ABRATES

Journal of Seed Science

ISSN 2317-1545
www.abrates.org.br/revista

ARTICLE

Journal of Seed Science, v.46, e202446024, 2024

http://dx.doi.org/10.1590/2317-1545v46282173

CqNLP1 promotes *Chenopodium quinoa* Willd. seed germination regulated by NO_3^-

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ABSTRACT: Quinoa (*Chenopodium quinoa*) has earned special attention worldwide due to its higher nutritional value and its adaptive ability to contrasting environments. Here, it was explored how quinoa seed germination is regulated. *CqNLP1* gene was identified and cloned due to its higher expression level in quinoa seeds. The role of *CqNLP1* in seed germination was studied based on model species as *Arabidopsis* sp. The function of NO_3^- during seed germination of quinoa was analyzed. The results showed *CqNLP1* gene can restore the germination rate of Arabidopsis mutant *nlp8-1* and *nlp8-2* strains, suggesting that *CqNLP1* gene plays an important role in promoting seed germination. Appropriate level of NO_3^- could improve the germination rate of quinoa seeds, promote the decomposition and utilization of soluble protein and ABA, increase the expression level of *CqNLP1* and *CYP707A2* during germination. The optimal NO_3^- concentration to promote seed germination is 1mM.

Index terms: ABA, *CYP707A2*, mutant strain, quinoa, soluble protein.

RESUMO: A quinoa (*Chenopodium quinoa*) tem ganhado atenção especial em todo o mundo devido ao seu maior valor nutricional e à sua capacidade de adaptação a diferentes ambientes. Nesse estudo, foi explorado como a germinação das sementes de quinoa é regulada. O gene *CqNLP1* foi identificado e clonado devido ao seu maior nível de expressão em sementes de quinoa. O papel do *CqNLP1* na germinação de sementes foi estudado com base em espécies modelo como *Arabidopsis* sp. A função do NO_3^- durante a germinação de sementes de quinoa foi analisada. Os resultados mostraram que o gene *CqNLP1* pode restaurar a taxa de germinação das cepas mutantes *nlp8-1* e *nlp8-2* de *Arabidopsis*, sugerindo que o gene *CqNLP1* desempenha um papel importante na promoção da germinação de sementes. O nível apropriado de NO_3^- poderia melhorar a taxa de germinação das sementes de quinoa, promover a decomposição e utilização de proteína solúvel e ABA, aumentar o nível de expressão de *CqNLP1* e *CYP707A2* durante a germinação. A concentração ideal de NO_3^- para promover a germinação das sementes é de 1 mM.

Termos de indexação: ABA, *CYP707A2*, cepa mutante, quinoa, proteína solúvel.

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Received: 01/11/2024.
Accepted: 07/15/2024.

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INTRODUCTION

Chenopodium is a genus of plants in the Amaranthaceae family and Chenopodaceae subfamily, it was originated in the Andes Mountains and has been cultivated for 7,000 years. Today, quinoa has earned special attention worldwide due to its higher nutritional value and its ability to adapt to contrasting environments (Hinojosa et al., 2018).

Seed germination is the first developmental process in plant life cycle and plays a crucial role in crop production and quality. Hao et al. (2022) demonstrated a transcriptomic and metabolomic landscape of quinoa during seed germination in a general way. In recent years, several studies have contributed to shedding light on the molecular networks underlying seed germination processes with model plants as Arabidopsis (Yan et al., 2016; Gu et al., 2019; Chen et al., 2020; Bai et al., 2020). However, the regulation of quinoa seed germination is largely unknown.

Seed germination is influenced by various environmental cues. In soil, NO_3^- is one of the main nitrogen sources for plants, and it is also an important signal molecule involved in seed dormancy interruption (Alboresi et al., 2005). A transcriptome analysis revealed that the environmental factors regulate a common downstream action related to seed dormancy interruption where abscisic acid (ABA) plays a key role (Finch-Savage et al., 2007; Glison et al., 2017). NODULE INCEPTION (NIN)-like protein, namely, NLP transcription factor actively participate in nitrogen response of plants (Konishi and Yanagisawa, 2011). In *Arabidopsis thaliana*, NLP8, namely AtNLP8 has proven to be a major regulator of nitrate-promoting seed germination, binding directly to the promoter of the *CYP707A2*, and reducing ABA levels (Yan et al., 2016). Considering the potential presented by quinoa and the benefits of seed germination for the production of offspring, it would be useful to explore how quinoa seed germination is regulated.

Herein, based on the *CqNLPs* (*NLP* genes in *Chenopodium quinoa*) expression profile in different quinoa tissues, we cloned *CqNLP1*, the homolog of *AtNLP8* genes and explored its function in quinoa seed germination.

MATERIAL AND METHODS

Arabidopsis (Col-0) seeds were available at the laboratory stock and quinoa line ZK7 (*Chenopodium quinoa*. ZK7) was provided by the College of Agronomy of Shanxi Agricultural University, China. *Arabidopsis thaliana* T-DNA insertion mutants *nlp8-1* (SALK_031064) were obtained from the AraShare (http://www.arashare.cn) and *nlp8-2* (SALK_025839) (Yan et al., 2016) was gifted by Professor Eiji Nambara, University of Toronto, Canada (all in Col-0 background, simply referred to by their gene abbreviations henceforth). The binary expression vector pNC-Cam2304-MCS35S containing *GUS* reporter gene (Figure 1) was provided by Yan Pu, Institute of Tropical Biotechnology, Chinese Academy of Tropical Agricultural Sciences, China.

CqNLPs expression profile analysis

Using the RNA-seq data (SRP226463, SRP116149) of quinoa which were downloaded from the SRA website (https://www.ncbi.nlm.nih.gov/sra/). We analyzed the expression profile of 9 *CqNLPs* (Zhu et al., 2021) in root, stem, leaf, flower and seed. Gene expression level was calculated as $\log_2$FPKM (fragments per kilobase of transcript per million reads mapped), and the expression heat map of *NLPs* gene was plotted by TBtools software.

RNA extraction and qRT-PCR analysis

Expression pattern of *CqNLP1* genes and *CYP707A2* was validated by qRT-PCR analysis with three biological replicates. Total RNA was extracted by the Trizol method (EasyPure® Plant RNA Kit), and RNA quality was detected by a micronucleic acid protein analyzer (scandrop100). The first strand of cDNA was synthesized using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix. Relative quantification of gene expression was calculated with primers (5'-TGTTTGCCATGCTCTTGAGG-3'; 3'-AAGGCAATCTGTGTGCATGG-5' for *CqNLP1* and 5'-GTCCTGAAGCCGCAAAGATAGTT-3'; 3'-CATCTCAACTAGAGTGTTGATGGAG-5' for *CYP707A2*) and normalized using *EF1α* as an internal standard with primers 5'-GTACGCATGGGTGCTTGACAAACTC-3'; 3' - ATCAGCCTGGGAGGTACCAGTAAT -5'.

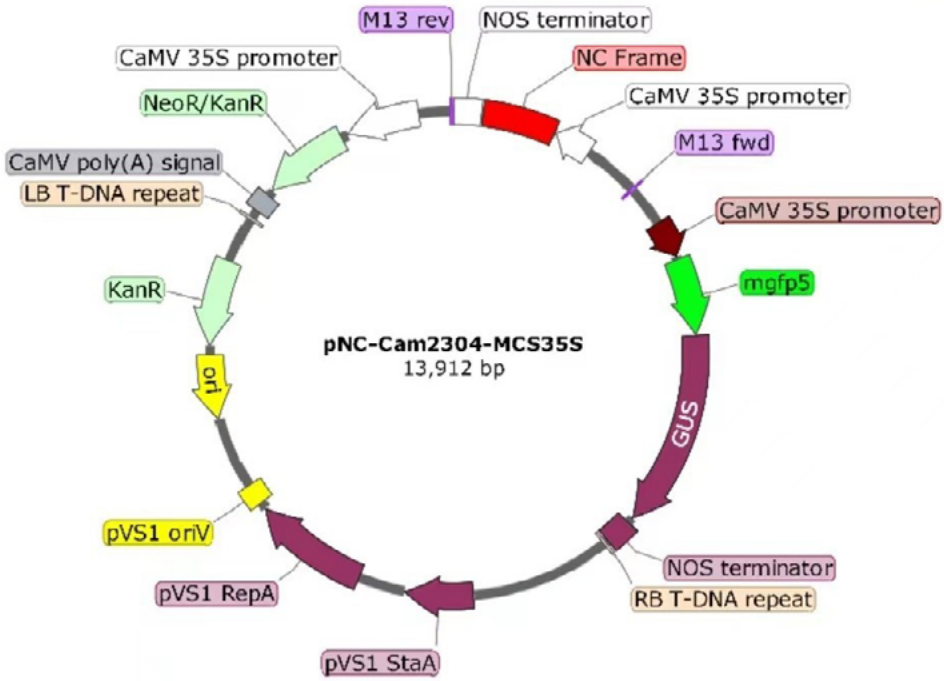

Figure 1. Map of vector pNC-Cam2304-MCS35S.

The reaction system was 10.0 µL, of which 2× SYBR Green Supermix 5.0 µL and 0.5 µL each of primers. The comparative Ct ($2^{-(\Delta\Delta Ct)}$) method was used to calculate the fold-changes in gene expression level.

CqNLP1 gene cloning

The coding sequence of the *CqNLP1* gene was obtained from the quinoa genome database (https://www.ncbi.nlm.nih.gov/nuccore/2496127099). Using the cDNA of quinoa seed as template, PCR was performed to obtain the full length of *CqNLP1* gene. the gene-specific primers (F1 5'-agtggtctctgtccagtcctATGGAATACTCCTTTTCTCCTAAGGA-3' and R1 (5'-ggtctcagcagaccacaagtCTAACAAATTCCAGGTATGAAACAACT-3') were designed according to the *CqNLP1* coding sequence (lowercase letters are splice sequences). NC Loving was used to construct the expression vector (Yan et al., 2020). A 2,808-bp PCR product was obtained and subsequently ligated into the plasmid pNC-Cam2304-MCS35S to produce the binary vector *Pro35S::CqNLP1*.

Arabidopsis mutants verification and functional reversion obtainment

CqNLP1 is the homolog of *AtNLP8* genes. Arabidopsis T-DNA insertion mutants *nlp8-1* and *nlp8-2* were adopted to explored *CqNLP1* function in seed germination. Arabidopsis mutants *nlp8-1* and *nlp8-2* were verified by PCR amplification. LP and RP are DNA-specific primers, and BP and RP are T-DNA insertion primers (Table 1). The *Pro35S::CqNLP1* construct was introduced into *nlp8-1* and *nlp8-2* mutants by the flower dip method of agrobacterium (*Agrobacterium tumefaciens*, GV3101) –mediated transformation (Clough and Bent, 1998). This generated *nlp8-1* and *nlp8-2* functional recovery lines (refer to *nlp8-1-CqNLP1* and *nlp8-2-CqNLP1* henceforth). The *nlp8-1-CqNLP1* and *nlp8-2-CqNLP1* were primarily selected on MS (Murashige and Skoog) medium containing 50 $mg.L^{-1}$ hygromycin B, and the presence of the transgene was further confirmed by GUS staining the inbred lines of the recovery lines.

Measurement of seed germination rate, soluble protein and ABA contents

Germination test

The *Arabidopsis* seeds of wild type, *nlp8-1* and *nlp8-2* mutants, *nlp8-1-CqNLP1* and *nlp8-2-CqNLP1* were used for

Table 1. *nlp8-1* and *nlp8-2* mutant identification primers.

Name	Primer name	Primer sequence
nlp8-1	LP	AGCTTGGAACAACAACTTCTAT
	RP	AGCATTAACCTCCATGAATGAT
nlp8-2	LP	TTCTGGCGTTAGGAATTTGA
	RP	ACTATAGCAATCTTGCTGGC
T-DNA	LB1.3	ATTTTGCCGATTTCGGAAC

germination test. Approximately 50 seeds were surface sterilized with 10% NaClO, rinsed thoroughly with sterilized water and sown on 10 cm petri dishes containing solid agarose culture medium. The petri dishes were then placed in a growth chamber under the conditions of 22 °C and 50% humidity. After 4 days, taking radicle protrusion as a criterion for germination, the seed germination rate was calculated (germination rate = number of germinated seeds / total seed × 100%).

Quinoa seed germination was investigated using a similar method. Approximately 50 quinoa seeds were surface sterilized with 10% NaClO, rinsed thoroughly with sterilized water and sown on 10 cm petri dishes covered with filter paper and soaked in the solutions of 1 mM of KNO_3, KCl, KH_2PO_4, K_2SO_4, or different concentrations of KNO_3 (1, 2.5, 5, 10, 20, 40, 50 mM), respectively. Plates were incubated at 27 °C, 70% humidity. The seed germination rate was counted every hour from 6 h to 12 h of incubation using sprout length reaching half of the seed length as the germination criterion, germination rate (%) = number of germinated seeds / total number of seeds × 100%.

Contents of soluble protein and ABA

The protein content in quinoa seeds was determined by Coomassie Brilliant Blue Staining method (Bradford, 1976; Sedmak and Grossberg, 1977). The seeds of different treatments of quinoa were frozen in liquid nitrogen. The content of ABA was determined by high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (HPLC-MS) and was conducted by SCI-TECH INNOVATION Co., Qingdao, China.

RESULTS

The investigation on RNA-seq data of quinoa revealed that CqNLPs presented varied expression patterns in different quinoa tissues. The expression level of *CqNLP1, CqNLP5, CqNLP6, CqNLP7* and *CqNLP8* were all higher in seeds than those in other tissues, this is especially true for *CqNLP1* (Figure 2A). qRT-PCR of *CqNLP1* further confirmed that *CqNLP1* expression level in seeds was the highest, which was 8.6, 1.8, 1.3 and 2.7 times higher than those of roots, stems, leaves and flowers, respectively (Figure 2B).

Because of the clear evidences for *CqNLP1* differential expression in different tissues, we validated its role in quinoa seed germination using Arabidopsis model. The full 2,808-bp of *CqNLP1* gene (NCBI Gene ID: MW915417) was ligated into the plasmid pNC-Cam2304-MCS35S to produce the binary vector Pro35S::*CqNLP1* (Figure 3). The Arabidopsis *nlp8-1* and *nlp8-2* mutant plants were verified by PCR and GUS staining (Figures 4A and 4B). We created recovery lines for these mutants by transforming them with the vector carrying *CqNLP1*. The growth profile of mutants and their recovery lines showed *CqNLP1* restored the leaf area, leaf number, and growth rate of the *nlp8-1* and *nlp8-2* mutant plants (Figure 4C). Further, *nlp8-1-CqNLP1* and *nlp8-2-CqNLP1* plants grew even better than the wild type. The germination rates of Arabidopsis wild type, *nlp8-1, nlp8-2* mutants and *nlp8-1-CqNLP1*, *nlp8-1-CqNLP2* were analyzed on the 4th day (Figure 5). The germination rates of *nlp8-1* and *nlp8-2* mutant were significantly smaller than WT and recovery lines, and that of recovery lines (*nlp8-1-CqNLP1* and *nlp8-1-CqNLP2*) were the highest.

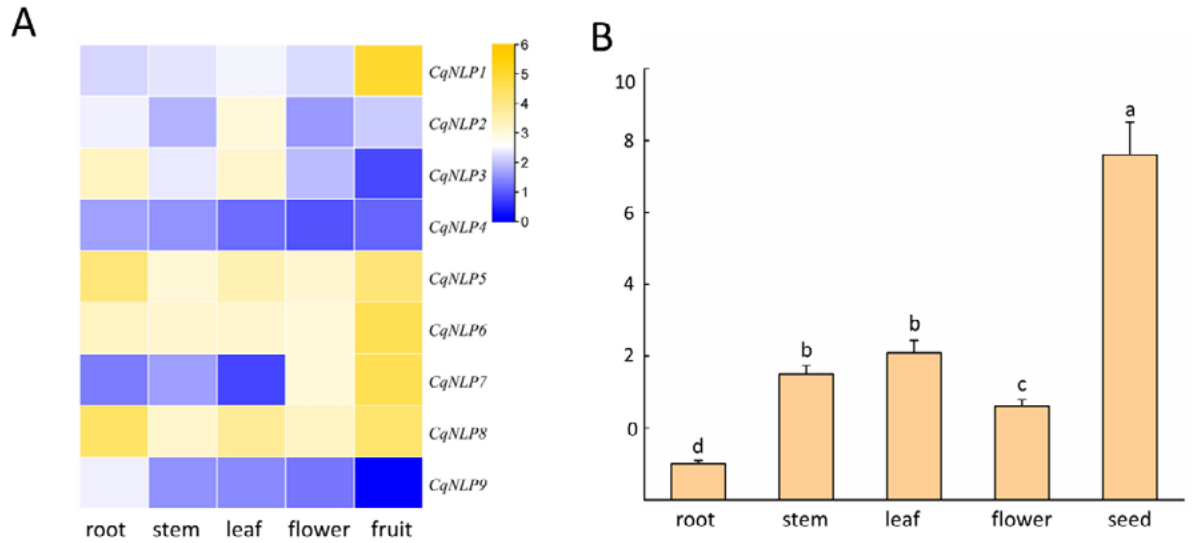

Figure 2. Expression level of *CqNLPs* in quinoa tissues. A is the heat map of *CqNLPs* expression profile in quinoa, B is the qRT-PCR results of *CqNLP1* gene. Different letters indicate significant difference among tissues at $p < 0.05$, according to Duncan's multiple range test.

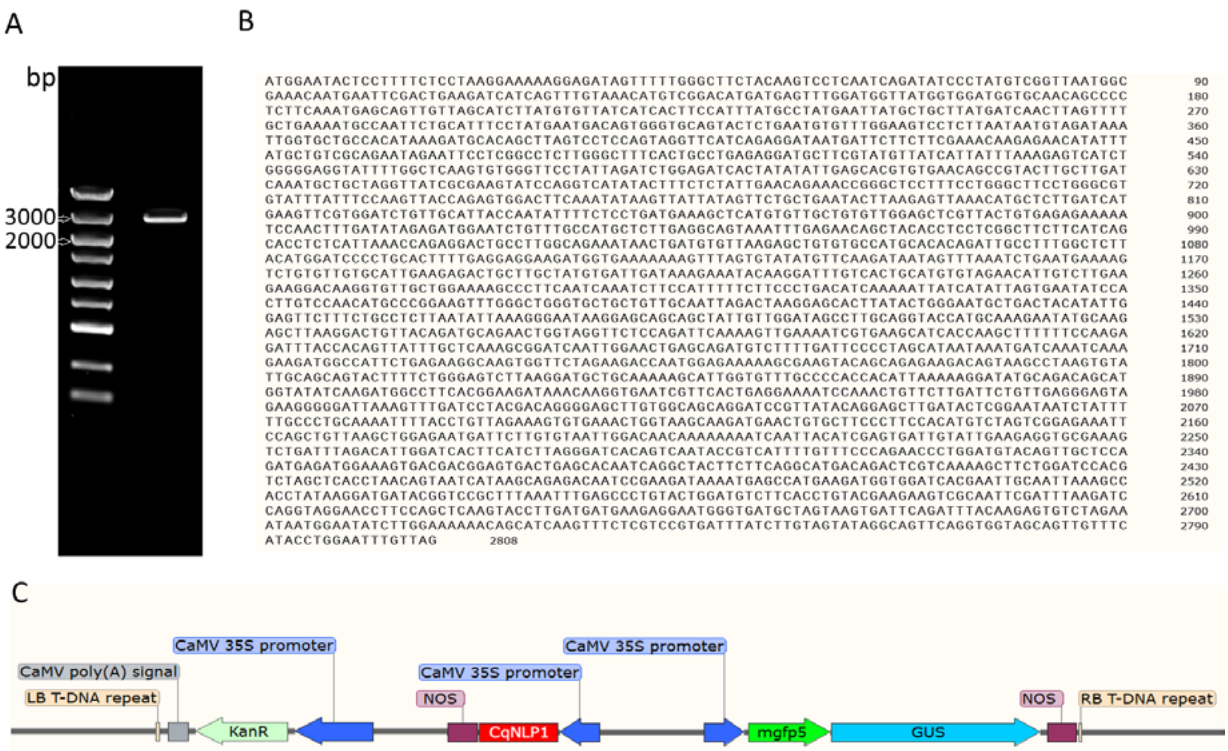

Figure 3. Binary vector construction for *CqNLP1*. A is PCR amplification of *CqNLP1*. M: 5000 bp DNA maker, B is the CDS sequence of *CqNLP1*, C is the construction of Pro35S::*CqNLP1*.

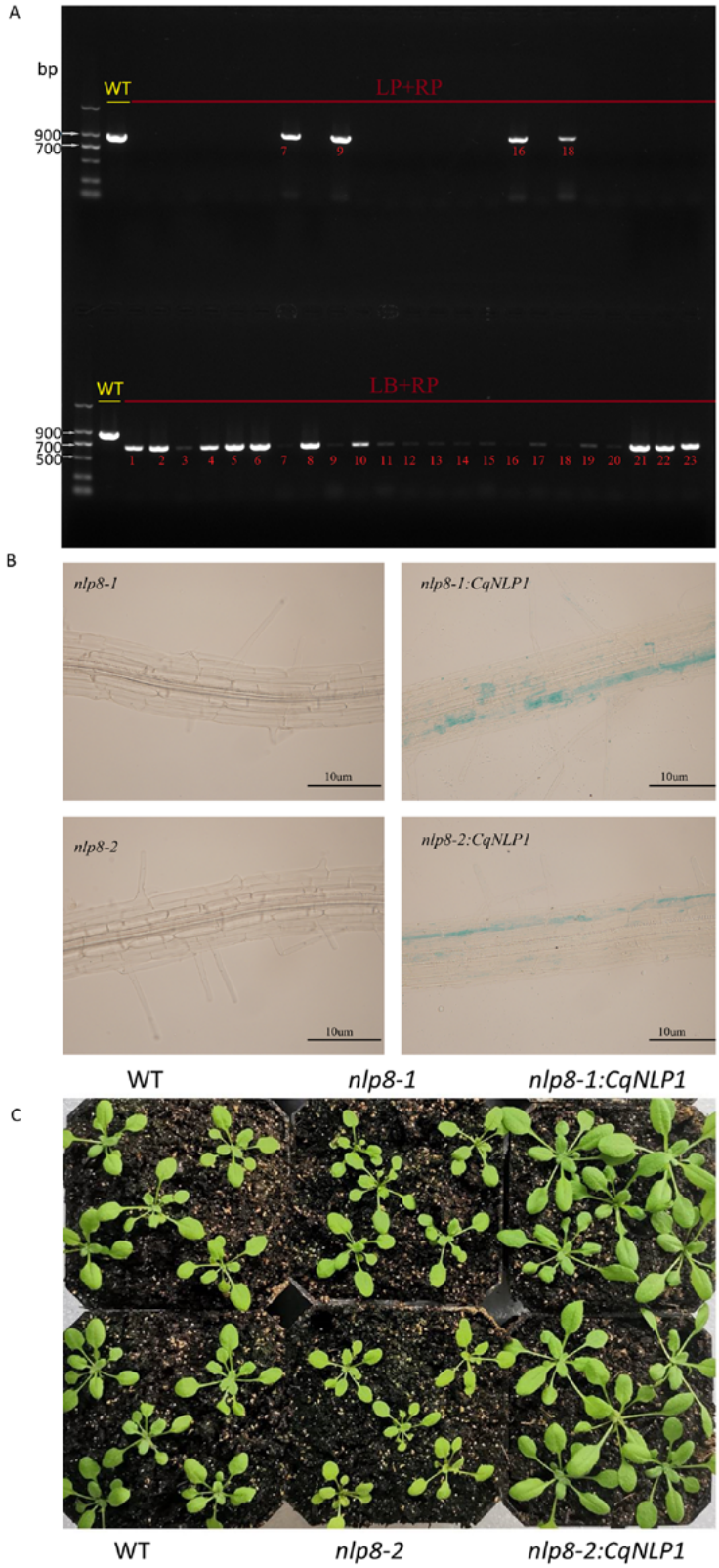

Figure 4. Nlp-2 mutant identification and recovery line selection. A is the PCR identification for mutant. WT is wild type plant, 1-23 is different mutant plants. LP: left primer, RP: right primer, LB: T-DNA left border primer. B is the GUS staining for *nlp8-1-CqNLP1, nlp8-1-CqNLP2*. C is the phenotype of WT, *nlp8-1, nlp8-2* mutants and *nlp8-1-CqNLP1, nlp8-1-CqNLP2*.

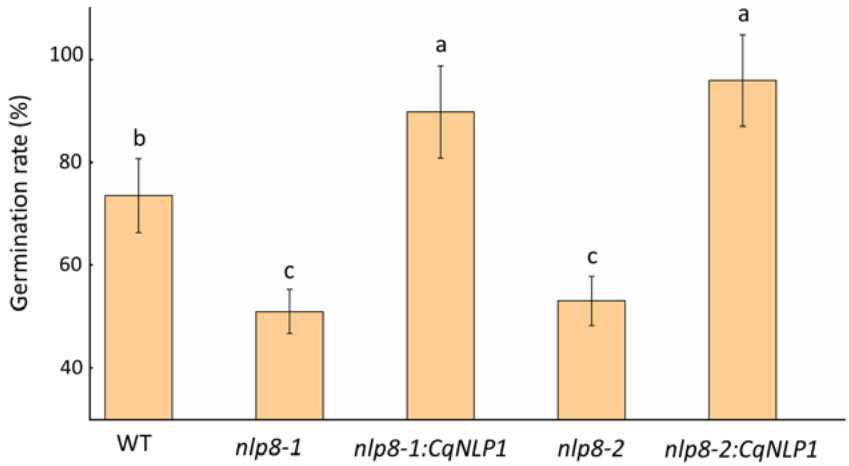

Figure 5. Seed germination rate of Arabidopsis in different lines. Different letters indicate significant difference among lines at $p<0.05$ according to Duncan's multiple range test.

Table 2. Germination rate of quinoa seeds treated with different types of potassium salt.

	Time (h)						
	6	7	8	9	10	11	12
Water	8±0.82 b	17.3±0.47 bc	35.3±0.47 b	46±0.82 b	61.3±0.47 b	65.3±0.47 bc	70±0.82 c
KNO_3	15.3±0.47 a	24±0.82 a	48±0.82 a	55.3±0.47 a	67.3±0.47 a	72.0±0.82 a	78±0.82 a
KCl	7.3±0.47 b	15.3±1.25 c	25.3±0.47 c	36±0.82 c	53.3±0.47 c	67.3±0.47 b	73.3±0.47 b
KH_2PO_4	9.3±0.47 b	19.3±0.47 b	35.3±0.94 b	43.3±0.47 b	63.3±0.47 b	64±0.82 c	72±0.8b2 c
K_2SO_4	2.7±0.47 c	11.3±0.47 d	18±0.82 d	29.3±0.94 d	53.3±0.47 c	60±0.82 d	73.3±0.47 b

Data are shown as means ± SD (n =3). Different letters in the same column indicate significant difference at $p<0.05$ according to Duncan's multiple range test. The concentration of KNO_3, KCl, KH_2PO_4 and K_2SO_4 was 1mM.

The effect of different types of potassium salt on quinoa seed germination was explored. It was showed the seed germination rate of quinoa under different treatments gradually increased after 6- to 12h of incubation, it was the highest under KNO_3 treatment at each time point that settled (Table 2). To further clarify the optimal concentration of NO_3^- for quinoa seed germination, a series KNO_3 concentrations were settled. The seed germination rate was the highest in 1 mM KNO_3 treatment except that under 2.5 mM KNO_3 treatment at 7 h (Table 3).

After 6h, 8h,10h and 13h incubation with KNO_3, soluble protein content in quinoa seeds was consistently significantly lower than that under water treatment. Under KCl treatment, soluble protein content was higher than that of water treatment at 6 h, 8 h and 13 h. After 13 h incubation with KH_2PO_4, soluble protein content was lower than that of the water treatment, while it was higher at 6 h, 8 h and 10 h. The lowest soluble protein content was presented under 1 mM KNO_3 treatment. That is, during the quinoa germination process, the soluble protein decomposition speed is faster under NO_3^- treatment and the optimal concentration for soluble protein decomposition is 1 mM (Figure 6).

Table 3. Germination rate of quinoa seeds treated with different concentrations of potassium nitrate.

	Time (h)						
	6	7	8	9	10	11	12
0 mM	8.0±0.82 c	16±0.82 b	35.3±0.94 b	46±0.82 b	61.3±0.47 b	65.3±0.94 b	70±0.82 d
1 mM	15.3±0.47 a	24±0.82 a	48±0.82 a	54.6±1.25 a	67.3±1.25 a	72±0.82 a	78±0.82 a
2.5 mM	12±1.63 b	22.7±0.94 a	38±0.82 b	46.7±0.47 b	60±0.82 b	70±0.82 a	76±0.82 b
5 mM	3.3±0.47 d	8±0.82 c	24±0.82 c	42±0.82 c	55.3±0.47 c	64±0.82 b	74±0.82 c
10 mM	4.7±0.47 d	6±0.82 c	12.7±0.94 e	29.3±1.67 e	43.3±0.94 d	51.3±0.47 d	63.3±0.47 e
20 mM	4±0.82 d	8.7±0.94 c	16.7±0.47 d	35.3±0.47 d	44±1.41 d	59.3±0.47 c	69.3±0.47 d
40 mM	4.7±0.47 d	7.3±0.47 c	12.7±1.25 e	34±0.82 d	38±0.82 e	52±0.82 d	62±0.82 e
50 mM	2.7±0.47 d	2.7±0.47 d	8±1.63 f	23.3±0.47 f	28±0.82 f	42±0.82 e	57.3±0.47 f

Data are shown as means ± SD(n =3). Different letters in the same column indicate significant difference at p<0.05 according to Duncan's multiple range test.

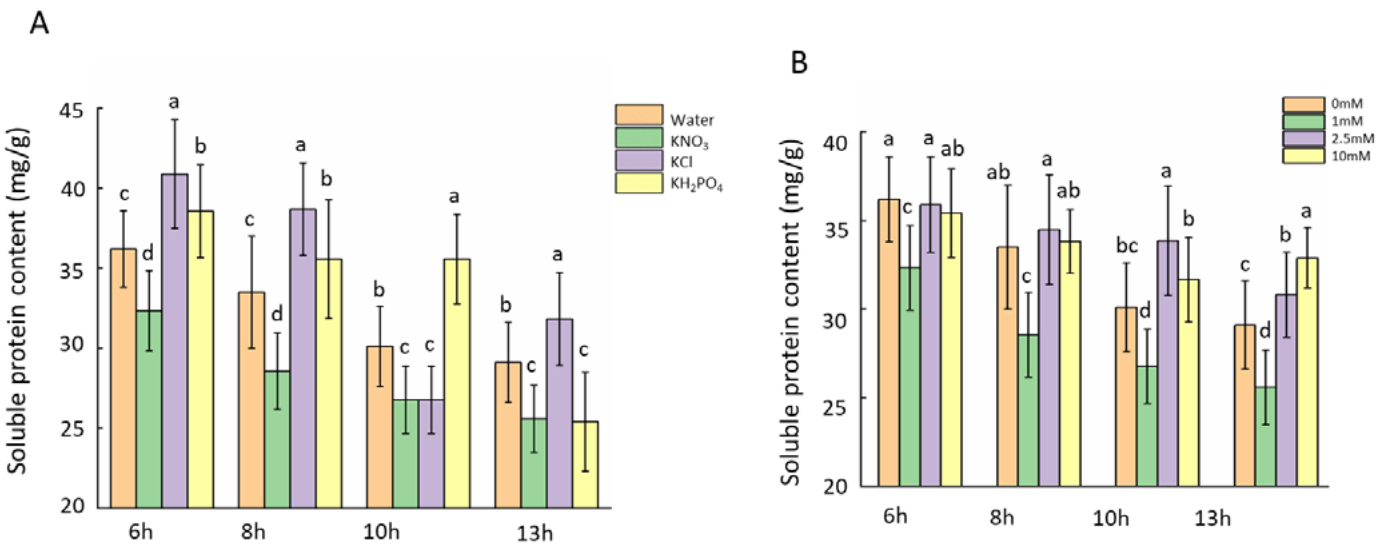

Figure 6. Contents of soluble protein in quinoa seeds. A shows soluble protein content treated with different types of potassium salt. B shows soluble protein content treated with different concentrations of potassium nitrate. Different letters indicate significant difference at the same time (p<0.05, Duncan's multiple range test).

ABA has an inhibitory effect on seed germination, so it must be degraded when germination occurs. Here, in all the treatments, ABA contents in quinoa seeds were lower under KNO_3 treatment compared with that of water treatment. It was observed that the optimal NO_3^- concentration for ABA degradation in quinoa seeds is 1 mM KNO_3 (Figure 7).

CYP707A2, coding ABA decomposing enzyme gene, plays a key role in ABA degradation. Here, after 12 h post-treatment, the expression level of *CYP707A2* was the highest under NO_3^- treatment group which was 2.3 times higher than the water treatment. It was lower in KCl, KH_2PO_4 and K_2SO_4 treatments compared with the water treatment. The concentration of 1mM NO_3^- maximized *CYP707A2* gene expression, more than twice that of water treatment (Figure 8). The expression pattern of *CqNLP1* is similar to that of *CYP707A2*.

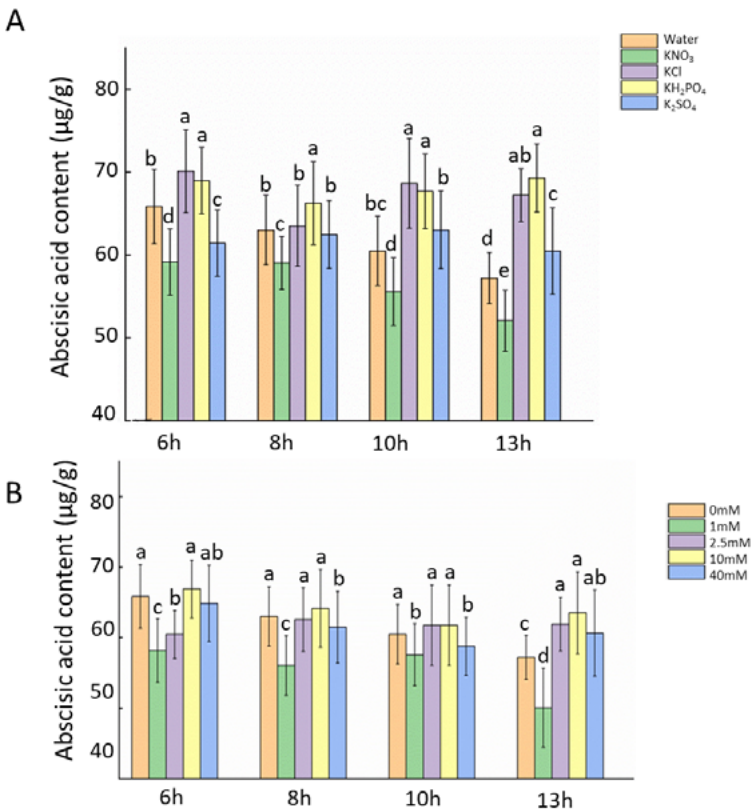

Figure 7. Contents of ABA in quinoa seeds treated with different types of potassium salts and different concentrations of potassium nitrate. A is the content of ABA quinoa seeds treated with different types of potassium salt. B is the contents of ABA of quinoa seeds treated with different concentrations of potassium nitrate. Different letters indicate significant difference at the same time ($p<0.05$, Duncan's multiple range test).

DISCUSSION

AtNLP8 gene has been confirmed to play a key role in Arabidopsis seed germination. In barley, *HvNLP2* was verified involved in nitrate signaling (Gao et al., 2022). Here, we found *CqNLP*, the homologous gene of *AtNLP8* exhibited significantly higher expression level in quinoa seeds indicating that *CqNLP1* gene might be closely connected to seed germination. It was then verified the role of *CqNLP1* in promoting seed germination by expressing it in two different *AtNLP8* mutants. The results showed that the germination rate of recovery lines was higher than others, where the presence of *CqNLP1* probably increased seed germination. It is documented that AtNLP8 binds directly to the promoter of *CYP707A2* gene and reduces ABA levels in a nitrate-dependent manner, thereby promoting seed germination (Yan et al., 2016). Sufficient studies have documented that ABA negatively regulate seed germination (Sano and Marion-Poll, 2021; Xu et al., 2021; Zhou et al., 2023). A transcriptomic study on seed germination of quinoa verified that during transition from quinoa dry seed to seedling, seed metabolism is reprogrammed with significant alteration of multiple phytohormones, especially ABA (Hao et al., 2022). Gao et al. (2023) found quinoa germination rate displayed lower when seeds treated with ABA at concentrations of 50, 100, and 200 μM. Therefore, it was speculated that the promotion function of *CqNLP1* in seed germination might related to the suppression of ABA level.

Studies have shown that NO_3^-, as a necessary signaling molecule, enables AtNLP8 directly bind to the downstream ABA- degrading enzyme gene and activate its expression, thereby reducing the content of ABA in seeds during germination and thus promoting seed germination (Yan et al., 2016). In this study, the content of soluble protein in quinoa was smaller in KNO_3 treatment and 1 mM NO_3^- is the favorable concentration for seed germination. We

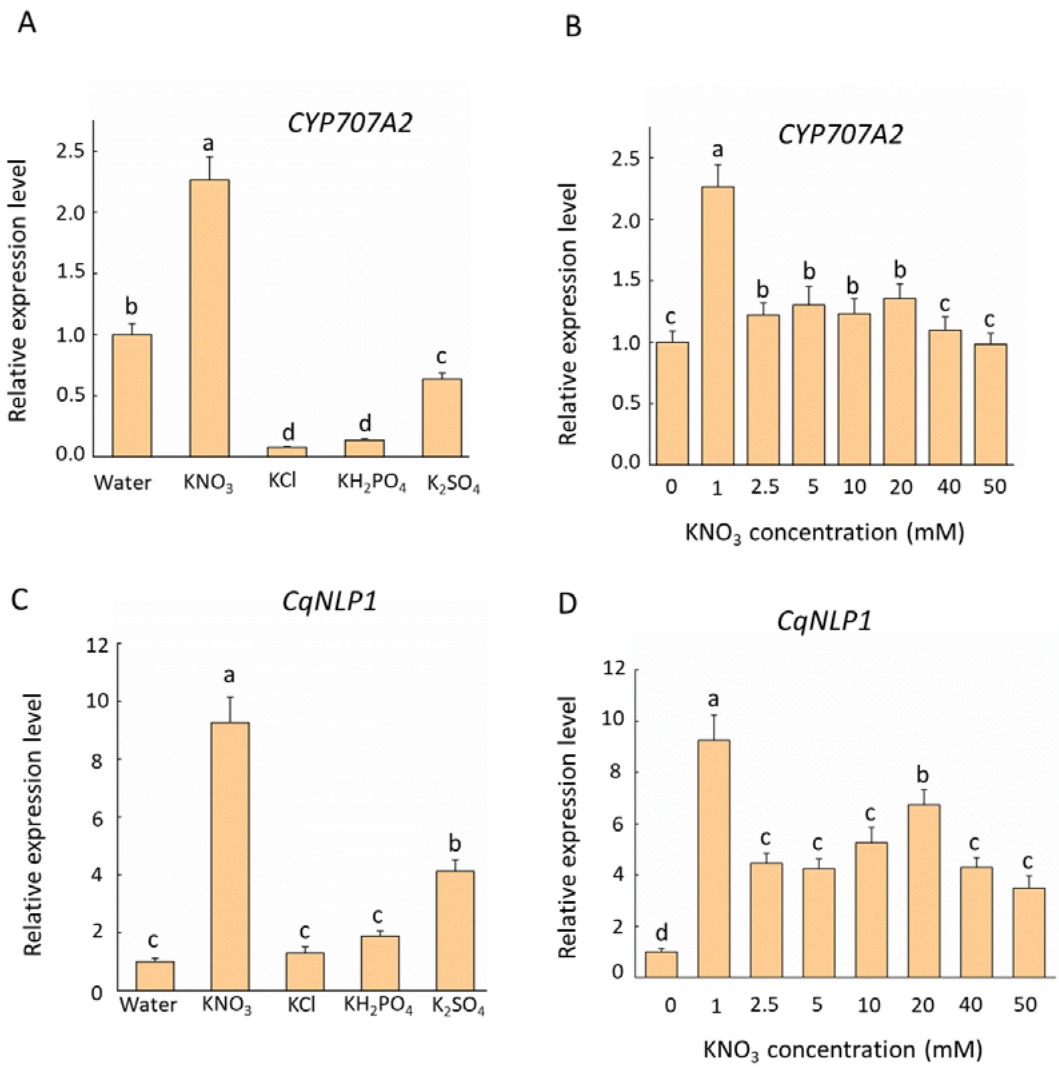

Figure 8. Relative expression of *CqCYP707A2* and *CqNLP1*. A and B show the expression profile of *CqCYP707A2* quinoa seeds treated with different types of potassium salt and different concentrations of potassium nitrate. C and D show the expression profile of *CqNLP1* with different types of potassium salt and different concentrations of potassium nitrate. Expression levels of *CqCYP707A2* and *CqNLP1* were analyzed 12h-post treatment. Different letters indicate significant difference at $p < 0.05$ according to Duncan's multiple range test.

hypothesize that the NO_3^- signaling pathway promotes the degradation of soluble protein during quinoa seed process and therefore provide nutrients for seed germination. The content of ABA in seeds regulates seed germination or dormancy. During seed maturation, ABA content in seeds is constantly induced and accumulated to maintain seed dormancy and inhibit seed germination, while during seed germination process, ABA content decreased continuously.

Herein, ABA content in quinoa seeds was consistent with the soluble protein content in KNO_3 treatment, and with 1 mM NO_3^-, ABA content was the lowest. Previous studies have revealed the role of *CYP707A2* gene in controlling seed ABA levels during seed germination (Matakiadis et al., 2009; Sasaki et al., 2015), and the present result agreed with their conclusion. We therefore proposed that NO_3^-, as a signaling molecule, acts as an upstream regulatory factor activating the expression of *CqNLP1* gene in quinoa, and further degrading ABA in seeds, relieving the inhibition of seed germination and promoting the quinoa seed germination. The mechanism is similar to Arabidopsis elucidated by Yan et al. (2016) (Figure 9). In the future, it would be interesting to explore the interaction model of *CqNLP1* and *CYP707A2*.

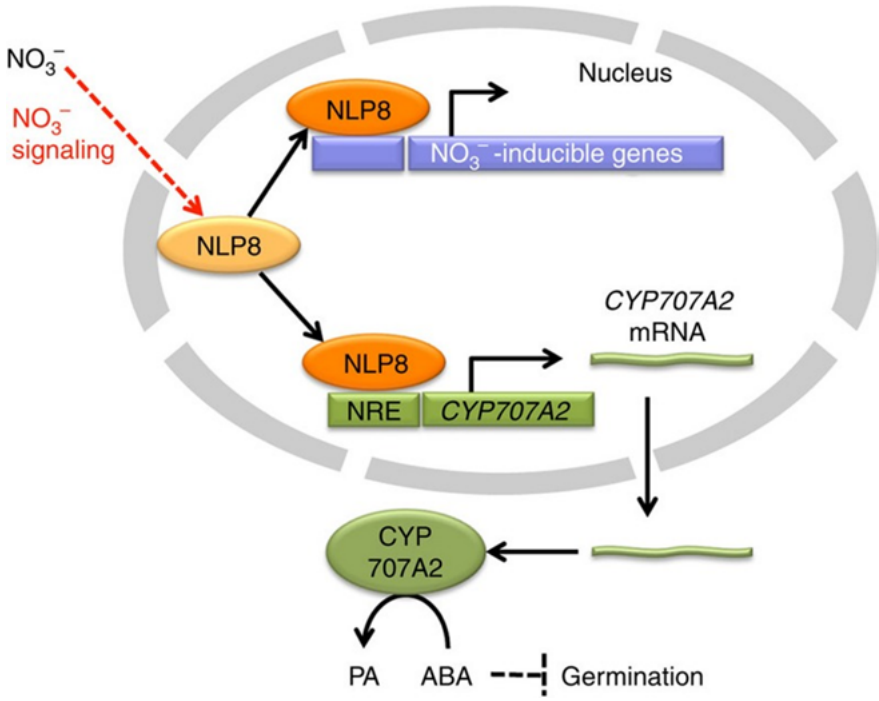

Figure 9. A proposed schematic model for NLP8 activity in regulating nitrate-promoted seed germination (Yan et al., 2016).

CONCLUSIONS

CqNLP1 gene can restore the germination rate of Arabidopsis mutant *nlp8-1* and *nlp8-2* strains, suggesting that *CqNLP1* gene plays an important role in promoting seed germination.

Among different types of potassium salt and water treatment, the germination rate of quinoa seeds was the highest in KNO_3 treatment. NO_3^- could promote the decomposition and utilization of soluble protein and ABA, increase the expression level of *CqNLP1* and *CYP707A2* during germination. The optimal NO_3^- concentration to promote seed germination is 1 mM.

ACKNOWLEDGMENTS

The research was supported by the Natural Science Foundation of Shanxi Province, China (202203021221122).We thank Dr. Chinjian Yang for kindly editing the manuscript.

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