

Adaptation of Cucumber Seedlings to Low Temperature Stress by Reducing Nitrate to Ammonium During It's Transportation

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Abstract

Background: Low temperature seriously depressed the uptake, translocation from root to shoot and metabolism of nitrate and ammonium in thermophilic plants such as cucumber, and the growth of plant was inhibited accordingly. However, there was no breakthrough in the effect of low temperature on nitrogen transport over the years.

Results: By using the non-invasive micro-test technology the net NO_3^- and NH_4^+ fluxes rate in root hair zone and vascular bundles of main root, stem, petiole, midrib, lateral vein, and shoot tip of cucumber seedlings under normal temperature (NT) and low temperature (LT) treatment were tested, respectively. Under LT treatment, the net NO_3^- flux rate in root hair zone and vascular bundles of cucumber seedlings decreased, while the net NH_4^+ flux rate in vascular bundles of midribs, lateral veins and shoot tips increased. In accordance with this, the relative expression of *CsNRT1.4a* in petioles and midribs was down-regulated, while the expressions of *CsAMT1.2a~1.2c* in midribs were up-regulated. The results of ^{15}N isotope tracing showed that compared with NT treatment, NO_3^- -N and NH_4^+ -N uptake of the seedlings under LT treatment decreased 78.1% and 58.8%, respectively, and the concentration and proportion of both NO_3^- -N and NH_4^+ -N distributed in the shoot decreased. Under LT treatment, the actual nitrate reductase activity (NRA_{act}) in roots didn't change significantly, while NRA_{act} in stems and petioles of LT treatment increased by 113.2% and 96.2%, respectively.

Conclusion: In summary, the higher net NH_4^+ flux rate in leaves and young tissues may be due to the higher NR_{act} in stems and petioles, which could reduce more NO_3^- to NH_4^+ so as to reduce the energy consumption in nitrogen transportation under low temperature.

Background

Cucumber (*Cucumis stivus* L.) is an important vegetable crop worldwide and model system for sex determination and vascular biology [1]. It is native to the tropics and is sensitive to low temperature [2]. Cucumber is widely cultivated in greenhouses in northern China during the winter and spring seasons. Low temperature is one of the major environmental factors that limit the development and productivity of cucumber [3].

Nitrogen (N) is the mineral nutrient required in the highest amount in plants [4]. It contributes approximately 2% of dry plant matter and exerts the greatest nutrient influence (up to 50%) on the growth and yield of plants under different environmental conditions [5-6]. And it is crucial for the biosynthesis of amino acids, proteins, nucleic acids, etc. [7]. The absorption and utilization of N by plants under normal temperatures have been clarified. Plant roots absorb N primarily as nitrate (NO_3^-) and ammonium (NH_4^+), especially NO_3^- for terrestrial plants [8]. The NO_3^- absorbed by plants should first be reduced to NH_4^+ before it could be metabolized. Reduction of NO_3^- to NH_4^+ is catalyzed by nitrate reductase (NR) and nitrite reductase (NiR) [9]. Among them, NR is considered to be the rate-limiting step in nitrogen

assimilation, and it catalyzes the nitrate-to-nitrite reduction process in plants [10]. It has been extensively demonstrated that NR and NiR activity can be detected in many tissues of plants (i.e. root, stem, cotyledon, inflorescence stalks, flowers, petiole, and leaf etc.) [11-14]. The main tissues that nitrate reduction took place in different plant species were different, mainly in the shoots, such as leaves, or petioles.

The absorption and transportation of nitrate and ammonium in plants were mediated by nitrate transporters (NRTs) and ammonium transporters (AMTs) respectively [15]. Four families of nitrate-transporting proteins have been identified so far: nitrate transporter 1 family (NRT1), nitrate transporter 2 family (NRT2), the chloride channel family (CLC), and slow anion channel-associated homologues (SLAC1/SLAH) [16]. And the ammonium transporter gene family of vascular plants consists of two clades, AMT and MEP [17]. Many studies have shown that regulation of nitrate uptake and transport is often highly correlated with changes in expression of relevant transporter genes [18-19].

In many crop species, especially those originated from tropical and subtropical region, low temperature restricts uptake capacity of the root and distribution of nutrient elements in the shoot, especially depressed the uptake, translocation and metabolism of NO_3^- and NH_4^+ , and then the plant growth and products were inhibited [20-21]. Low-temperature stress decreased plant height, root length, leaf area, dry mass accumulation and the strong seedling index, chlorophyll contents, photosynthesis, leaf and root nutrient contents, antioxidant enzymatic activities, and hormone accumulation in cucumber seedling [22]. Low root zone temperatures decreased the fresh weight, chlorophyll content, and antioxidant activity compared to optimum temperature, while the phenols of shoot and NR activity increased [23]. Long term (2-4 weeks) exposure to low root-zone temperature induced nitrate accumulation in roots and inhibited N transport via xylem [24]. Suboptimal root-zone temperatures led to a significant reduction in nitrate uptake rate [25].

The uptake of inorganic nitrogen forms was favoured by warm temperatures, especially nitrate [26]. Lowering growth temperature decreased N translocation more strongly than uptake, as the result, the N concentration in root increased [27]. However, previous studies mainly focused on the changes in physiological characteristics under low temperature, for example, the activities of nitrogen assimilation enzymes, and N concentrations in different plant tissues, etc. The effects of low temperature on the expression of *NRTs* and *AMTs* in higher plants were largely unknown. Furthermore, stable nitrogen isotope has always been the most important method in the study of nitrogen absorption and transportation. This method could only study the absorption and distribution of N in a period of time, and it is difficult to monitor the dynamic transport of nitrogen. So, there was no effective technology to study where nitrogen transport is stuck at low temperature before.

In recent years the Non-invasive Micro-test Technology (NMT) provides a new way to detect ion velocity in living plant tissue more directly [28]. The development and application of NO_3^- and NH_4^+ sensors for NMT provide convenience for the detection of NO_3^- and NH_4^+ uptake and transportation in plants by intuitively monitoring the net flow rate of the ions [29-30]. The purpose of this study, therefore, was to

study the effects of low temperature on the absorption and transportation of NO_3^- and NH_4^+ on the premise that the plant is regarded as a whole by using the new NMT technology, combined with ^{15}N isotope tracing and qPCR technology.

We found that low temperature reduced the net NO_3^- flux rate in root hair zone and vascular bundles of cucumber seedlings, while enhanced the net NH_4^+ flux rate in vascular bundles of midribs, lateral veins and shoot tips. In order to further understand the regulation of nitrogen transportation by low temperature, the uptake and distribution of $^{15}\text{N}\text{-NO}_3^-$ and $^{15}\text{N}\text{-NH}_4^+$, NR and NiR activities and gene expressions, relative expression of nitrate transporter (NRT) and ammonium transporter (AMT) were all measured under normal temperature (NT) treatment and low temperature (LT) treatment respectively. The results could help us to further understand the absorption and transport mechanism of nitrogen in thermophilic plants under low temperature, and provide technical support for the forms of nitrogen application in order to improve the growth and yield of the plant.

Methods

Experimental design

All experiments in this study were conducted in controlled-environment chambers (Memmert ICH L260). The seeds of cucumber (*Cucumis stivus* L.) cultivar 'Xintai Mici', which was bought from the China Vegetable Seed Technology CO., LTD (Beijing) were incubated in darkness until germination at 28°C and then grown on vermiculite-sand mixture [1:2, volume/volume (V/V)] supplied with half-strength modified Hoagland nutrient solutions at 26°C/17°C (day/night) [25]. The photosynthetic photon flux density (PPFD), photoperiod, and relative humidity (RH) were 350 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 12 h/d, and 70%–80%, respectively. When the cotyledons of seedlings fully expanded, the seedlings were supplied with whole strength modified Hoagland nutrient solution (pH=6.0) containing 4 mM $\text{Ca}(\text{NO}_3)_2$, 5 mM KNO_3 , 1 mM NH_4NO_3 , 1 mM KH_2PO_4 , 2 mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 40 μM EDTA-Fe, 4 μM H_3BO_3 , 2 μM $\text{MnSO}_4\cdot 4\text{H}_2\text{O}$, 2 μM $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, 1 μM $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, and 0.5 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$. When the second leaves fully expanded, the seedlings were used for the following experiments.

Net fluxes rate of NO_3^- and NH_4^+ , activities of NR and NiR, gene expression: The seedlings were divided into two groups, exposed to normal temperature (NT: 26 °C) and low temperature (LT: 8°C) for 5 hours separately. During the treatment, the light intensity and RH were the same as the seedling growth conditions. Then the seedlings were harvested for the tests.

Uptake of NO_3^- and NH_4^+ : NO_3^- and NH_4^+ uptake was measured according to the method described by Garnett et al. [31], with some modifications. Briefly, the seedlings were transplanted into rectangular hydroponic containers and supplied with whole-strength modified Hoagland nutrient solution one day prior the test. The containers were supplied with air bubblers to ensure adequate oxygen supply. On

sampling days, plants were transferred to the same solution supplemented with ^{15}N -labelled NO_3^- or NH_4^+ . The treatments were as follows:

(1) NT: 26 °C ^{15}N -labelled NO_3^- (^{15}N 25%)

(2) LT: 8 °C ^{15}N -labelled NO_3^- (^{15}N 25%)

(3) NT: 26 °C ^{15}N -labelled NH_4^+ (^{15}N 100%)

(4) LT: 8 °C ^{15}N -labelled NH_4^+ (^{15}N 100%)

After 5 hours of exposure, the seedlings were harvested for the determination of ^{15}N content.

Net fluxes rate of NO_3^- and NH_4^+ measurement

Net NO_3^- and NH_4^+ flux rate in root hair zone, main root, stem, petiole, midrib, lateral vein, and shoot tip were detected using the non-invasive micro-test (NMT) system (Younger USA LLC, Amherst, MA 01002, USA) by Xuyue (Beijing) Sci. & Tech. Co., Ltd., Beijing, China. The method referred to Lei et al. [32] with some modification. Samples were immersed in a petri dish containing 10-20 mL solution (1.625 mM $\text{Ca}(\text{NO}_3)_2$, 0.25 mM NH_4NO_3 , 0.1 mM MgSO_4 , 0.3 mM MES; pH=6.0) for 10 min to reduce the influence of incision exudate on ion fluxes, then transferred to another petri dish containing fresh solution for steady-state NO_3^- and NH_4^+ fluxes measurement. The measuring sites were pointed out in Fig. 1.

NO_3^- and NH_4^+ uptakes measurement

NO_3^- and NH_4^+ uptakes were determined following the protocols described by Garnett et al.[31]. Five hours after the treatment, roots were rinsed for 2 min in the identical and unlabeled modified Hoagland nutrient solution. The root surface was dried with absorbent paper. In addition, root, stem, cotyledon, 1st petiole, 1st blade, 2nd petiole, 2nd blade, and shoot tip were also sampled. All the samples were defoliated at 105°C, and dried to constant weight at 55°C. Then the samples were weighted and ground to a fine powder. Total nitrogen and ^{15}N contents were measured using Continuous Flow-Isotope Ratio Mass Spectroscopy (CF-IRMS) united with vario PYRO cube with IsoPrime 100 [33].

Detection of NR and NiR activities

Root, stem, cotyledon, 1st petiole, 1st blade, 2nd petiole, 2nd blade, and shoot tip were sampled for the measurement of the nitrate reductase activity (NRA) and nitrite reductase activity (NiRA) according to Glaab [34] and Rajasekhar & Mohr [35], respectively.

Quantitative real-time PCR analysis

The samples were excised and frozen in liquid nitrogen and stored at -80°C for the detection of gene expressions of nitrate transporter family genes (*CsNRT1.1*, *CsNRT1.2a~CsNRT1.2c*, *CsNRT1.3*, *CsNRT1.4a~CsNRT1.4b*, *CsNRT1.5a~CsNRT1.5c*, *CsNRT1.7~CsNRT1.10*), chloride channel protein family genes (*CsCLCa~CsCLCg*), slow anion channel-associated homologues (*CsSLAH1~CsSLAH4*), ammonium transporter family genes (*CsAMT1.2a~CsAMT1.2c*, *CsAMT2*, *CsAMT3.3*), NR family genes (*CsNR1~CsNR3*), and NiR gene (*CsNiR*).

Total RNA was extracted using RNeasy Pure Plant Kit (TANGEN, Beijing, China) according to the manufacturer's instructions. The concentration of RNA was quantified by spectrophotometrical measurement at $\lambda = 260$ nm, and its integrity was checked on agarose gels [25]. First strand cDNA was synthesized using FastQuant RT Kit (TANGEN, Beijing, China) according to the manufacturer's instructions. The cDNA was then analyzed by qRT-PCR using Hieff qPCR SYBR Green Master Mix (11203ES03, YEASEN) on ABI 7500 Real Time PCR System (Applied Biosystems) [36]. Transcripts of the *TIP41* (*PP2A phosphatase activator*, GW881871) were used to standardize the cDNA samples for different genes, because its expression is insensitive to low temperature [37]. Specific primers were designed using the Primer Premier 5 software [38] and the cucumber genome database [1]. Oligonucleotides list was described in Additional file 1.

Data analysis

Analysis of variance (two-way ANOVA) was performed using Least-Significant Difference (LSD) test. All statistically significant differences were identified as $P < 0.05$, and Graphpad Prism 5 was applied for graphical presentation.

Results

Net NO_3^- and NH_4^+ flux rate

In our first set of analysis, we found that LT (8°C) treatment significantly depressed the net NO_3^- flux rate in root hair zones and in the vascular bundle of other detection sites. The net NO_3^- influx rate in the root hair zone of LT (8°C) treatment was reduced to 19.3% of the NT (26°C) treatment. The net NO_3^- efflux rate in the vascular bundles of main roots, stems, petioles, midribs, lateral veins, and shoot tips were reduced to 36.2%, 11.7%, 11.0%, 21.5%, 7.6%, and 23.1% of the NT (26°C) treatment, respectively (Fig. 2). This indicated that low temperature inhibited the uptake and upward transport of nitrate.

However, compared with the net NO_3^- flux rate, the change of net NH_4^+ flux rate under LT treatment was different. The net NH_4^+ influx rate in the root hair zone of LT (8°C) treatment was reduced to 68.7% of the

NT (26 °C) treatment. The net NH_4^+ efflux rate in the vascular bundles of main roots, stems and petioles under LT (8°C) treatment were reduced to 37.6%, 9.4%, and 14.5% of NT (26 °C) treatment, respectively (Fig. 3). While the net NH_4^+ flux rate in the vascular bundles of midribs, lateral veins and shoot tips increased to 160.9%, 303.0%, and 164.1% of NT (26 °C) treatment, respectively.

Compared with the net NO_3^- flux rate, the net NH_4^+ flux rate of detection sites were much lower under NT treatment, but significantly higher in lateral vein and shoot tip under LT treatment. This indicated that the inhibition of net NO_3^- flux rate at low temperature was more serious than that of net NH_4^+ flux rate.

N uptake per plant, N concentration and N distribution in different tissues of the seedling

In order to further explore the above phenomenon, the effects of low temperature on the uptake and distribution of NO_3^- and NH_4^+ in plants was studied by isotope tracer method. Compared with NT treatment, NO_3^- -N, NH_4^+ -N and total N uptake per plant under LT treatment decreased 78.1%, 58.8% and 72.6%, respectively (Table 1). After LT treatment, the ratio of NO_3^- -N/total N decreased significantly, while the ratio of NH_4^+ -N/total N increased significantly. This indicated that low temperature decreased NO_3^- and NH_4^+ uptake significantly, especially NO_3^- .

After 5 hours of LT treatment, the NO_3^- -N contents in the root, stem, cotyledon, 1st petiole, 1st blade, 2nd petiole, 2nd blade, and shoot tip were reduced to 33.8%, 13.7%, 15.8%, 6.8%, 18.8%, 8.2%, 14.4%, and 10.6% of NT treatment, respectively. While the NH_4^+ -N contents in the root, stem, cotyledon, 1st petiole, 1st blade, 2nd petiole, 2nd blade, and shoot tip were reduced to 78.3%, 43.0%, 29.7%, 23.4%, 30.4%, 24.6%, 23.7%, and 11.0% of NT treatment, respectively (Fig. 4). This result was consistent with Fig. 2-3.

Table 1 NO_3^- -N, NH_4^+ -N, and total N uptake of per cucumber seedlings exposed to 26°C and 8°C for 5 h.

Treatment	NO_3^- -N uptake (μmol per plant)	NH_4^+ -N uptake (μmol per plant)	Total N uptake (μmol per plant)	NO_3^- -N/total N (%)	NH_4^+ -N/total N (%)
NT(26°C)	229.60 ± 12.13 a	92.13 ± 6.60 a	321.73 ± 18.73 a	71.37 ± 3.78 a	28.63 ± 1.86 b
LT(8°C)	50.33 ± 3.00 b	37.93 ± 2.33 b	88.27 ± 5.33 b	57.02 ± 3.36 b	42.98 ± 2.53 a

Note: Total N refers to NO_3^- -N plus NH_4^+ -N. Values were means ± SE (n = 3). Different lowercase letters indicate significant differences ($P < 0.05$).

As shown in Fig. 5, exposure of cucumber seedlings to low temperature resulted in a significant increase in not only NO_3^- -N (24.8%) but also NH_4^+ -N (26.0%) distribution proportion in roots. In other words, LT

treatment significantly reduced the distribution ratio of NO_3^- -N and NH_4^+ -N in the shoot. This result indicated that low temperature inhibited the transportation of NO_3^- and NH_4^+ from root to shoot, and resulted in the accumulation of nitrogen in root.

The distribution proportion of NO_3^- -N and NH_4^+ -N in blade, stem, cotyledon, and other aerial tissues all decreased under LT treatment. Among the detection aerial tissues, the proportion of NO_3^- -N distribution in stems, 1st petioles, 1st blades, 2nd petioles, 2nd blades and shoot tips of LT treatment was 61.8%, 32.7%, 85.4%, 38.2%, 66.9%, and 47.6% of that of NT treatment, respectively. The NH_4^+ -N distribution ratios in stems, 1st petioles, 1st blades, 2nd petioles, 2nd blades, and shoot tips treated with 8°C was 103.5%, 59.2%, 73.3%, 61.0% 58.7%, and 26.2% of those treated with 26°C, respectively. This indicated that low temperature decreased the NO_3^- -N distribution in petioles, stems and shoot tips more seriously than that in blades. While the effects of low temperature on the NH_4^+ -N distribution in stems and petioles were almost the same, except that the proportion of NH_4^+ -N distribution in stems increased under low temperature.

Gene expression of *CsNRTs*, *CsCLCs*, *CsSLAHs*, and *CsAMTs* in petioles and midribs

The effects of low temperature on the transcription levels of *CsNRTs*, *CsCLCs*, and *CsAMTs* were shown in Fig.6. The results showed that exposure of cucumber seedlings to low temperature decreased the relative expression of *CsNRT1.4a* in petioles and midribs, and *CsAMT3.3* in midribs, whereas significantly enhanced the expression levels of *CsNRT1.1*, *CsNRT1.3*, *CsNRT1.7*, *CsNRT1.8*, *CsCLCa*, *CsCLCe*, *CsAMT1.2c* in petioles and midribs, *CsNRT1.2b*, *CsAMT1.2a*, and *CsAMT1.2b* in midribs.

The expression of *CsNRT1.2a*, *CsNRT1.5a*, *CsNRT1.10*, *CsCLCc*, *CsCLCd*, *CsAMT2*, and *CsAMT3.3* in petioles and midribs, *CsNRT1.2b*, *CsNRT1.4a*, *CsNRT1.4b*, *CsCLCa*, *CsCLCb*, *CsAMT1.2a* and *CsAMT1.2b* in petioles were not significantly affected by low temperature (Fig. 6). The relative expression of *CsNRT1.2c*, *CsNRT1.5b*, *CsNRT1.5c*, *CsNRT1.9*, *CsSLAH1~4*, *CsCLCf*, *CsCLCg*, *CsAMT1.1a*, and *CsAMT1.1b* in petioles and midribs of the seedlings were much lower than the genes shown in Fig. 6. So we didn't mention their relative expressions in this article.

NRA_{max} , NRA_{act} , NiRA in stems and petioles, gene expressions of *CsNRs* and *CsNiR*

NR and NiR catalyze the nitrate-to-nitrite and nitrite-to-ammonium reduction process in plants, respectively [10, 39]. NRA_{max} can reflect the amount of enzyme protein indirectly, and NRA_{act} indicates actual NR activity in situ [40]. After 5 hours LT treatment, NRA_{max} in roots were depressed significantly (61.5% of NT treatment), while NRA_{max} in stems, petioles and midribs increased significantly (Fig. 7A). There was no significant difference in NRA_{max} in blades between the two treatments.

Compared with NT treatment, NRA_{act} in stems and petioles of LT treatment increased by 113.2% and 96.2%, respectively (Fig. 7B). While NRA_{act} in midribs and blades of LT treatment decreased significantly.

There was no significant difference in NRA_{act} in roots between NT and LT treatment. Except for midribs, NiR activity in roots, stems, petioles and blades was not significantly decreased by LT treatment (Fig. 7C).

Cucumis sativus has 3 NR family genes (*CsNR1*, *CsNR2*, and *CsNR3*) according to Reda et al. [41]. Among them, the relative expression of *CsNR1* in roots were much higher than that in other organs (Fig. 8A). Its expression in roots, stems, petioles, and midribs of cucumber were down-regulated by low temperature significantly. While its expression in blades was not affected significantly by LT treatment. The relative expression of *CsNR2* in leaves (petioles, midribs and blades) were higher than that in roots and stems, and low temperature up-regulated the expression of *CsNR2* in leaves (Fig. 8B). The relative expression of *CsNR2* in roots was down-regulated by LT treatment, while the expression in stems was not affected significantly. High expression of *CsNR3* was also found in leaves too, especially in blades. The relative expression of *CsNR3* in blade of NT and LT treatment was 284 and 355 times higher than that in stems, respectively. LT treatment increased its expression in all the detected tissues significantly (Fig. 8C). These results suggested that *CsNR1* may be the dominant gene of NR in cucumber roots, and *CsNR3* may be the dominant gene in cucumber leaves. *CsNR2* and *CsNR3* may play a leading role together in stem and petiole.

Compared with the *CsNRs*, the difference of relative expression of *CsNiR* in different tissues was small (Fig. 8D). LT treatment enhanced the expression of *CsNiR* in petioles, midribs, and blades. The highest expression of *CsNiR* was observed in roots in both NT and LT treatment, but not affected by low temperature.

Discussion

NO_3^- -N and NH_4^+ -N are the main nitrogen sources of cucumber. Compared with single NO_3^- -N source or single NH_4^+ -N source, compound nitrogen source is more conducive to the nitrogen absorption and growth of plants [42]. Plant preference for NO_3^- -N or NH_4^+ -N is related to species and influenced by environmental conditions and growth stages [43-45]. Cucumber is a temperature sensitive protected horticultural crop. The response of nitrogen uptake and transportation to low temperature is one of the important manifestations of temperature reduction affecting plant growth.

Low temperature inhibited NO_3^- absorption more than NH_4^+

The results on *Ceratonia siliqua* showed that root temperature affected the kinetic parameters of nitrate uptake more than those of ammonium uptake [46]. For barley plants, Q10 temperature coefficients for NO_3^- was quite bigger than that for NH_4^+ [47]. Under low temperature the NO_3^- uptake in *Secale cereale* and *Brassica napus* reduced [48]. Our result showed that compared with the cucumber seedlings grown under suitable temperature (26°C), the NO_3^- and NH_4^+ absorbed by cucumber seedlings under low temperature (8°C) decreased significantly, especially NO_3^- (Table 1), indicating that the inhibition of low

temperature on NO_3^- absorption was greater than NH_4^+ absorption. Decreasing air temperature could severely inhibit total N absorption, mainly in NO_3^- -N.

This may be due to the fact that, cucumber prefers to absorb NO_3^- -N rather than NH_4^+ -N under normal environmental conditions. The uptake of NO_3^- is energy dependent [49]. And the energy requirements for absorption and assimilation of NO_3^- are several fold higher than those of NH_4^+ [50]. With the occurrence of low temperature stress, the energy absorbed and utilized by leaves decreased significantly [51]. So after LT treatment, the uptake of NO_3^- by roots would be severely inhibited due to limited energy.

Low temperature inhibited NO_3^- and NH_4^+ upward transportation and reduced N concentrations in the shoot

The transport of nitrate was induced by NO_3^- , regulated by the feedback of cell nitrogen level, and promoted by photosynthesis [52]. Previous studies showed that low temperature severely reduced xylem sap transport in cucumber [53]. Laine reported that low temperature decreased xylem N translocation, and resulted N accumulation in the roots of *Secale cereale* and *Brassica napus* [48]. Our results conformed that low temperature not only inhibited the uptake of NO_3^- -N and NH_4^+ -N, but also inhibited the upward transportation of them. Compared with NT treatment, the distribution proportion of NO_3^- -N and NH_4^+ -N in the shoot under LT treatment decreased from 74.0% and 70.4% to 45.2% and 44.3%, respectively (Fig. 5). It means that under low temperature a greater proportion of NO_3^- -N and NH_4^+ -N absorbed by cucumber seedlings were accumulated in the root, instead of being transported to the shoot. This indicated that low temperature seriously inhibited the transportation of nitrogen from root to shoot. Our results also showed that the inhibition degree of NO_3^- -N upward transportation was almost the same as that of NH_4^+ -N by low temperature.

Low temperature significantly depressed the NO_3^- -N and NH_4^+ -N concentrations in all the detection sites of cucumber seedlings (Fig. 4). Under 26°C treatment, the NO_3^- -N concentrations in the detection sites of cucumber seedlings were significantly higher than NH_4^+ -N concentrations, indicating that NO_3^- -N is the main nitrogen form used by cucumber seedlings. This was the same as most terrestrial plants [8]. Recently Anwar [22] reported that low temperature reduced N content in roots of cucumber seedlings, but didn't reduce N contents in the shoot significantly. This may be due to the different detection methods. The total N contents were detected in his paper, while the ^{15}N concentrations were detected in our experiment.

Under LT treatment the NO_3^- -N concentrations in root, cotyledon and blade of cucumber seedlings were significantly higher than NH_4^+ -N concentrations, while the NO_3^- -N concentrations in stems and petioles (1st petiole and 2nd petiole) were significantly lower than NH_4^+ -N concentrations (Fig. 4). In contrast, under low temperature the NH_4^+ -N distribution in stems increased from 3.68% to 3.81%, while the NO_3^- -N

distribution in stems decreased from 4.63% to 2.86% (Fig. 5). Compared with NT treatment, under low temperature the decrease of NH_4^+ -N distribution in the 1st petioles and 2nd petioles (40.8%, and 38.6%, respectively) was significantly lower than that of NO_3^- -N (92.9%, and 91.6%, respectively) (Fig. 5). This indicated that the distribution of NO_3^- -N in vascular bundles was more significantly affected by low temperature than NH_4^+ -N.

Low temperature enhanced the net NH_4^+ efflux rate in the vascular bundles of midrib, lateral vein and shoot tip of cucumber seedlings

The net NO_3^- and NH_4^+ flux rate detected by NMT method showed that the NO_3^- flux rate in the vascular bundles of cucumber seedlings was reduced significantly under low temperature. And the net NH_4^+ flux rate in the vascular bundles of main root, stem and petiole decreased sharply as expected. What we found interesting was that, the net NH_4^+ flux rate in midrib, lateral vein, and shoot tip of cucumber seedlings increased significantly under low temperature (Fig. 4), which was inverse change in net NO_3^- flux rate, and was not consistent with the distribution proportion of $^{15}\text{N-NH}_4^+$ in leaf and shoot tip (Fig. 5).

Low temperature increased the NR activity in stems and petioles

Under low temperature, on the premise that the total amount of NO_3^- -N and NH_4^+ -N absorbed by the seedlings and transported from root to shoot decreased, how to explain the increase of net NH_4^+ flux rate in midrib, lateral vein and shoot tip? Then, the morphological changes of nitrogen in the process of transport were studied..

NR was the key rate limiting enzyme in nitrate reduction [10]. In higher plants the activity of NR is regulated at both phosphorylation and transcriptional level [54]. Under low temperature, NRA_{max} in roots decreased, while NRA_{act} didn't change significantly, indicating that low temperature reduced the amount of enzyme protein, but had no significant effect on the apparent activity of the enzyme (Fig. 7). Therefore, the amount of NR protein may be redundant in root. NRA_{act} and NRA_{max} in stems and petioles were both increased significantly, indicating that the change of enzyme protein content was consistent with the change of enzyme apparent activity, and NRA_{act} in stems and petioles were regulated by low temperature at transcriptional level mainly. Compared with LT treatment, under low temperature NRA_{max} in midribs and blades didn't decrease, while NRA_{act} decreased significantly in both midribs and blades. This indicated that the regulation of low temperature on NRA_{act} in midrib and blade may be mainly through protein phosphorylation. In all, low temperature had no effect on NRA_{act} in roots, but significantly increased NRA_{act} in stems and petioles of cucumber seedlings. This may result in the fact that more NO_3^- was reduced to NH_4^+ during its transportation in stems and petioles.

The qPCR analysis of NR gene expression and NRA_{max} analysis showed that *CsNR1* may be the dominant gene of NR in cucumber roots (Fig. 7A and Fig. 8A). *CsNR3* may be the dominant gene in

cucumber leaves (Fig. 7A and Fig. 8C). In stems and petioles *CsNR2* and *CsNR3* may play a leading role together (Fig. 7A and Fig. 8B-C).

Different changes of NRT and AMT family gene expression patterns under low temperature

Nitrate uptake by plants is regulated by transcriptional regulation [55]. Two environmental conditions, temperature and nutrient concentration, were found to significantly influence the gene expression of nutrient transporters [56]. With the increase of temperature, the relative expression of AMT family genes showed regular changes, while the maximum expression of different genes in AMT family emerged at different temperatures. There are few studies on the function of cucumber nitrogen transporter so far [57-59]. And little information about the regulatory pathways that involved in the effect of low temperature on the expression of these genes has been reported.

In this experiment, the relative expression of 34 nitrogen transporter genes in petioles and midribs of cucumber seedlings treated with 26°C and 8°C respectively were detected. *NRT1.1* (NPF6.3) was regarded to be a dual-affinity nitrate transporter participated in nitrate absorption and transport [60-61]. Our results showed that the expression of *CsNRT1.1* in midribs of cucumber seedlings was up-regulated by low temperature, indicating that *CsNRT1.1* may not be the dominant gene in nitrate transportation in cucumber. *AtNRT1.8* in *Arabidopsis* was related to stress-induced nitrate redistribution [62]. The relative expression of *CsNRT1.8* in petioles and midribs were up-regulated by low temperature. This may allow more nitrate to be transported to the root, thus reducing the net ion flux rate in petiole and midrib. *AtNRT1.4* and *AtNRT1.7* family genes were responsible for nitrate flow to petioles and leaves [63-64]. In cucumber, we found two homologous genes of *AtNRT1.4* from NCBI, *CsNRT1.4a* and *CsNRT1.4b*. After 5 h of LT treatment the expression of *CsNRT1.4a* in petioles and midribs was down-regulated, while the expression of *CsNRT1.4b* was up-regulated. The different response of their relative expression to low temperature may be due to their different functions. *CsNRT1.7* was involved in nitrate recycling in cucumber [57]. Under low temperature *CsNRT1.7* in petioles and midribs was up-regulated. This may reduce nitrate up-transport to the leaves to some extent.

AtCLCs were reported to play a role in nitrate assimilation of plants. *AtCLCa* and *AtCLCe* have been shown to be critical for nitrate transport into the vacuoles [65-66]. The relative expression of *CsCLCa* and *CsCLCe* in petioles and midribs of cucumber seedlings were significantly up-regulated caused by low temperature. This may lead to more nitrate storage in vacuoles under low temperature.

The AMT1 subfamily of *Arabidopsis* plays an important role in the stage of ammonium absorption [67]. And the MEP subfamily (*AtAMT2*) may play a role in the transport of ammonium from apoplast to symplast [68]. In our experiment up-regulated *CsAMT1.2a~1.2c* in midribs possibly contributed to the higher net NH_4^+ flux rate under low temperature.

Biological significance of the increase of net NH_4^+ fluxes in the vigorous growing tissue under low temperature

Plants will transfer nutrients to young tissues and seeds when unsuitable environment comes [69]. This is the result of the long evolution of plants. In this experiment, on the premise that the absorption and upward transportation of NH_4^+ decreased under low temperature, the net NH_4^+ flux rate in midribs, lateral veins and shoot tips increased significantly. This could be due to the transformation of NO_3^- during transportation. More NO_3^- was reduced to NH_4^+ during its upward transportation under low temperature. This could greatly reduce the energy consumption in the transportation process. According to Han et al. [70], under low temperature stress, the content of NO_3^- -N and the NR activities in tomato leaves significantly decreased, while the NH_4^+ -N content significantly increased. Under drought stress, the NH_4^+ nutrition can limit the effect of water deficit by osmotic adjustment and can limit oxidative damage [71]. So, assumed that NH_4^+ plays a role in the prevention of stress-induced peroxidation, the increase of NH_4^+ content in leaves and young tissues is not only beneficial to the utilization of nitrogen nutrition, but also to the improvement of stress tolerance of plants. Kant [72] believed that improving nitrate uptake and transport would enhance plant growth, resulting in improved crop yields. In the future, research on improving the nitrogen nutrition status of plants by improving the ratio of NO_3^- -N: NH_4^+ -N under low temperature needs to be carried out.

Conclusion

In conclusion, we found that low temperature reduced the uptake and distribution of $^{15}\text{N-NH}_4^+$ and $^{15}\text{N-NO}_3^-$ in leaves and shoot tips of cucumber seedlings, reduced the net NO_3^- flux rate in root hair zone and vascular bundles of cucumber seedlings, while enhanced the net NH_4^+ flux rate in vascular bundles of midribs, lateral veins and shoot tips, as presented in Fig. 9. In line with this, the relative expression of *CsNRT1.4a* in petioles and midribs was down-regulated, while the expressions of *CsAMT1.2a~1.2c* in midribs were up-regulated by low temperature. So, the higher net NH_4^+ flux rate in leaves and young tissues may be due to the higher NR_{act} in stems and petioles, which was mainly regulated at transcriptional level by low temperature. Our results provided first evidence that cucumber seedlings reduced energy consumption of nitrate transportation by reducing more NO_3^- to NH_4^+ under low temperature. Given the importance of cucumber as a vegetable crop in greenhouse, this study may not only help further understand the low temperature tolerance of thermophilic plants, but also help improve the winter cultivation techniques of protected vegetables in greenhouse.

Abbreviations

NT: normal temperature, 26 °C; LT: low temperature, 8°C; NMT: the non-invasive micro-test technology; NR: nitrate reductase; NiR: nitrite reductase; NRA: the nitrate reductase activity; NiRA: the nitrite reductase activity; NRA_{act} : the actual nitrate reductase activity; NRA_{max} : the maximum nitrate reductase activity; NRT: nitrate transporter; NRT1: nitrate transporter 1 family; NRT2: nitrate transporter 2 family; CLC: the

chloride channel family; SLAH: slow anion channel-associated homologues; AMT: ammonium transporter.

Declarations

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Availability of data and materials

The datasets supporting the results of this article are included within the article and the additional files.

Authors' contributions

XCY, YSL and YML conceived and designed the research. YML performed most of the experiments. LQB, MTS, and JW made important comments on design of the trial, the article writing, and the revisions. YSL, YML, LQB and MTS analyzed the data. YML wrote the first draft of the manuscript. XCY and YSL improved the first draft of the manuscript. All of the authors read and approved the final manuscript.

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

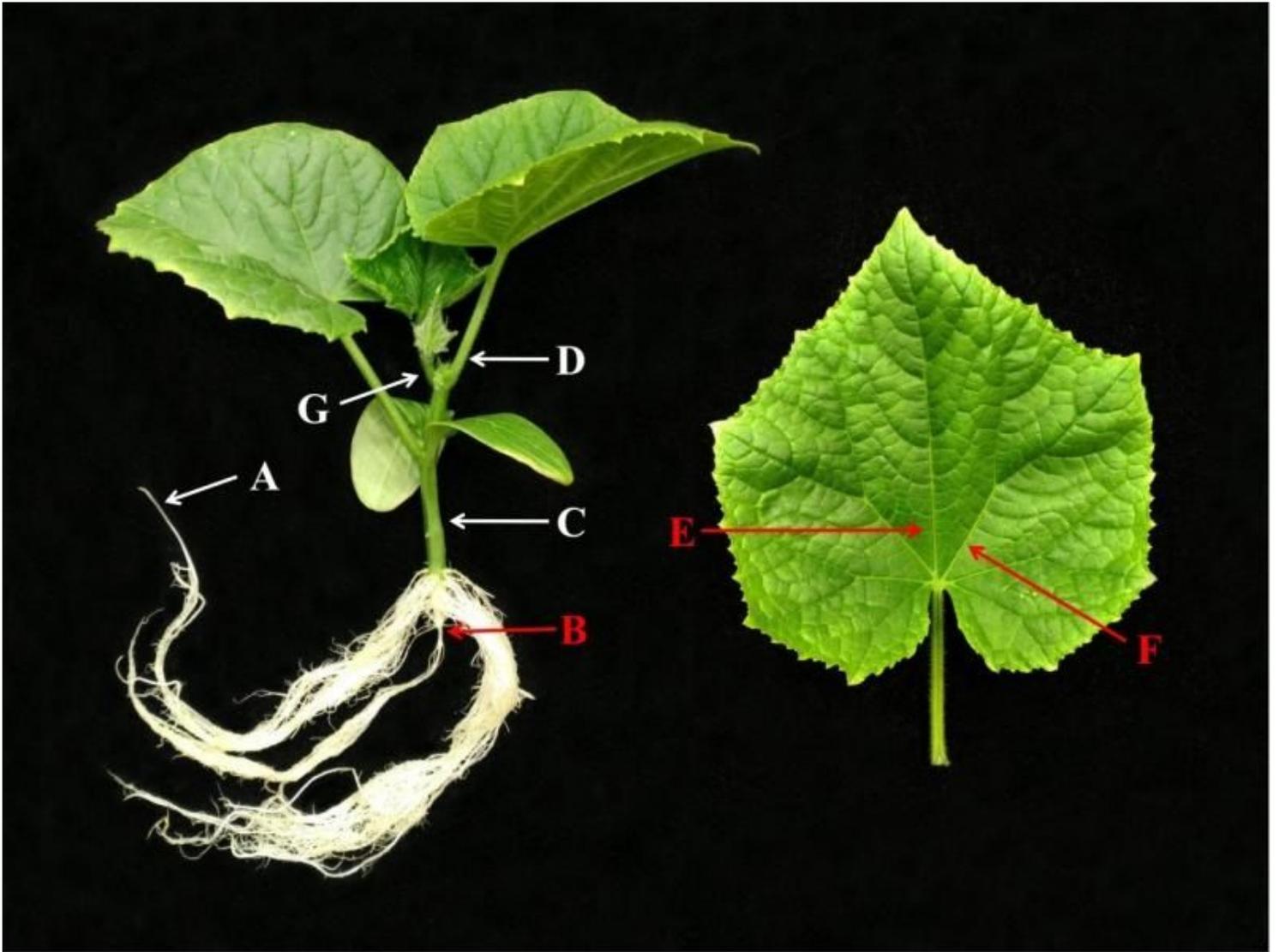


Figure 1

transferred to another petri dish containing fresh solution for steady-state NO_3^- and NH_4^+ fluxes measurement. The measuring sites were pointed out in Fig. 1.

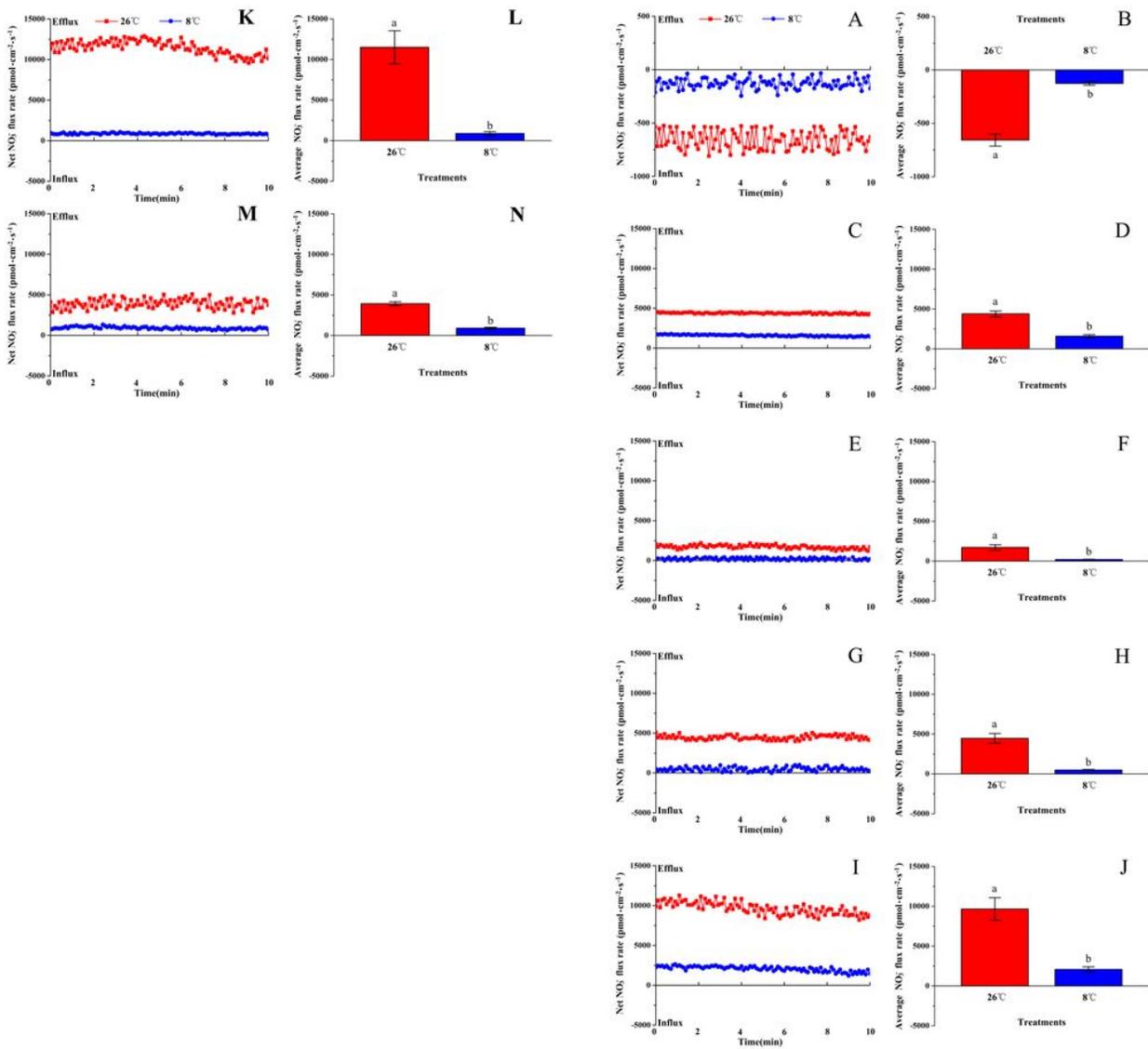


Figure 2

The net NO₃⁻ efflux rate in the vascular bundles of main roots, stems, petioles, midribs, lateral veins, and shoot tips were reduced to 36.2%, 11.7%, 11.0%, 21.5%, 7.6%, and 23.1% of the NT (26 °C) treatment, respectively (Fig. 2).

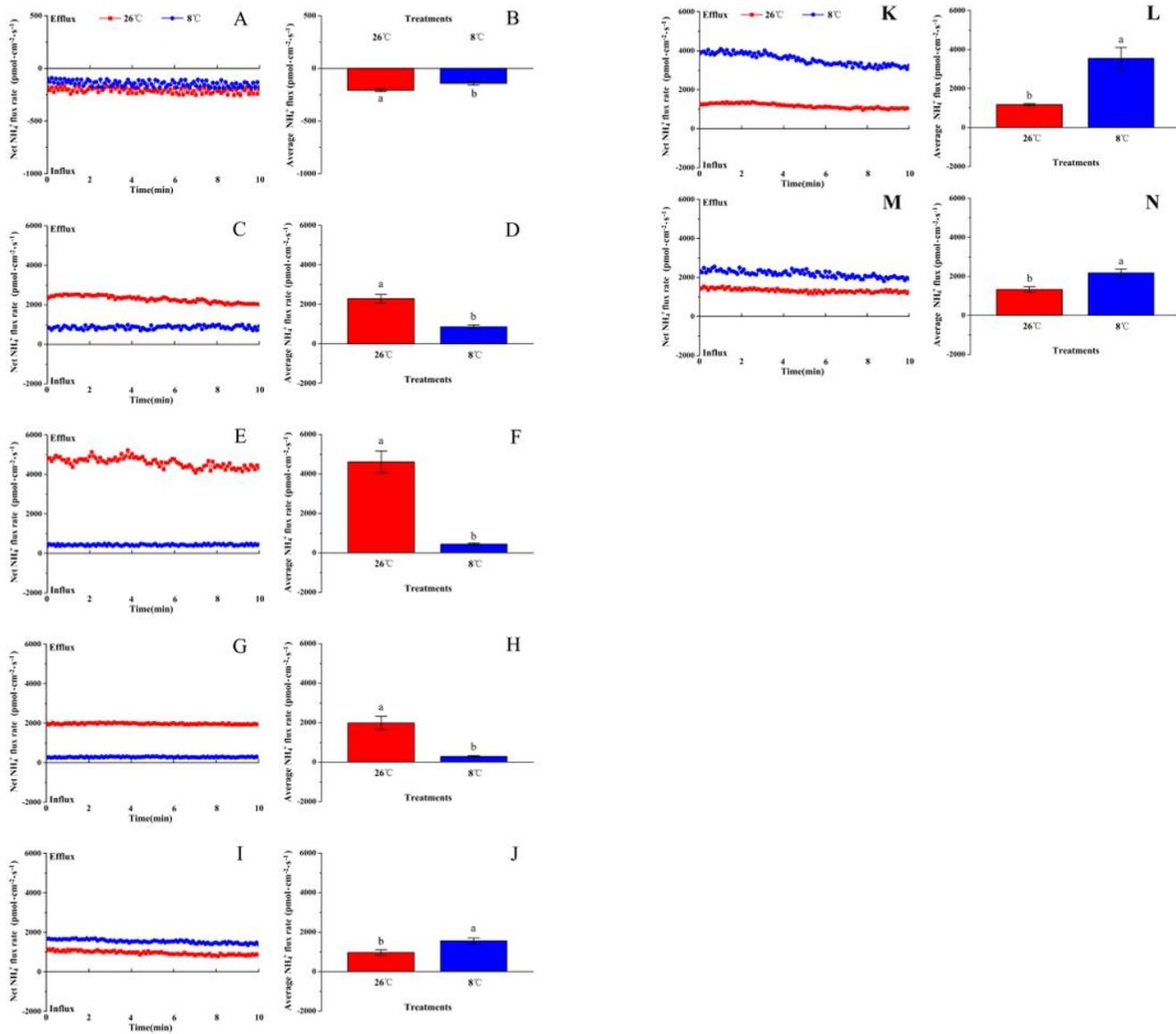


Figure 3

stems and petioles under LT (8°C) treatment were reduced to 37.6%, 9.4%, and 14.5% of NT (26 °C) treatment, respectively (Fig. 3).

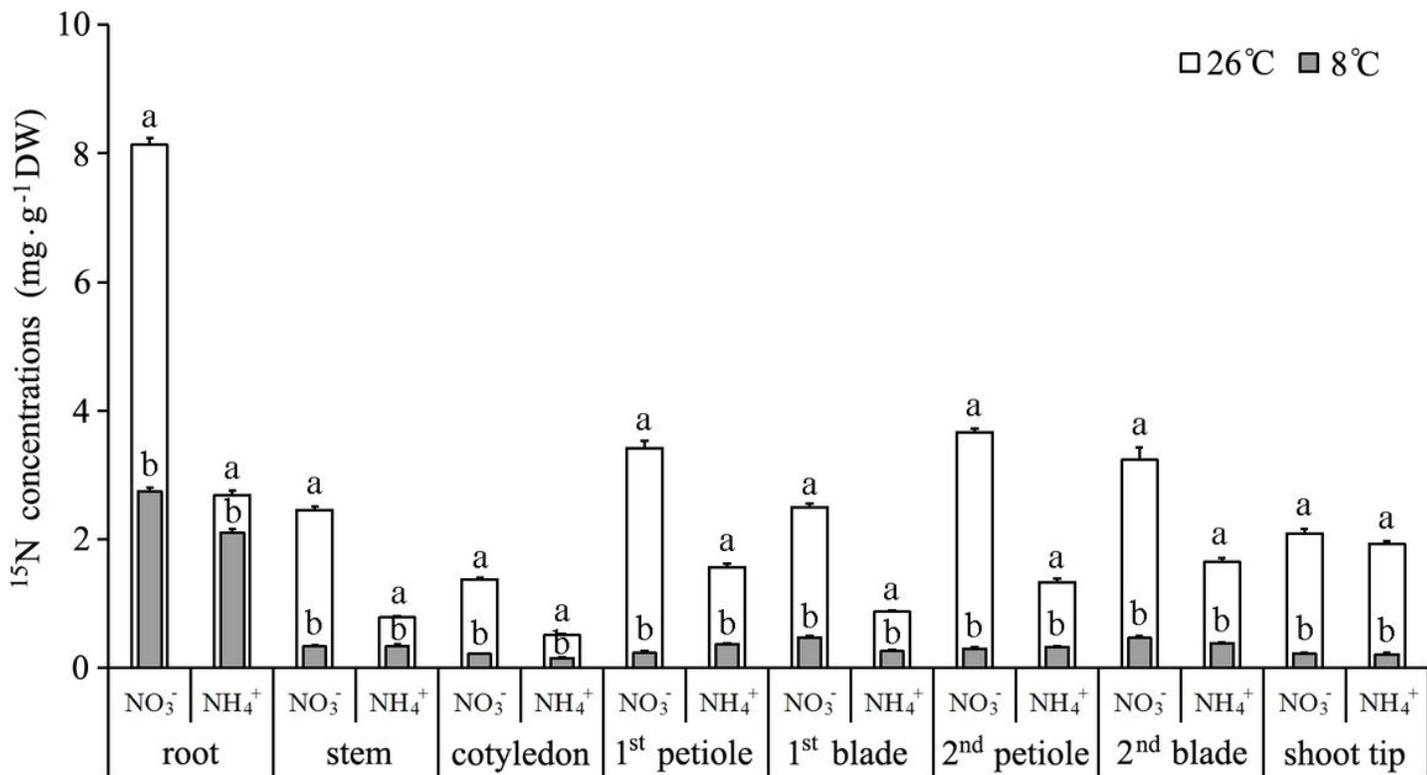


Figure 4

While the NH₄⁺-N contents in the root, stem, cotyledon, 1st petiole, 1st blade, 2nd petiole, 2nd blade, and shoot tip were reduced to 78.3%, 43.0%, 29.7%, 23.4%, 30.4%, 24.6%, 23.7%, and 11.0% of NT treatment, respectively (Fig. 4).

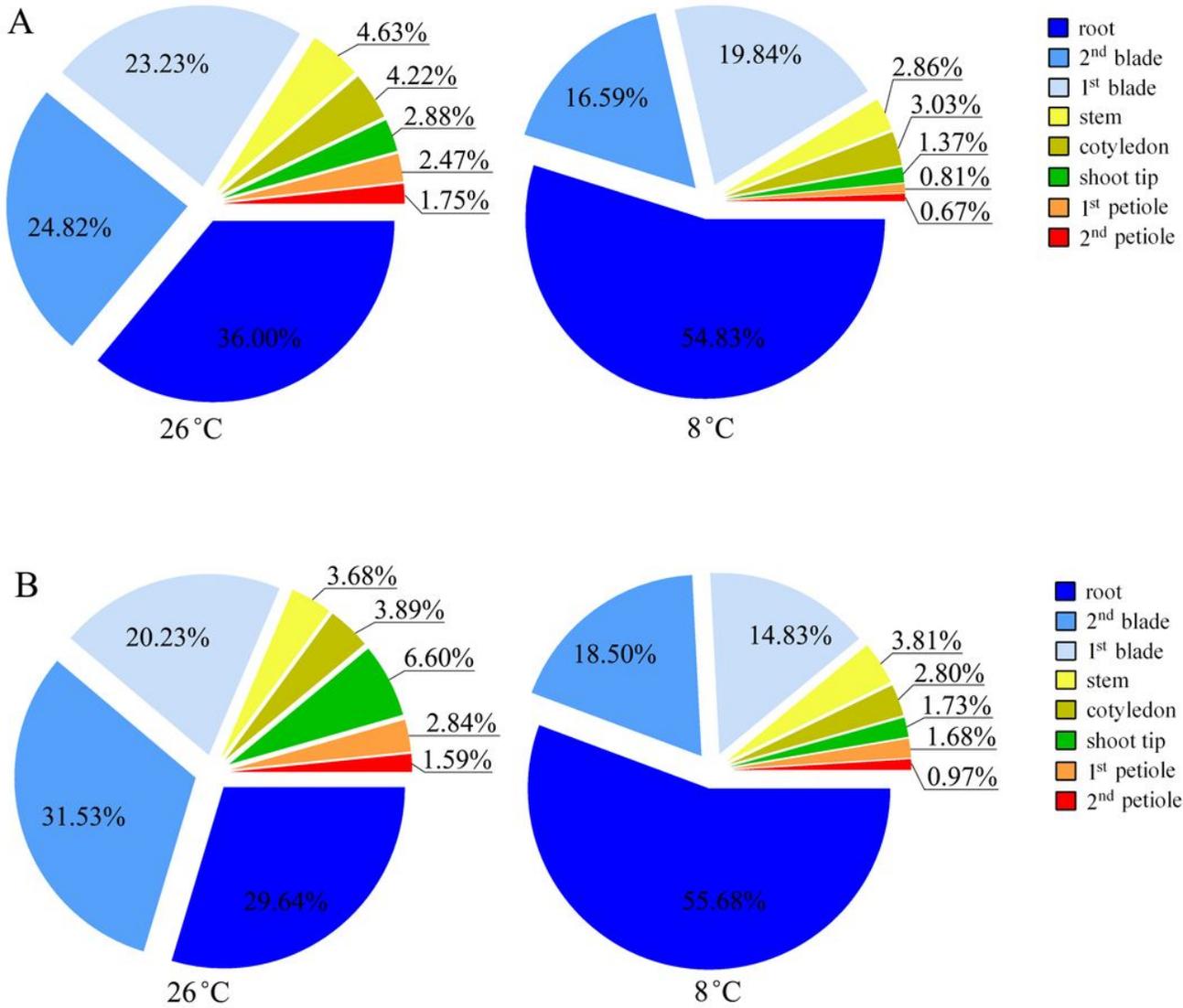


Figure 5

As shown in Fig. 5, exposure of cucumber seedlings to low temperature resulted in a significant increase in not only NO₃-N (24.8%) but also NH₄⁺-N (26.0%) distribution proportion in roots.

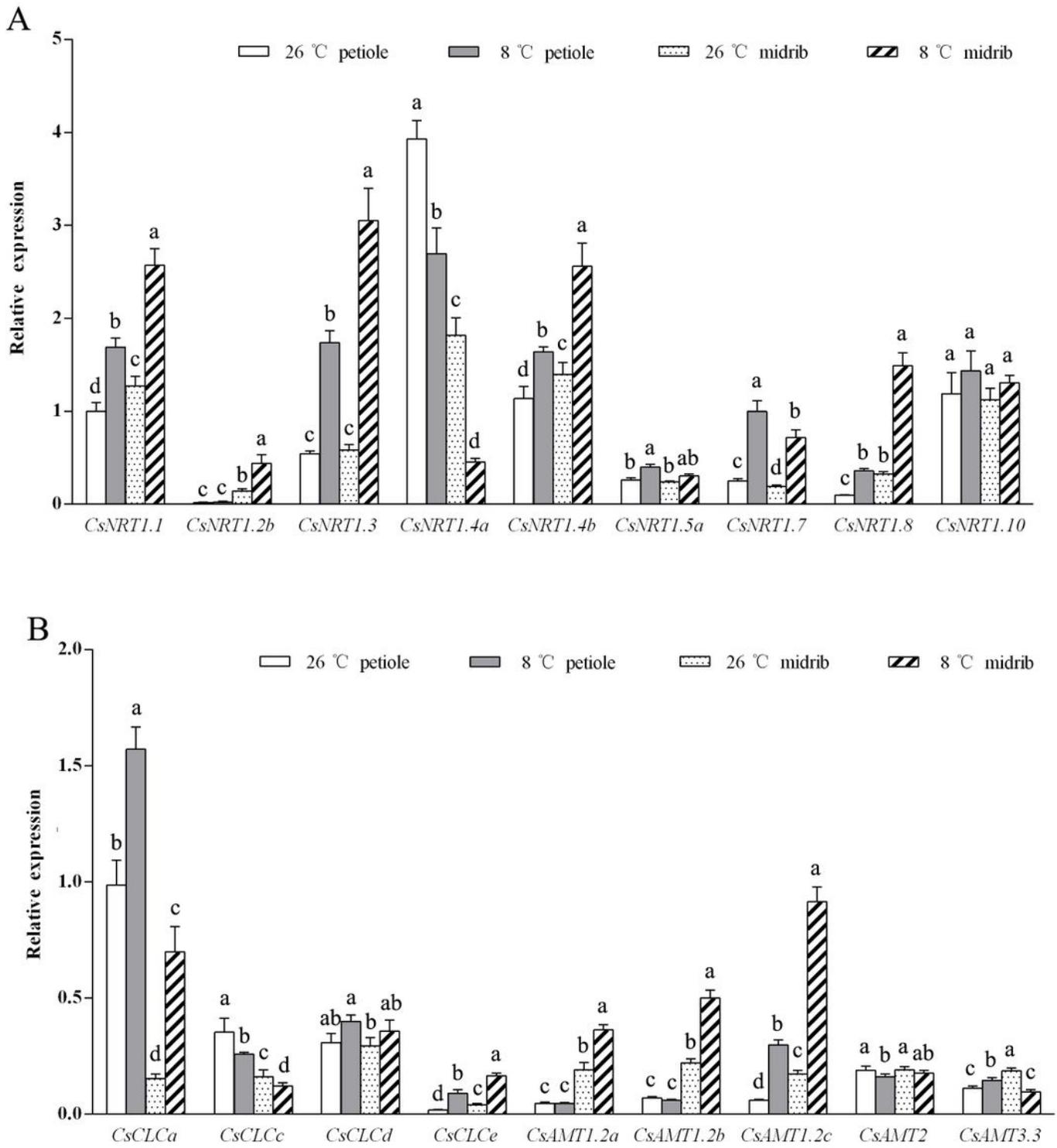


Figure 6

The effects of low temperature on the transcription levels of CsNRTs, CsCLCs, and CsAMTs were shown in Fig.6.

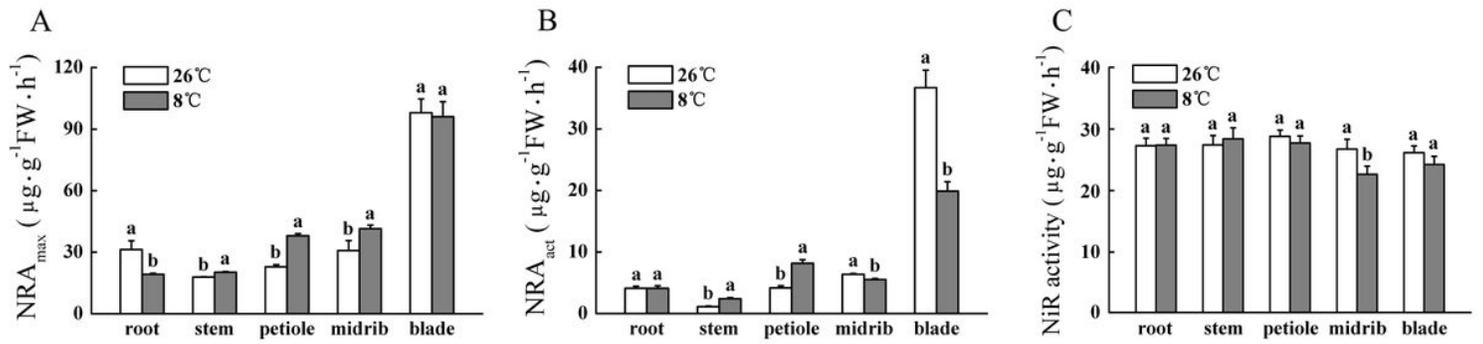


Figure 7

Except for midribs, NiR activity in roots, stems, petioles and blades was not significantly decreased by LT treatment (Fig. 7C).

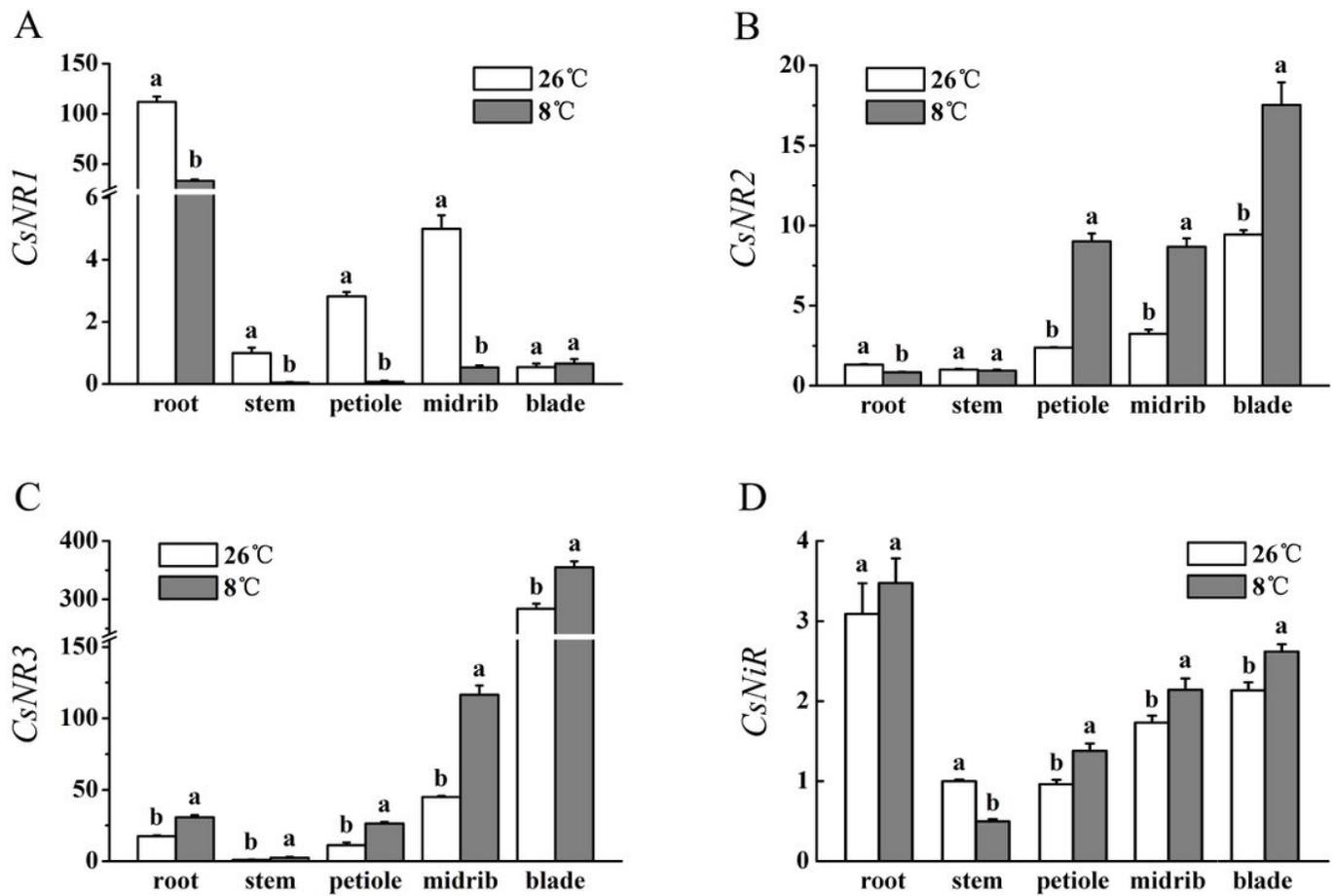


Figure 8

Compared with the CsNRs, the difference of relative expression of CsNiR in different tissues was small

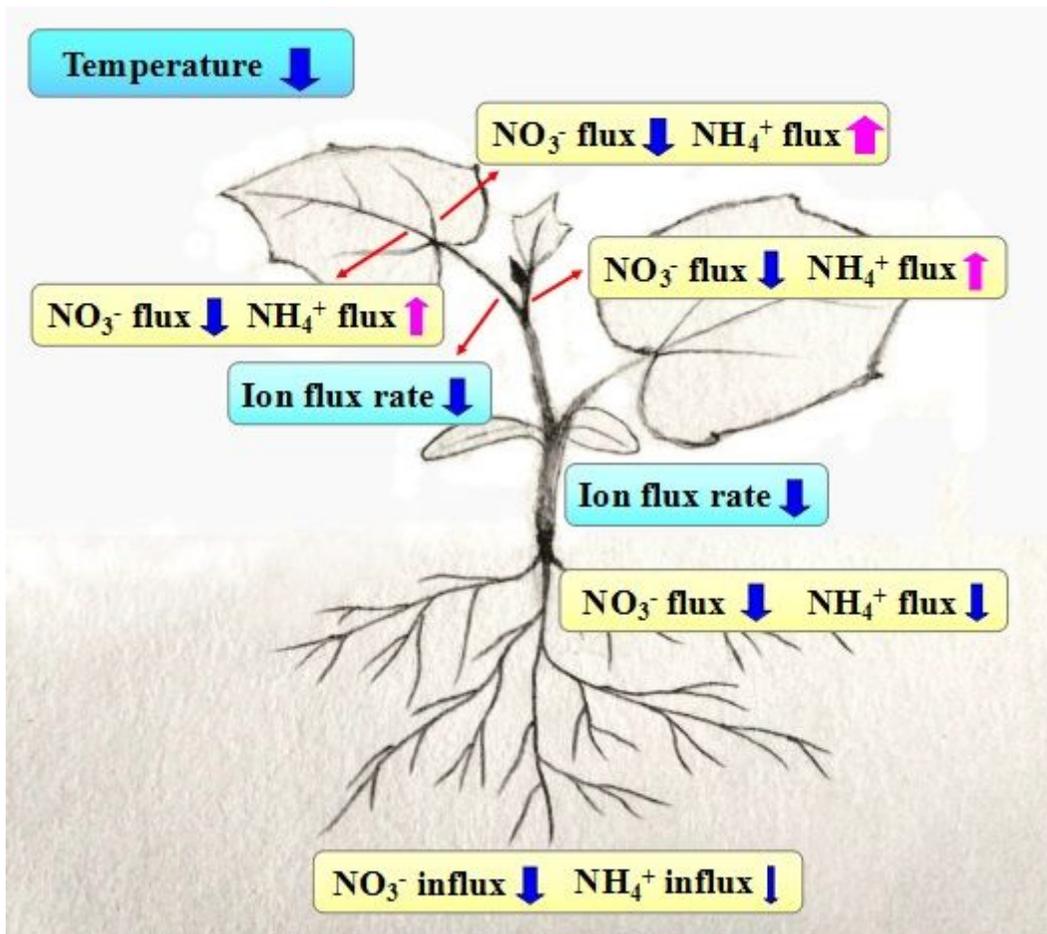


Figure 9

The net NH_4^+ flux rate in vascular bundles of midribs, lateral veins and shoot tips, as presented in Fig. 9.

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