGG Pathway functional annotation. First count the number of genes per function or per pathway for all target gene annotations corresponding to the selected miRNA, and then a hypergeometric test was applied to find the number of genes corresponding to GO or KEGG pathway in the annotation library (all genes with functional annotation, or all genes with functional

annotation of The p-value ≤ 0.05 was calculated as the threshold value, and functions satisfying this condition were defined as those that were significantly enriched in miRNAs-mRNA pairs. Functional significance enrichment analysis was able to identify the main biological functions of miRNAs-mRNA relationships.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis

Upstream primers for miRNAs were designed using miRNAs Design V1.01 (see Table 5 in Appendix A for details) with the universal reverse transcription primer: 3'– CAGCATAGGTCACGTCCCAGGCTCCAT AAGCGTGACCTATGCTGTTCAAG – 5' and the universal downstream primer: 5'– AGT GCAGGGTCCGAGGTATT – 3'. The first strand cDNA synthesis of stem-loop miRNAs was performed using MR101 kit (Vazyme Biotech Co., Ltd). The reaction system is 2μl 5×gDNA Wiper Mix, RNase-free ddH_2O to 10μl, and mix by pipette. React at 42°C for 2 min to remove genomic DNA. After the reaction, 1μl Stem-loop Primer (2μM), 2μl 10×RT Mix,2μl HiScript Enzyme Mix, and finally make up 20μl with RNase-free ddH_2O. After mixing, proceed as follows: 25°C for 5min, 50°C for 15min,85°C for 5min. The synthesis of the first cDNA strand is completed. The selected internal reference gene was elf1-α, and qRT-PCR was performed using VAZYME (Nanjing) MQ101-01 kit. For miRNAs, expression levels were detected by stem-loop RT-PCR using miRNA- specific stem-loop primers. Prepare the following mix in a qPCR tube: 10.0 μl 2 × miRNAs Universal SYBR qPCR Master Mix,0.4 μl Specific Primer (10 μM),0.4 μl mQ Primer R (10 μM),1 μl Template DNA/cDNA with RNase-free ddH_2O. Make up to 20.0 μl. The qRT-PCR reaction was performed in three biological replicates: 5 min at 95°C for 40 cycles of 10 s at 95°C and 30 s at 60°C. Melting curve analysis was performed to determine product specificity. Finally, the fold change in miRNA expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method.

The miRNA data were uploaded to the GEO database (GEO: GSE199457).

Analysis of luciferase binding site detection

We used histoculture seedlings of the nitrogen-efficient variety Yanshu4 and the nitrogen-inefficient potato Atlantic, extracted total RNA, obtained the first clone of the *StNiR* sequence by PCR amplification (see Table 6 in Appendix A, Appendix C and Appendix D for details) and predicted its binding site to *stu-miR396-5p* via the miranda online website.

The pCAMBIA1300-LUC-NiR and pCAMBIA1300-35S-396-5p expression vectors were constructed (see Appendix C for details) and transformed into Agrobacterium strains. 2–4 week old tobacco plants were selected for luciferase assay using the Dual-Luciferase Assay System, Promega Inc. The luciferase activity was analysed on a Promega luminescence detector. A total of four test groups were set up, namely test group 1: NiR-luc bound to miR396-5p, test group 2: NiR-luc bound to 1300-35S-X, test group 3:1300-luc bound to NiR-luc and test group 4:1300-luc bound to 1300-35S-X, where 1300-35S-X was the control for miR396-5p and 1300-luc was a control for NiR-luc. Finally, according to the fluorescence value measured by the dual reporter system, the fluorescence value of the target plasmid/control plasmid (that

is, the F/R value) is calculated and the ratio relative to the control is calculated, and the significance analysis and bar graph display are performed.

Declarations

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Authors' contributions

The original idea was conceived by Han Yuzhu. Zhang Jingying designed the experimental protocol. Lu Yue obtained plant samples and extracted RNA samples for small RNA sequencing. Han Zhijun compiled the data. Han Zhongcai planted experimental materials and assisted in obtaining samples, while Li Shuang, Zhang Jiayue and Ma Haoran carried out the experimental work. Lu Yue, Zhang Jingying wrote the manuscript. All authors read, edited, and approved the final manuscript.

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Availability of data and materials

Experimental materials Yanshu4 (Y) and Atlantic (D) were obtained from Jilin Provincial Vegetable and Flower Research Institute and we have got the permission to use. Experimental research and field studies comply with Jilin Agricultural University guidelines. The data content has been submitted to GEO database on 2022.3.25, and will be publicly released on 2024.2.28. The following links allow you to view the uploaded data:

To review GEO accession GSE199457:

Go to https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE199457.

Enter token unedcscqzpahxet into the box.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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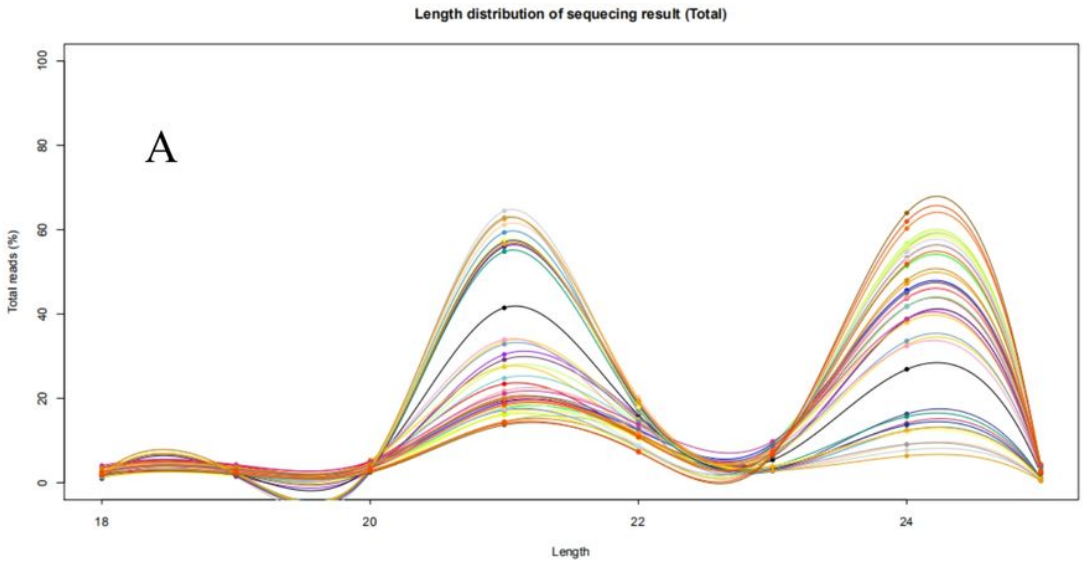
Figures

Fig. 1

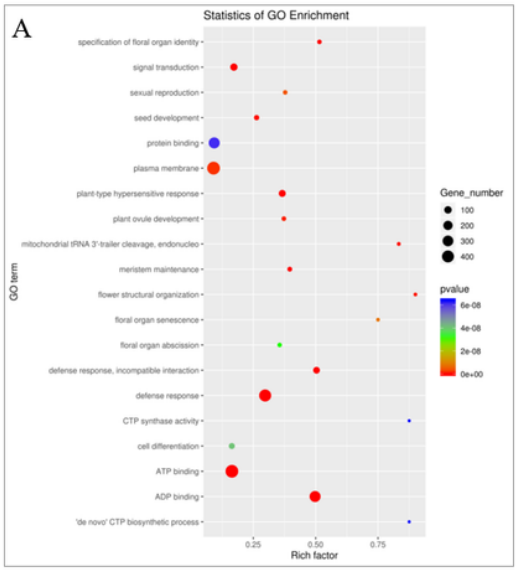
A



Length distribution of miRNAs

Figure 1

Length distribution of miRNAs



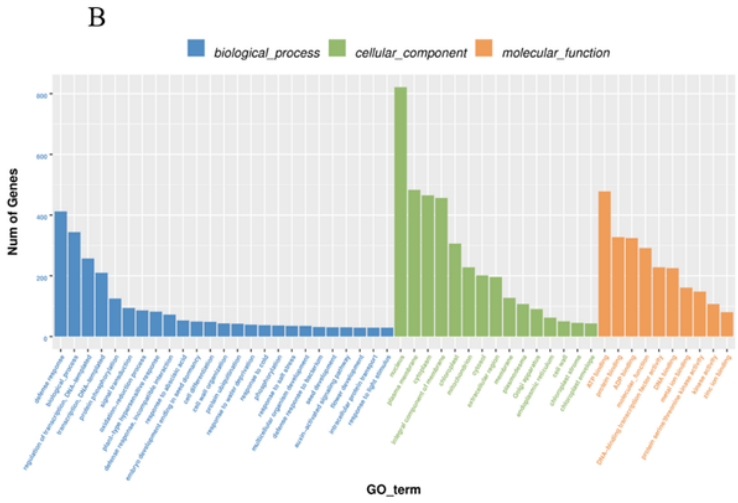


Figure 2

GO enrichment analysis of target genes corresponding to differential miRNAs

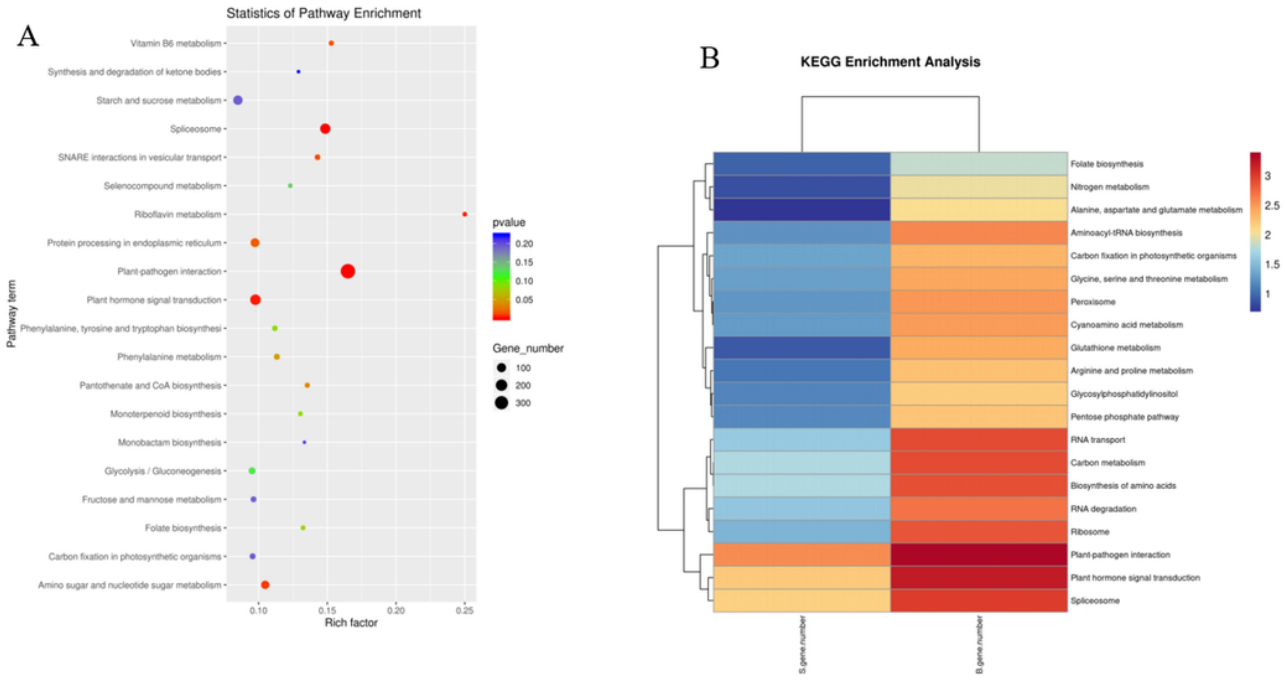


Figure 3

Differences miRNAs corresponding target gene KEGG enrichment analysis

number is the number of genes matched to a single KEGG with significant differences, B gene number is the number of genes matched to a single KEGG

Figure 4

Expression of differential miRNAs under no N application treatment

HN: excess N application; LN: no N application; Y: Yanshu4; D: Atlantic; S: seedling stage; A: budding stage

Figure 5

Expression of differential miRNAs under excessive N application treatment

HN: excess N application; LN: no N application; Y: Yanshu4; D: Atlantic; S: seedling stage; A: budding stage

Figure 6

Expression of differential miRNAs in Atlantic

HN: excess N application; LN: no N application; Y: Yanshu4; D: Atlantic; S: seedling stage; A: budding stage

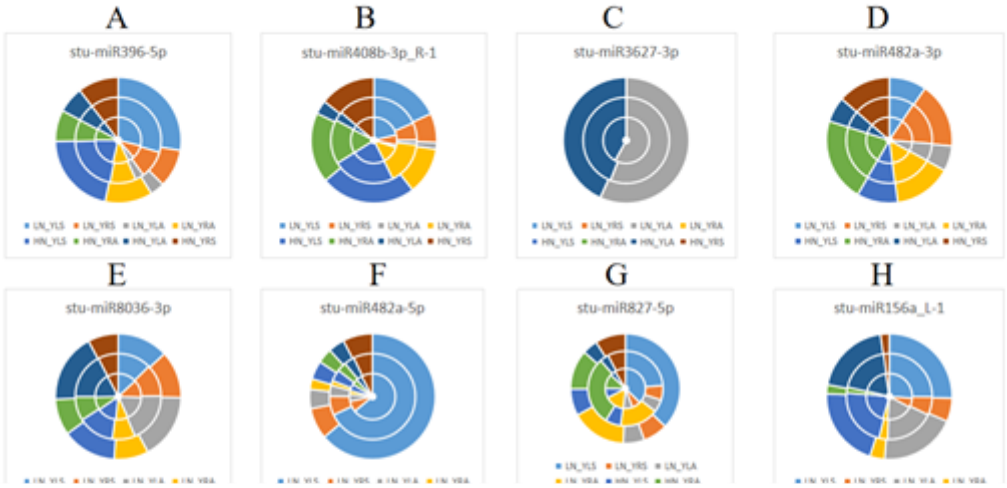


Figure 7

Expression of differential miRNAs in Yanshu4

HN: excess N application; LN: no N application; Y: Yanshu4; D: Atlantic; S: seedling stage; A: budding stage

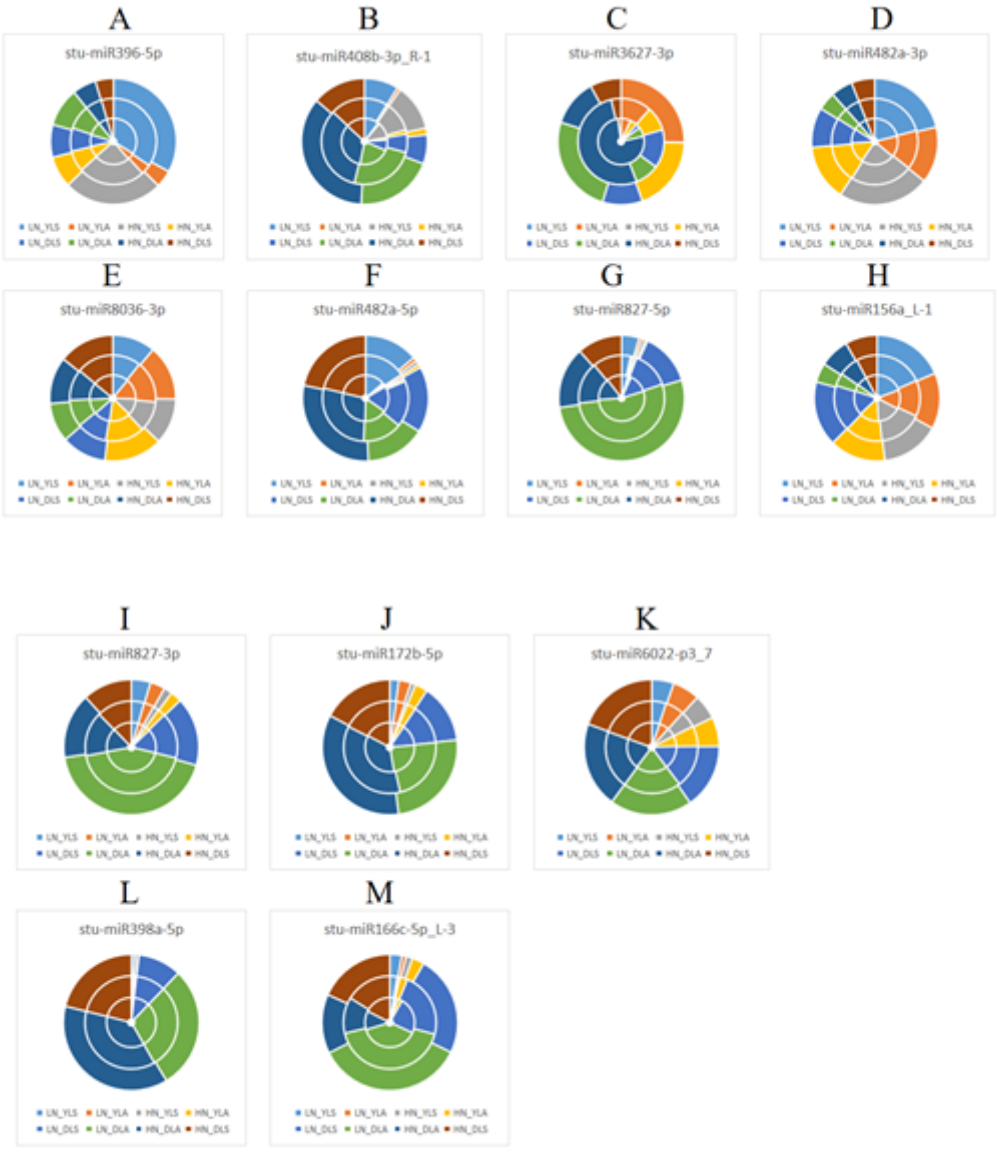


Figure 8

Expression of differential miRNAs in leaves of two potato species

HN: excess N application; LN:no N application; Y: Yanshu4; D: Atlantic; S: seedling stage; A: budding stage

Figure 9

Expression of differential miRNAs in the roots of two potato species

HN: excess N application; LN: no N application; Y: Yanshu4; D: Atlantic; S: seedling stage; A: budding stage

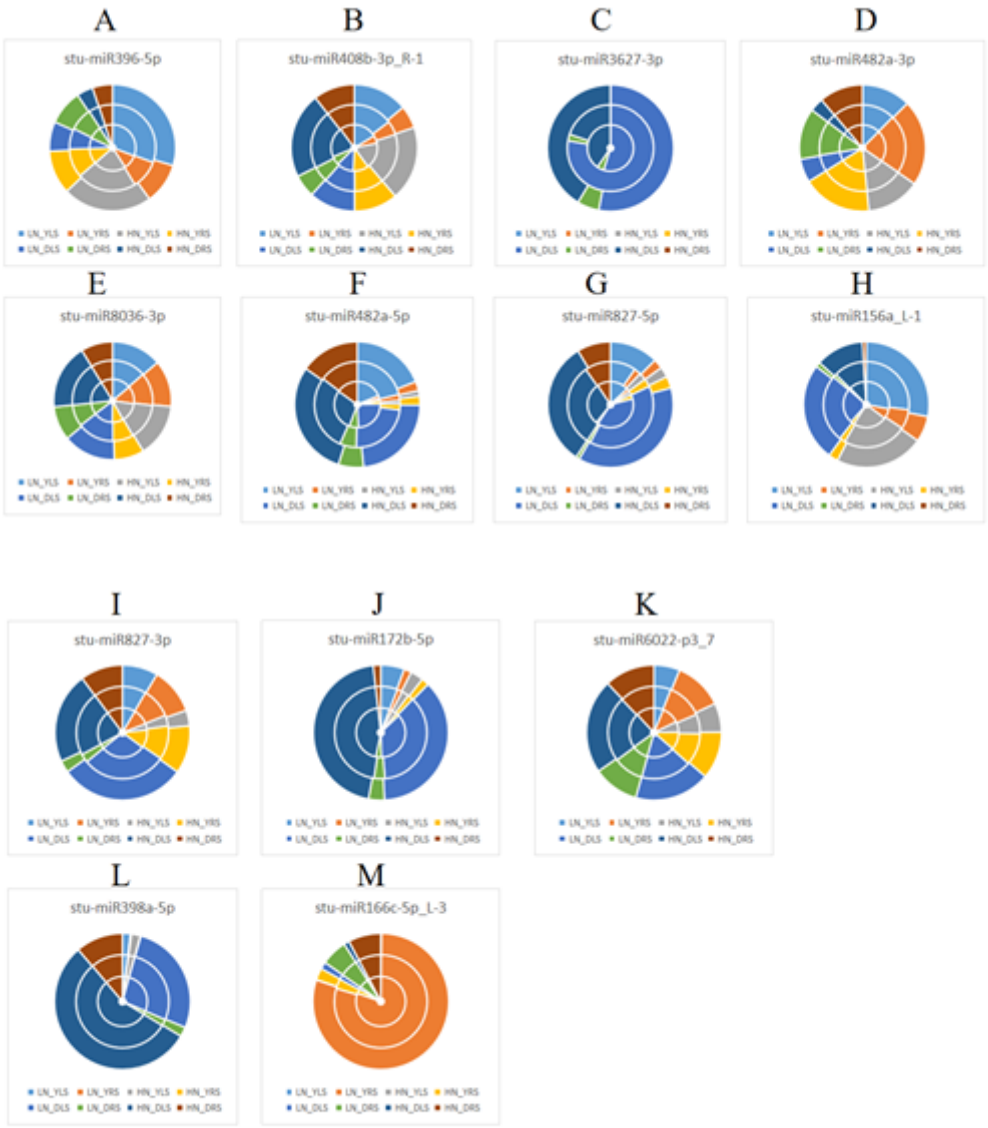


Figure 10

Expression of differential miRNAs at seedling stage in two potato species

HN: excess N application; LN: no N application; Y: Yanshu4; D: Atlantic; S: seedling stage; A: budding stage

Figure 11

Expression of differential miRNAs in two potatoes at budding stage

HN: excess N application; LN: no N application; Y: Yanshu4; D: Atlantic; S: seedling stage; A: budding stage

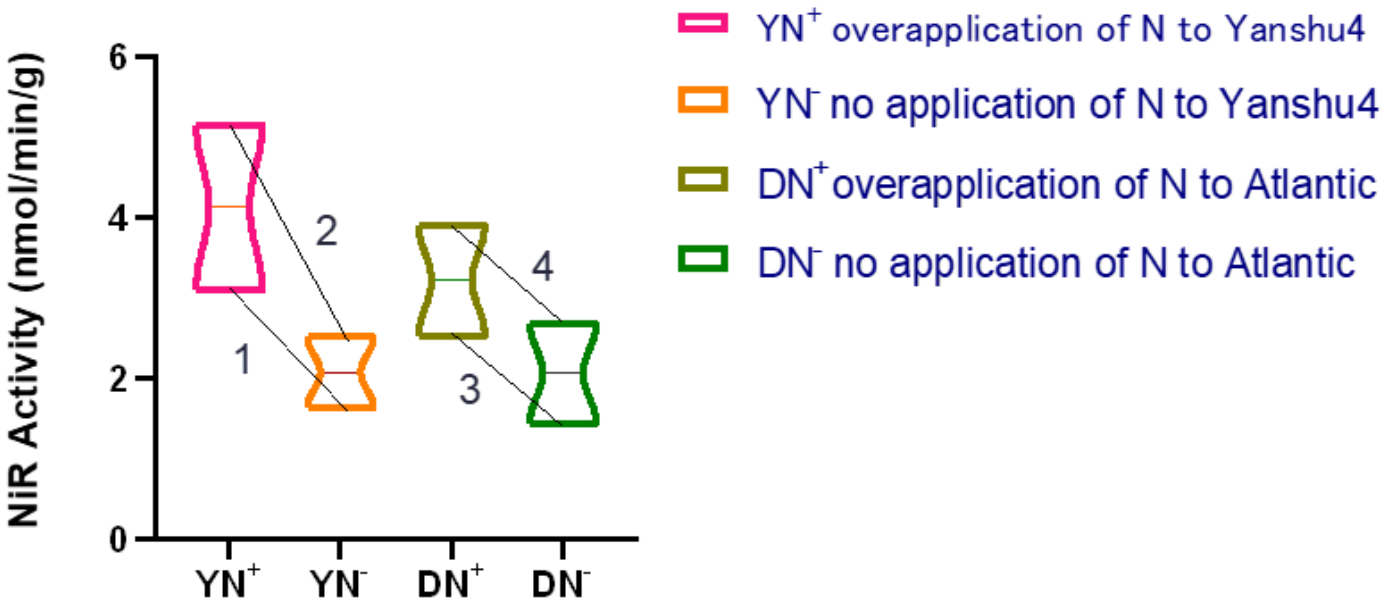


Figure 12

Analysis of nitrite reductase (NiR) activity of N-efficient and N-inefficient potatoes under N stress

1 and 3 seedling stage, 2 and 4 budding stage

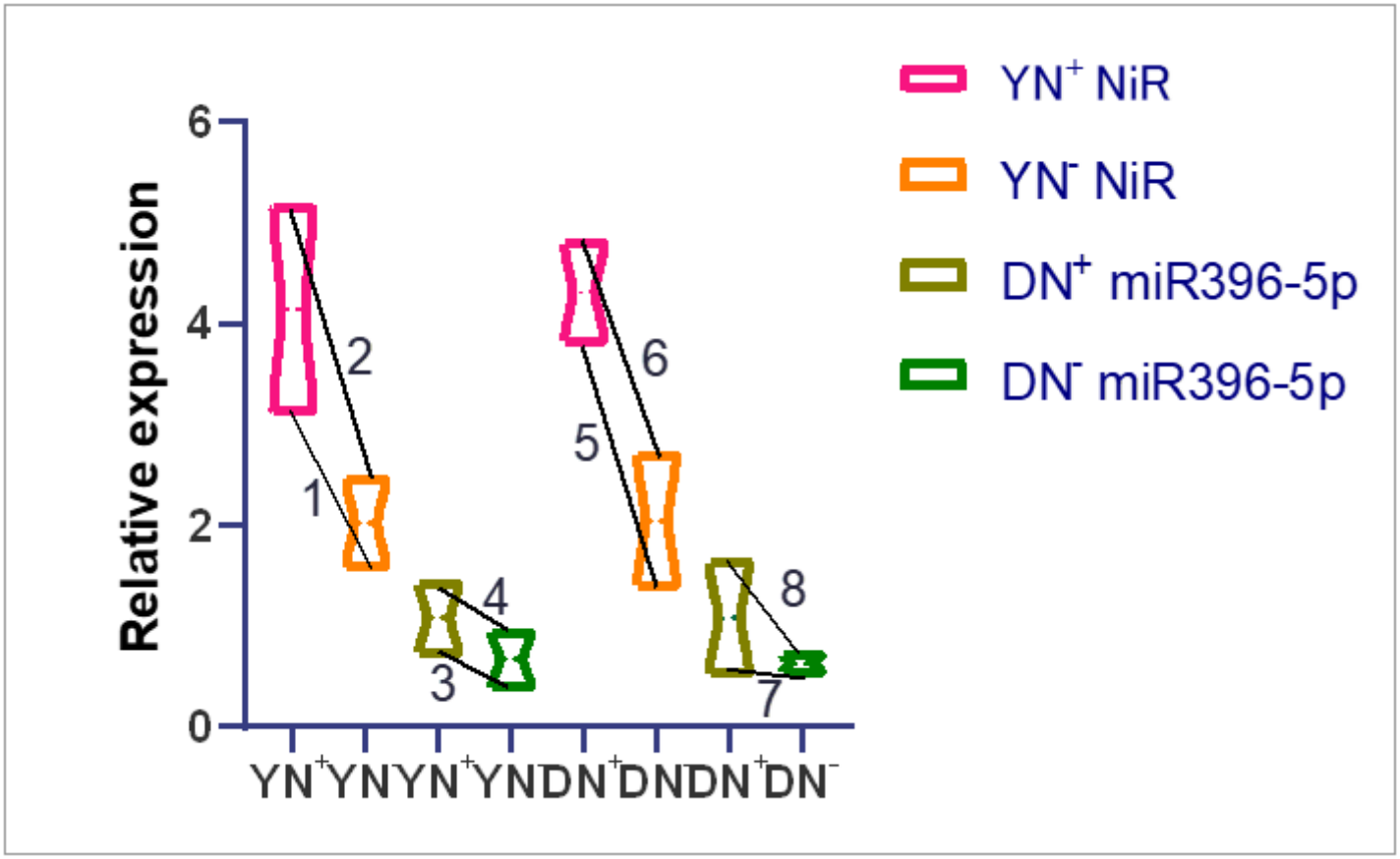


Figure 13

Relative expression analysis of N-efficient Yanshu4 and N-inefficient Atlantic nitrite reductase (NiR) and miR396-5p under N stress

YN^+: N overapplication in Yanshu4; YN^-: N unapplication in Yanshu4; DN^+: N overapplication in Atlantic; DN^-: N unapplication in Atlantic; 1, 4, 5, 8 seedling stage; 2, 3, 6, 7 budding stage

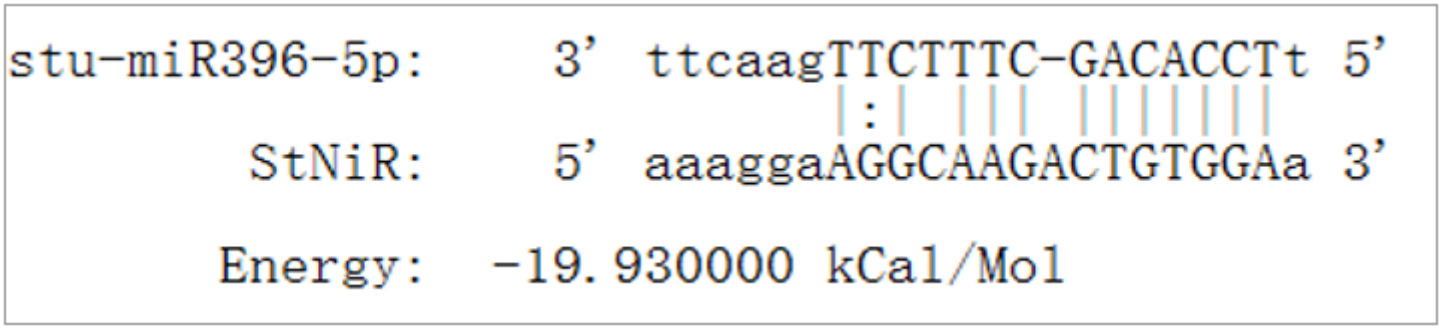


Figure 14

stu-miR396-5p predicted to bind to the *StNiR* binding site

Energy is the energy threshold, which is negative and gets tighter as it gets smaller this site has Energy= -1.

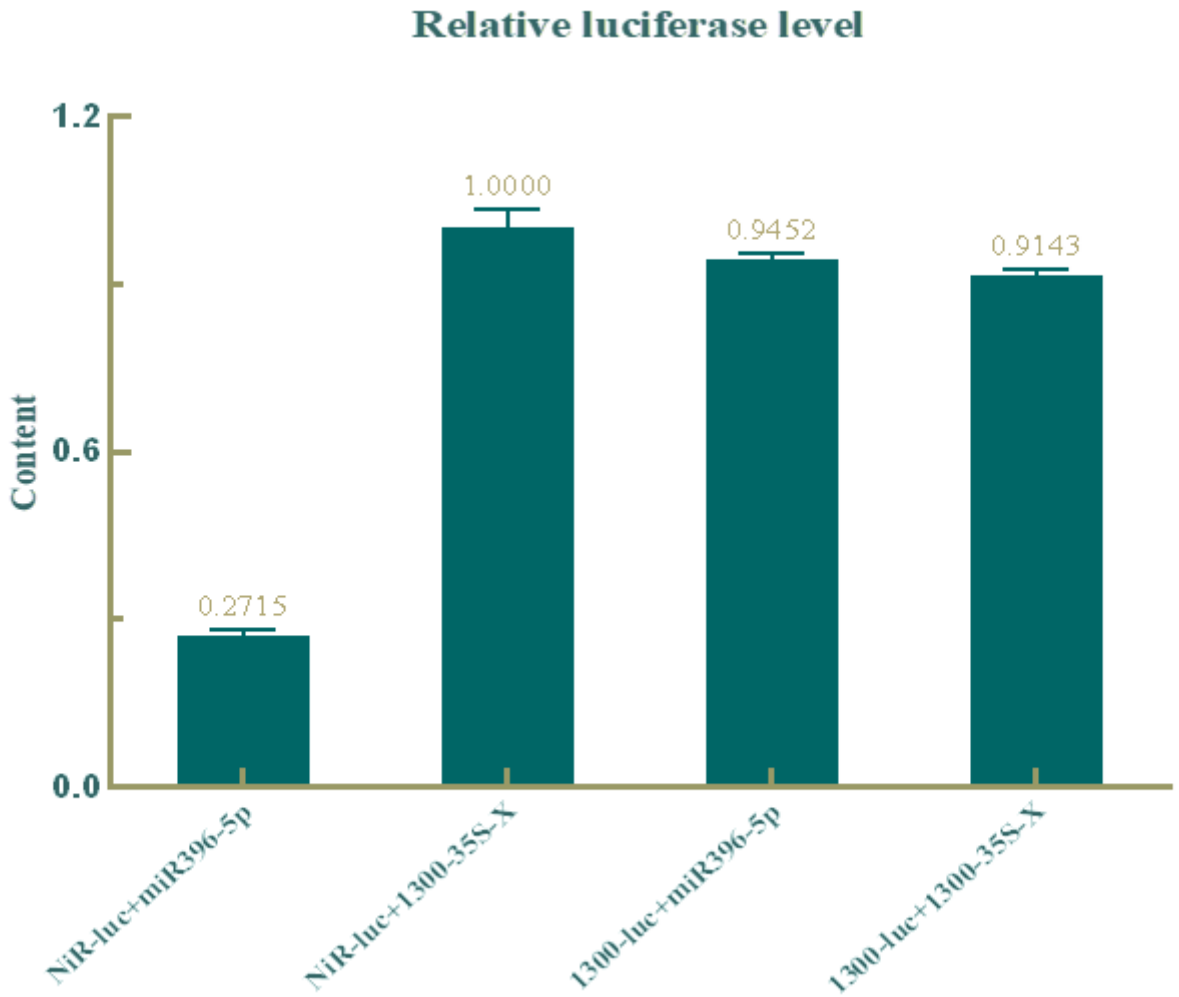


Figure 15

Validation of *stu-miR396-5p* binding site to *StNiR*

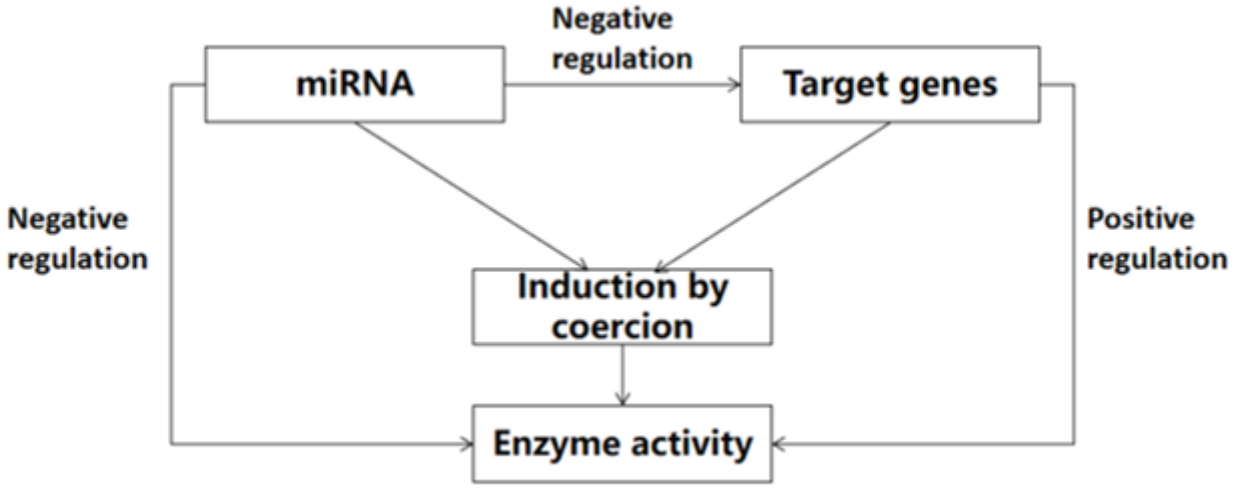


Figure 16

miRNA and target gene regulatory strategies

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Appendix.docx