

IDENTIFICATION OF INDEX FOR DETECTING LOW-NITROGEN TOLERANCE IN CUCUMBER

LIANXUE FAN^{1,2,3}, YALIN DU^{1,2,3}, DONGXUE GUO^{1,2}, CHUNYU TIAN^{1,2} AND TAO WU^{1,2*}

¹Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (Northeast Region), Ministry of Agriculture, Northeast Agricultural University, 59 Mucai Street, Harbin 150030, China;

²Horticulture College, Northeast Agricultural University, 59 Mucai Street, Harbin 150030, China

³These authors are contributed equally to this work

*Corresponding author's email: wutao@neau.edu.cn

Abstract

The selection of cucumber (*Cucumis sativus* L.) cultivars with high capability for low-nitrogen (N) tolerance can reduce the production costs and environmental damage caused by N fertilization. The specific biomarker for the objective detection of low-N tolerance in cucumber has not yet been identified. In this study, cucumber chalcone synthase (*CsCHS*, Csa3G600020) was identified by Solexa sequencing. *CsCHS* exhibited a consistent upregulated expression pattern owing to low-N tolerance in two cucumber cultivars with different capabilities for low-N tolerance. Results of real-time quantitative polymerase chain reaction further showed that *CsCHS* had a consistent upregulated expression pattern induced by low-N tolerance in cucumber cultivars with different ecotypes and developmental stages. By referring to *CsCHS* expression, nitrate (NO₃⁻) concentrations below 5 mM were identified as low-N tolerance in cucumber plants. Overall, results revealed that *CsCHS* expression was a stable index for detecting low-N tolerance in cucumber.

Key words: Cucumber; Fertilizer; Nitrate; RT-qPCR; Solexa sequencing.

Introduction

The oversupply of nitrate (NO₃⁻) in cucumber products, although rather acute, causes environmental pollution, reduced product quality, and high costs (Peoples *et al.*, 1995; Wang *et al.*, 2018). The detection of NO₃⁻ fertilizer conditions in a cucumber cultivation environment is important for the accurate and timely input of NO₃⁻ fertilizer without wastage. In this process, the rapid and accurate detection of the NO₃⁻ condition is crucial. The detection of soil nitrogen (N) content does not consider the preferences and demands of crops for different types of N, causing certain limitations and unreliability. Inductively coupled plasma mass spectrometry, atomic absorption spectrometry, and X-ray fluorescence analyses have been recently used to determine the actual amounts of various elements (Salt *et al.*, 2008). However, the sample preparation involved is an impractical and time-consuming process. An efficient, scientific, and accurate technique of N fertilizer detection during crop cultivation is expected to address the above problems.

Different crops or cultivars may possess similar resistance mechanisms against N tolerance, and measuring the expression of N-tolerance-responsive genes can effectively determine the N status of plants (Kamiya *et al.*, 2012). The molecular mechanism of low-N tolerance in cucumber is currently unknown. However, with the help of cucumber genome information (Huang *et al.*, 2009), genes involved in different stresses in plants such as the *chalcone synthase* gene (*CHS*) (Stewart *et al.*, 2001; Dao *et al.*, 2011; Misyura *et al.*, 2012) can be used as candidate markers for detecting the NO₃⁻ status of cucumber plants.

CHS encodes chalcone synthase, a necessary enzyme in the first important step in flavonoid biosynthesis (Winkel-Shirley, 2001). The role of *CHS* as a sensor of stress tolerance in plants has been widely studied (Stewart *et al.*, 2001; Dao *et al.*, 2011; Misyura *et al.*, 2012). The accumulation of *Arabidopsis* (*Arabidopsis thaliana*) *CHS*

mRNA responds to high-intensity light (Feinbaum & Ausubela, 1988). *CHS* is also induced by N-starvation in rice (*Oryza sativa* L.) (Chen *et al.*, 2003). In cucumber, the *chalcone synthase* gene (*CsCHS*) responds to aphid resistance and early N deficiency (Liang *et al.*, 2015; Zhao *et al.*, 2015). Although *CHS* has been widely examined using genetic, biochemical, and molecular approaches (Abe & Morita, 2010), the use of *CHS* expression as a stable index for low-N tolerance in cucumber plants has not been investigated.

In this work, we aimed to identify the stable molecular index for the detection of low-N tolerance status in cucumber. We used Solexa sequencing and real-time quantitative polymerase chain reaction (RT-qPCR) on two cucumber cultivars with different capabilities for low-N tolerance. *CsCHS* was found to exhibit a consistent upregulated expression pattern of low-N tolerance in cucumber cultivars with different ecotypes, developmental stages, and tissues. The application of *CsCHS* expression as a stable index for low-N tolerance detection in cucumber was described and discussed.

Materials and Methods

Plant material: Eight cucumber cultivars were used (Table 1). The capabilities for low-N tolerance of two of the eight cultivars were identified (D0328 with high capability for low-N tolerance and D0422 with low capability for low-N tolerance) in a previous study (Yu *et al.*, 2011).

Low-N treatment: Cucumber plants were supplied with high-N nutrients (14 mM NO₃⁻) containing 1.512 mM NaH₂PO₄·2H₂O, 0.257 mM Na₂HPO₄·12H₂O, 1.5 mM MgSO₄·7H₂O, 4 mM Ca(NO₃)₂·4H₂O, 6 mM KNO₃, 8.6 μM C₁₀H₁₂FeN₂NaO₈·3H₂O, 10.3 μM MnSO₄, 1 μM CuSO₄·5H₂O, 30 μM H₃BO₃, 24 nM Na₆Mo₇O₂₄·4H₂O, and 130 nM CoCl₂·6H₂O (Fujiwara *et al.*, 1992). For plants grown under low-N conditions (3 mM NO₃⁻), NO₃⁻

was supplied as 1 mM KNO₃ and 1 mM Ca(NO₃)₂·4H₂O. Nutrient solutions (120 mL) were provided twice a week to every pot.

The seeds of eight cucumber cultivars (Table 1) were first sown in a tray containing vermiculite supplied with different N nutrients (3 and 14 mM NO₃⁻), as mentioned above. After three weeks, individual plants were transferred to pots when three to four leaves emerged (diameter = 30 cm; height = 50 cm), containing a vermiculite with one plant per pot. Pots were placed in an artificial intelligence climate chamber under the following conditions: 28 °C/18 °C (12 h day/12 h night), 900 μmol/(m²·s) photon flux density, and 75% relative humidity. Leaf (third leaf) samples of eight cultivars grown under low and high N conditions were collected at 63 d, frozen in liquid N₂, and stored at -80 °C for RNA extraction and Solexa sequencing. The root, stem, and leaf tissues of D0328 plants grown under low- and high-N conditions were also harvested simultaneously for RNA extraction. D0328 cucumber plants were also treated under different N conditions (1, 3, 5, 7, and 14 mM NO₃⁻). The leaves of these plants were also harvested simultaneously at 37 d (vegetative stage) and 63 d (reproductive stage) and stored at -80 °C until use for RNA extraction, RT-qPCR analysis, and biochemical analysis.

RNA extraction and transcriptome profile analysis:

The total RNA of cucumber samples were extracted using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. For transcriptome profile analysis, total RNA from 10 representatives of D0328 and D0422 was pooled from equivalent sources. RNA samples were subsequently sent for Solexa sequencing to Beijing Novogene, a commercial sequencing facility. The candidate genes used for low-N tolerance detection in cucumber were identified based on the following criteria: (1) if the log₂ fold change of genes was more than or equal to one fold, (2) if the *p* value was less than 10%, and (3) if the genes exhibited a similar expression pattern between D0328 and D0422 induced by low-N tolerance.

cDNA synthesis and RT-qPCR analysis: Approximately 1 μg of the total pooled RNA was used for cDNA synthesis by using the RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Canada) in accordance with the manufacturer's instructions. The cDNA was then diluted 10 times and used as a template for RT-qPCR analysis. The gene-specific primers of RT-qPCR are listed in Table 2. For RT-qPCR, 20 μL of samples were run in triplicate on an ABI Prism 7000 Sequence Detection System and Applied Biosystems software using a tenfold dilution of 1 μL of cDNA and SYBR Green PCR Master Mix (Applied Biosystems). Thermal cycling was performed at an initial denaturation step at 95°C for 1 min followed by 40 cycles at 95°C for 15 s and annealing at 52°C for 15 s and at 72°C for 45 s. The relative quantitation of gene expression was calculated and normalized to *elongation factor 1α* (*EF1α*, forward: 5'-CCTTGGTGTCAAGCAGATGA-3'; reverse: 5'-TGAAGACACCTCCTTGATGATTT-3'). Three biological and three technical replicates were included in the RT-qPCR.

Biochemical analysis: Superoxide dismutase (SOD; EC 1.15.1.1) activity in leaves at different stages was measured by its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT; Beauchamp & Fridovich, 1971). One unit of enzyme activity was defined as the amount of enzyme required to result in 50% inhibition of the rate of NBT reduction at 560 nm. Peroxidase (POD; EC 1.11.1.7) activity in leaves at different stages was determined by the peroxidase/ guaiacol method (Kaur-Sawhney *et al.*, 1981) and involved the measurement of the rate of peroxidative oxidation of guaiacol by H₂O₂ released in enzyme extracts from leaf as one unit of enzyme activity. Catalase (CAT; EC 1.11.1.6) activity in leaves with different stages was estimated by the ultraviolet spectrophotometric method of Aebi (1984). Malondialdehyde (MDA) content was measured with thiobarbituric acid chromatometry (Yu & Sinnhuber, 1957).

Table 1. Cultivars used for gene expression analysis response to low N tolerance in cucumber (*Cucumis sativus* L.).

Germplasm name	Ecotype	Source
D0328	European greenhouse	Harbin, Heilongjiang, China
D0422	European greenhouse	Harbin, Heilongjiang, China
Dongnong 808 (DN808)	Southern China	Harbin, Heilongjiang, China
Ebai 06-7 (EB)	Pickled cucumber	Harbin, Heilongjiang, China
Jiza 8 (JZ8)	Southern China	Jilin, Changchun, China
Jiza 16 (JZ16)	Southern China	Jilin, Changchun, China
Jinyou 101 (JY101)	North China	Tianjin, China
Jinlv 11 (JL11)	North China	Tianjin, China

Table 2. Primer sequences for RT-qPCR.

Gene name	Gene ID	Forward primer(5'-3')	Reverse primer(5'-3')
<i>CsZHD5</i>	Csa1G043040	ACAGAGAATGCCTTCGGAAC	AGGTTGGCTGGAGAAGAAGA
<i>CsGols1</i>	Csa3G680120	CTCCTCCTACTCCATTTGCC	CCACTTTGACGCGATTAAGA
<i>CsFTSH6</i>	Csa5G636530	TCTGTTGAGCTCCACTGGTC	TACGACAATCGACTCGAAGC
<i>CsPRI</i>	Csa1G420360	AGAAATACGAAAGGATGGG	AGGGTCGTTACCCAGATAG
<i>CsCHS</i>	Csa3G600020	CGTTGTACGAGTTGGTTTG	AGTCCCCTCCCTCAAATG
<i>CsGRP5</i>	Csa7G336450	CCAAATTGTTCAITGGCTTTG	GCCTCCATAGGTCAGGAAGT

Results

Identification of candidate genes used for low-N-tolerance detection in cucumber by Solexa sequencing:

To identify the stable genes used for low-N-tolerance detection in cucumber, the leaves of cucumber D0328 and D0422 were analyzed for transcriptome profile analysis by Solexa sequencing. A total of 268 genes exhibiting similar expression patterns in both cultivars under low-N tolerance were identified (Fig. 1A). Among these genes, 168 and 100 were upregulated and downregulated in D0328 by low-N tolerance, respectively, whereas 165 and 103 genes showed upregulated and downregulated expression in D0422 by low-N tolerance, respectively. The 165 simultaneously upregulated genes in both D0328 and D0422 were further investigated. These genes were classified into different functional categories defined according to their putative annotations (Fig. 1B). The main functional groups of these concurrent upregulated genes were involved in molecular functions, cation binding, biological regulation, calcium ion binding, fatty acid biosynthesis, reactive oxygen species metabolism, and other processes (Fig. 1B), such as Csa2G035460 encoding Cytosol aminopeptidase family protein, Csa5G636530 encoding ATP-dependent zinc metalloprotease FtsH, and Csa4G0646909 encoding cis-epoxycarotenoid dioxygenase. Pathway analysis revealed that these genes were involved in 20 pathways, such as the biosynthesis of amino acids, biosynthesis of secondary metabolites, carbon fixation in photosynthetic organisms, flavonoid biosynthesis, nitrogen metabolism, and plant hormone signal transduction (Fig. 1C). Among these processes, the biosynthesis of secondary metabolites followed by the biosynthesis of amino acids are regarded as the most important common pathways related to low-N tolerance in cucumber (Zhao *et al.*, 2015). These results can contribute to understanding the common molecular mechanism of low-N tolerance in cucumber and suggested that the genes involved in these common pathways can be candidate genes used for low-N-tolerance detection in cucumber plants (Zhao *et al.*, 2015).

CsCHS expression as a stable index for detecting low-N tolerance in cucumber: To identify and verify the candidate stable genes for low-N tolerance detection, RT-qPCR was performed using samples prepared from eight cucumber cultivars belonging to different ecotypes (Table 1). Six genes with consistently high \log_2 fold change (>3) in both cultivars (D0328 and D0422) were selected for further identification. Results showed that among all cucumber cultivars, *CsCHS* (Csa3G600020) exhibited a consistent upregulated expression pattern (Fig. 2A). The expression of *CsFTSH6* (Csa5G636530) involved in photosynthesis was upregulated only by low-N tolerance in three cucumber cultivars and was not consistent in these three cultivars (Fig. 3). The expression levels of *CsGols1* (Csa3G680120), *CsZHD5* (Csa1G043040), and *CsGRP5* (Csa7G336450), which were involved in the responding to multiple stresses, the regulation of floral architecture and leaf development,

and the responses to abscisic and salicylic acids (de Oliveira *et al.*, 1990; Taji *et al.*, 2002; Ottow *et al.*, 2005; Nishizawa *et al.*, 2006; Park *et al.*, 2008; Hong *et al.*, 2010), were inconsistent in different cucumber cultivars despite their significant upregulation by low-N tolerance in all cucumber cultivars (Fig. 3). The expression of *CsPRI* (Csa1G420360) was induced by high N condition. Results revealed that in terms of the stability of gene expression, *CsCHS* met the criteria of gene identification for low-N tolerance detection in cucumber, and its expression may be suitable as a stable index for low-N-tolerance detection in cucumber.

Cucumber plant does not use mineral forms of N absorbed via roots from the soil in their reproductive stage; nutrients are withdrawn from the vegetative parts and transferred into generative parts of the plant at this stage of plant development. To exhibit a clearer picture of the physiological processes and draw more reliable conclusions, *CsCHS* expression at the vegetative stage was detected by RT-qPCR, and the upregulated expression pattern of *CsCHS* remained at this stage under low N compared with high N (Fig. 2B).

Oxidative stress, which is the rapid and excessive accumulation of reactive oxygen species in plant cells, is due to many environmental stresses such as salinity, drought, temperature or heavy metals damage, cell death, and nutrient deficiency (Allen, 1995; Mittler, 2002). To rule out the possibility that the increased *CsCHS* gene expression was due to the response of plants to oxidative stress occurring under low N, the antioxidant defense system comprising enzymes such as SOD, POD, CAT, and MDA content was determined (Fig. 4). The activities of three antioxidant enzymes (SOD, POD, and CAT) under low-N were similar to those under high N at different stages (Figs. 4A–C), and MDA content failed to change because of low- and high-N treatments (Fig. 4D). These data indicated that *CsCHS* expression was induced by low N rather than oxidative stress. *CsCHS* expression pattern can be strictly related to low-N-tolerance effects in cucumber and be suitable for evaluating the NO_3^- fertilizer status of cucumber plants.

We further examined *CsCHS* expression in different tissues. *CsCHS* was significantly upregulated in root and leaf tissues but not differentially expressed in the stem of cucumber D0328 plants (Fig. 5).

To identify the application of *CsCHS* expression as a stable index for low-N-tolerance detection in cucumber, *CsCHS* expression was investigated the effects of different N concentrations. *CsCHS* was found to be significantly upregulated at NO_3^- concentrations of 1, 3, and 5 mM but not differentially expressed under high NO_3^- conditions (7 mM) compared with 14 mM NO_3^- as the control (Fig. 6). These results revealed that *CsCHS* expression can be used as a reliable index for low-N-tolerance detection in cucumber and that cucumber plants suffered from low-N tolerance at NO_3^- concentrations below 5 mM.

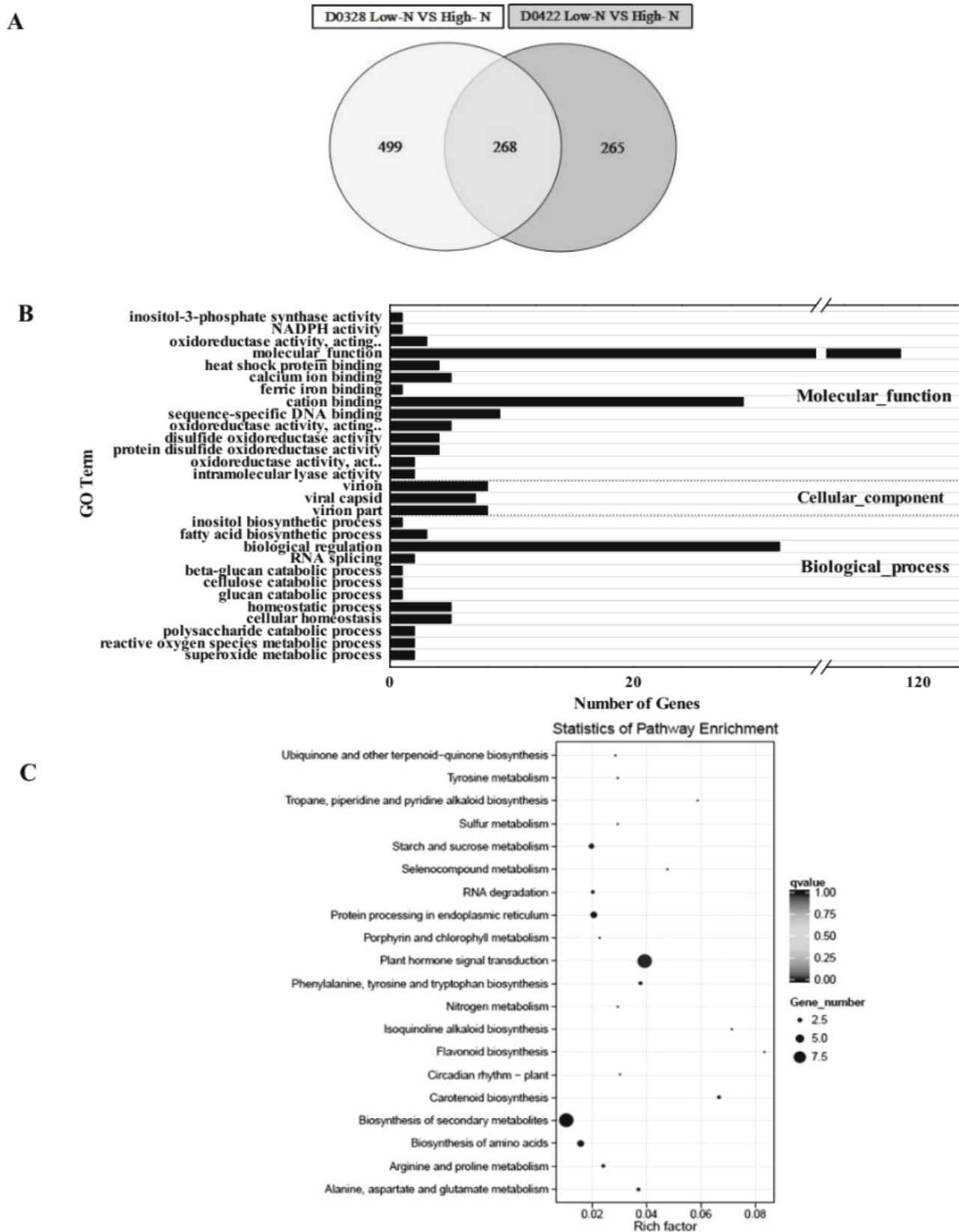


Fig. 1. Transcriptome profile analysis of low-N tolerance related genes in both D0328 and D0422 by Solexa sequencing. (A) Candidate genes identified from D0328 and D0422 under low- and high-N conditions by Solexa sequencing. Source leaves of cucumber plants grown for 63 d under low- and high-N condition were analyzed based on their transcriptome profiles. White and grey genes were specifically differentially expressed in D0328 or D0422 plants. Overlap genes of two colors were specifically differentially expressed in both D0328 and D0422 plants. Log₂ fold change ≥ 1 , p value $< 10\%$; (B) GO analysis of candidate genes with consistent upregulation expression pattern. The abscissa of the bar plot represents number of genes within each GO category. All processes listed had enrichment p values < 0.05 ; (C) the main KEGG pathways of candidate genes with consistent upregulation expression pattern. Gene number indicates the number of differentially expressed genes enriched in each pathways. All pathways listed had enrichment p values < 0.05 .

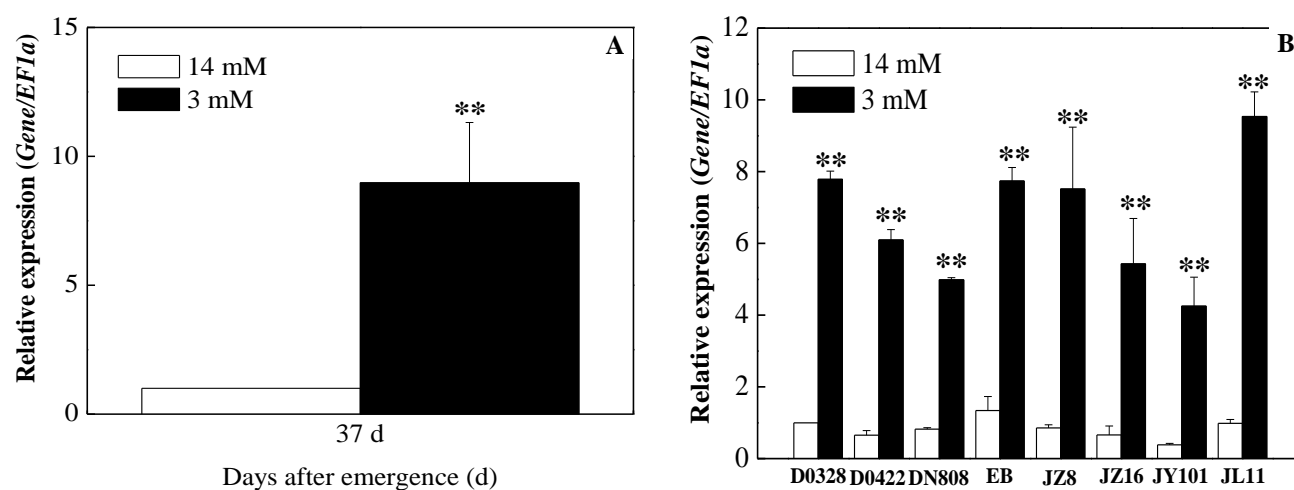


Fig. 2. Expression patterns of *CsCHS* by RT-qPCR. (A) Relative expression of *CsCHS* in different cucumber cultivars. Eight cucumber plants were grown for 63 d under low (3 mM NO_3^-) and high N (14 mM NO_3^-) conditions; (B) Expression patterns of *CsCHS* in different N concentrations at vegetative stage. D0328 plants were grown for 37 d under low- and high-N conditions. The mRNA levels were normalized to the level of *EF1a* and expressed as a ratio relative to the value at 14 mM NO_3^- . ‘**’ represent significant differences at the 0.05 and 0.01 level on the basis of *t* test.

Discussion

CHS encodes chalcone synthase, a necessary enzyme in the first important step in flavonoid biosynthesis (Winkel-Shirley, 2001). The relationship between the flavonoid pathway and N deficiency has been verified. For instance, the regulation and production of the flavonoid pathway in *Arabidopsis* was upregulated by N limitation (Olsen *et al.*, 2009). Flavonol accumulation in the leaves of mature tomato (*Lycopersicon esculentum* cv. Chaser) plants has been found to increase significantly in response to N stress (Stewart *et al.*, 2001). *CHS* is also induced by N starvation in rice and cucumber seedlings (Chen *et al.*, 2003; Zhao *et al.*, 2015). On the basis of available information and evidence, we inferred that when cucumber plants were stressed due to low N, the expression of *CsCHS* (a key enzyme in flavonoid biosynthesis) is upregulated. The elevated flavonoid pathway production subsequently protected against low N tolerance in cucumber plants. The stable and consistent upregulated expression pattern of *CsCHS* under low-N tolerance among different cucumber ecotypes and developmental stages (Fig. 2) also suggested that this regulation mechanism was universal in cucumber plants. In addition, by using the expression data of *CsCHS* under different N levels, we found that cucumber plants suffered from low-N stress at NO_3^- concentrations below 5 mM (Fig. 6). In numerous experiments on low-N tolerance in cucumber, 0–5 mM NO_3^- is frequently used as treatment for N deficiency or low N; those on high N tolerance, approximately 10–14 mM is frequently used (Matsumoto & Tamura, 1981; Martinez *et al.*, 1994; Hunt & McNeil, 1998).

CsCHS was related to the flavonoid production pathway (Fig. 1C), which is induced by a wide range of environmental stimuli, including various nutrient deficiencies (Li *et al.*, 1993; Dixon & Paiva, 1995; Leyva *et al.*, 1995; Stewart *et al.*, 2001). A comparison of tomato leaf extracts from control and N-starved plants

shows an increase in anthocyanin, particularly petunidin, as well as increased levels of the flavonol conjugate quercetin-3-O-glucoside in N-deficient plants (Bongue-Bartelsman & Phillips, 1995). The expression of *CHS* is also greatly increased under N deprivation. Gene Ontology and pathway analysis of candidate genes within two cucumber cultivars under low-N tolerance revealed that the pathways of plant hormone signal transduction, the biosynthesis of secondary metabolites, and the biosynthesis of amino acids can be common defense mechanism against low-N effects in cucumber (Figs. 1B and 1C). The data in this study were obtained from source leaves of cucumber plants during the reproductive stage, whereas the findings of Zhao *et al.*, (2015) are from leaves of cucumber plants during the seedling stage. The common pathway between the present study and that of Zhao *et al.*, (2015) is highly significant for low-N tolerance in cucumber. All these results suggested that some common processes in plant such as the biosynthesis of secondary metabolites act as protectant during low-N stress.

Overall, the data obtained in the current study proved that *CsCHS* expression was a reliable stable index for low-N tolerance in cucumber, which can help select new varieties or cultivars for cucumber breeding under low-N tolerance. *CsCHS* was first identified by Solexa sequencing and found to exhibit a consistent upregulated expression pattern owing to low-N tolerance in two cucumber cultivars with different capabilities for low-N tolerance. RT-qPCR results further showed that *CsCHS* had a consistent upregulated expression pattern induced by low-N tolerance in six other cucumber cultivars with different ecotypes and at two developmental stages. By using *CsCHS* expression, NO_3^- concentration below 5 mM was identified as low-N tolerance in cucumber plants. Therefore, *CsCHS* expression pattern can be strictly related to low-N tolerance effects in cucumber and be used to evaluate the NO_3^- fertilizer status of cucumber plants.

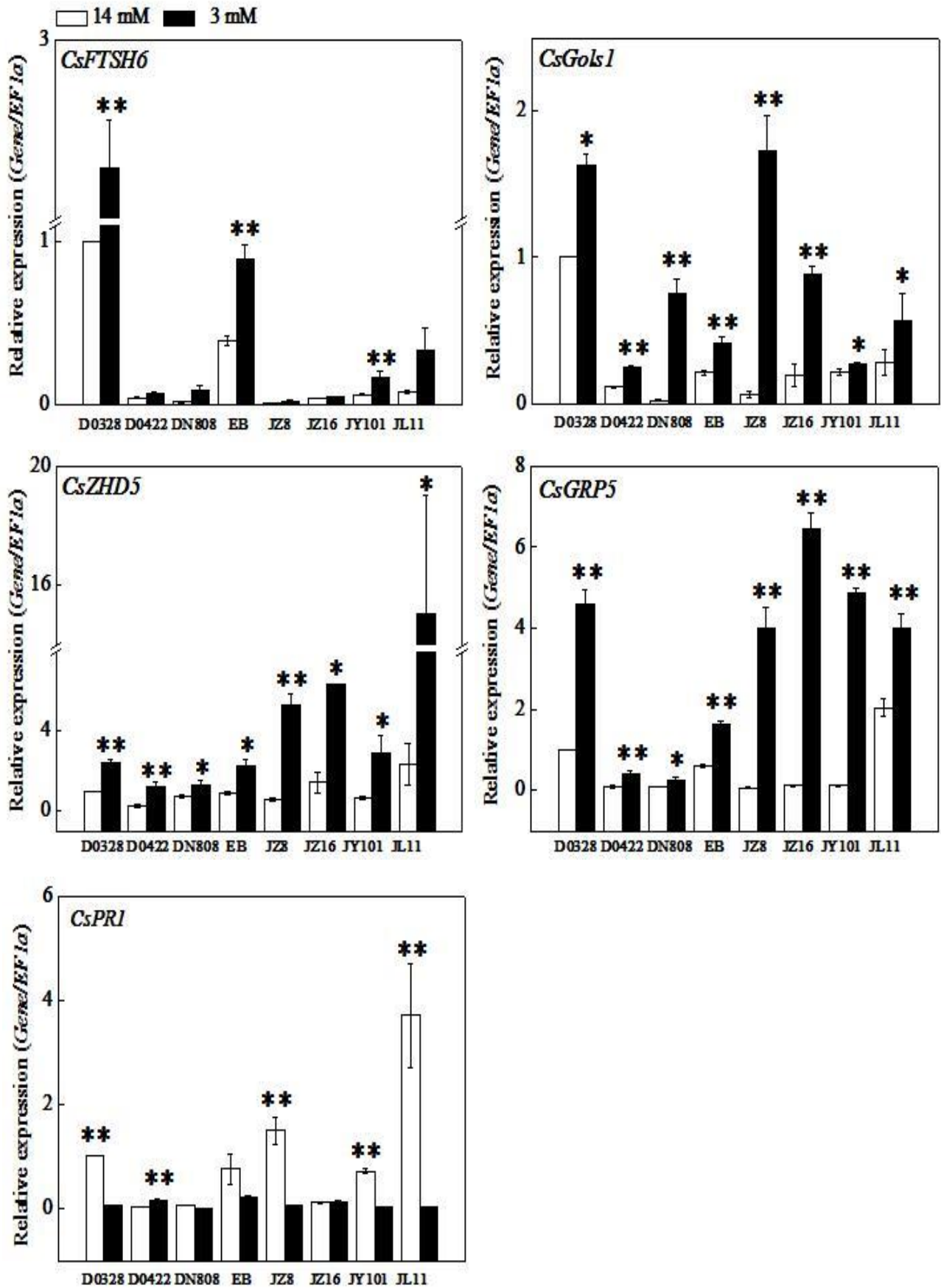


Fig. 3. Expression patterns of other candidate genes by RT-qPCR. Plants were grown for 63 d under low-N (3 mM NO₃⁻) and high-N (14 mM NO₃⁻) conditions. Total RNA was prepared from the source leaves of plants. The mRNA levels were normalized to the level of *EF1α* and expressed as a ratio relative to the value at 14 mM NO₃⁻. * and ** represent significant differences at the 0.05 and 0.01 level on the basis of *t* test.

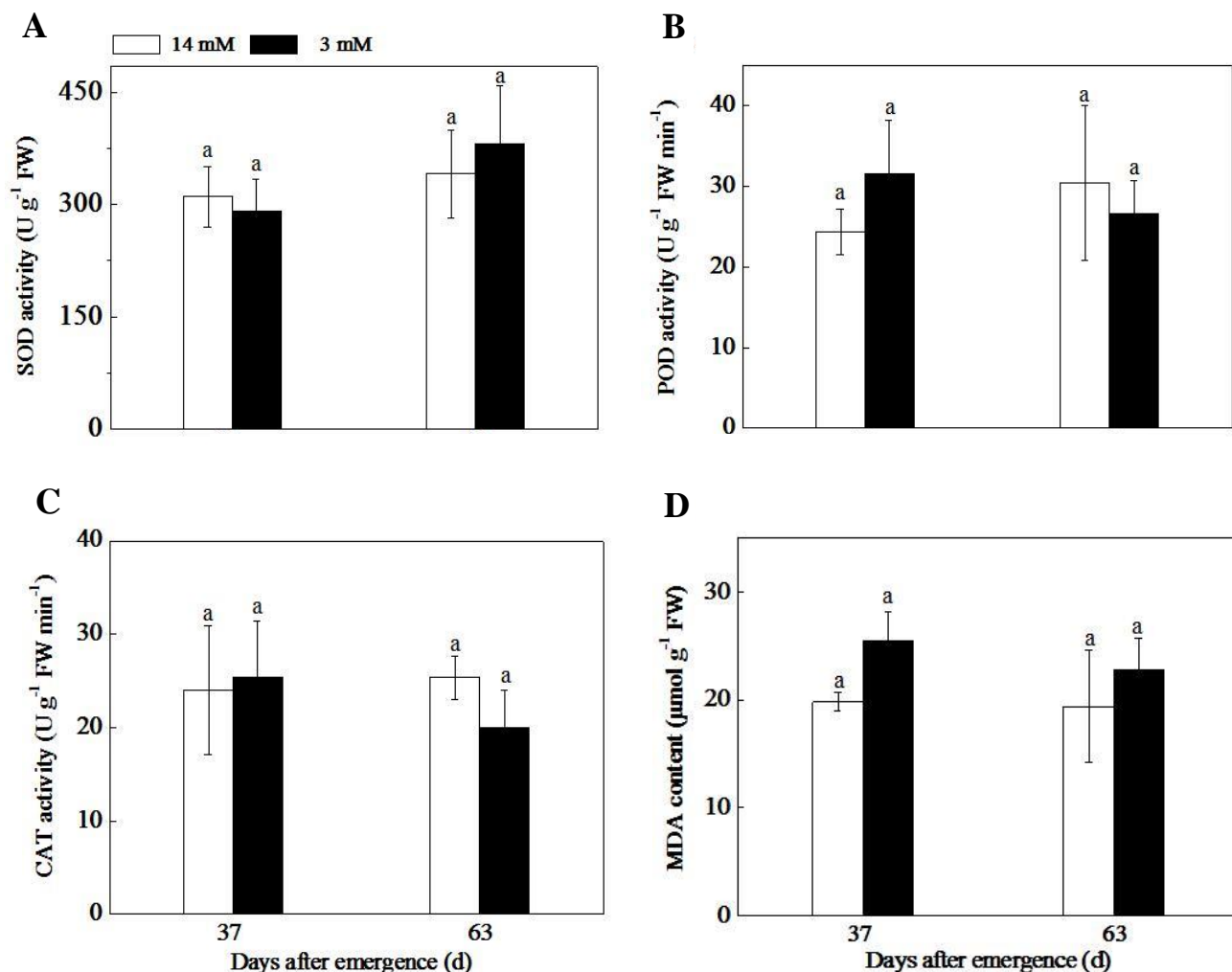


Fig. 4. Antioxidant enzymes activity and MDA content analysis. Leaves of D0328 plants grown for 37 d and 63 d under low- and high-N condition were collected and determined. (A) SOD activity in leaves at different stages under low- and high-N condition. (B) POD activity in leaves at different stages under low- and high-N condition. (C) CAT activity in leaves at different stages under low- and high-N condition. (D) MDA content in leaves at different stages under low- and high-N condition. Different letters represent significant differences at the 0.05 level on the basis of Tukey's test.

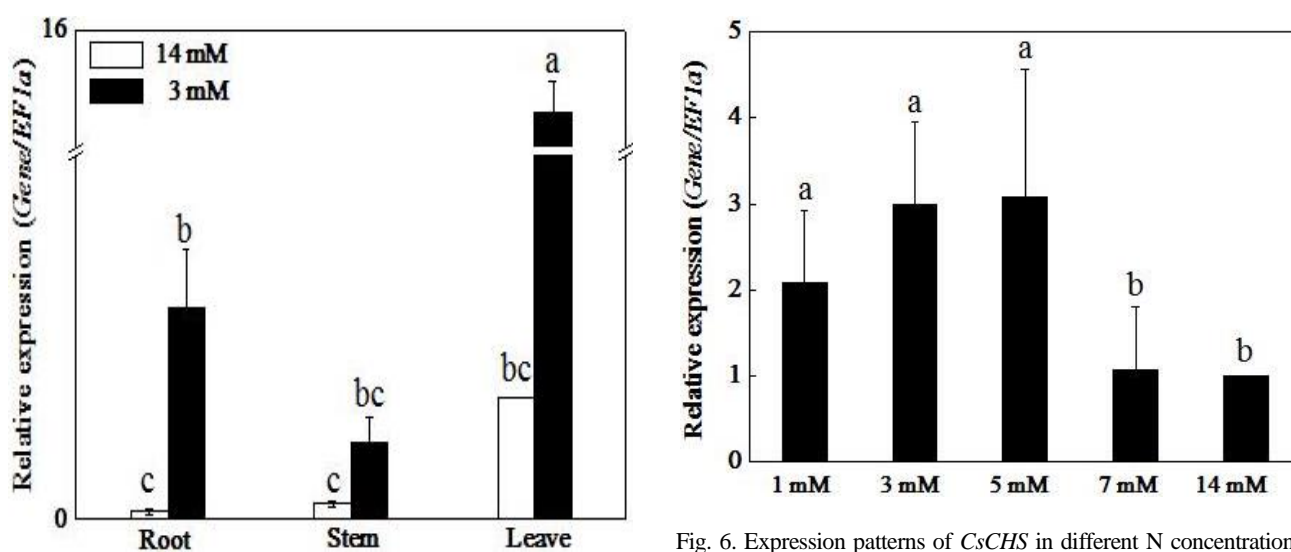


Fig. 5. Expression patterns of *CsCHS* in different tissues. D0328 plants were grown for 63 d under low (3 mM NO₃⁻) and high N (14 mM NO₃⁻) conditions. Different letters represent significant differences at the 0.05 level on the basis of Tukey's test.

Fig. 6. Expression patterns of *CsCHS* in different N concentration. D0328 plants were grown for 63 d under 1, 3, 5, 7, 14 mM NO₃⁻ conditions. Total RNA was extracted from the leaves. The mRNA levels were normalized to the level of *EF1α* and expressed as a ratio relative to the value at 14 mM NO₃⁻. Different letters represent significant differences at the 0.05 level on the basis of Tukey's test.

Acknowledgments

This work was supported by National Natural Science Foundation of China (31101545); Program for New Century Excellent Talents in Heilongjiang Provincial University (1452G03); University Nursing Program for Young Scholars with Creative Talents in Heilongjiang Province (UNPYSCT-2015001); 'Academic backbone' Project of Northeast Agricultural University (2016); 'Young Talents' Project of Northeast Agricultural University (14QC07); Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (Northeast Region), and Ministry of Agriculture/Northeast Agricultural University (neauhc201601).

References

- Abe, I. and H. Morita. 2010. Structure and function of the chalcone synthase superfamily of plant type III polyketide synthases. *Nat. Prod. Rep.*, 27(6): 809-838.
- Aebi, H. 1984. Catalase *In vitro*. *Methods Enzymol.*, 105(105):121-126.
- Allen, R.D. 1995. Dissection of oxidative stress tolerance using transgenic plants. *Plant Physiol.*, 107(4): 1049-1054.
- Beauchamp, C. and I. Fridovich. 1971. Superoxide dismutase: improved assay and an assay applicable to acrylamide gels. *Anal. Biochem.*, 44(1): 276-287.
- Bongue-Bartelsman, M. and D.A. Phillips. 1995. Nitrogen stress regulates gene expression of enzymes in the flavonoid biosynthetic pathway of tomato. *Plant Physiol. Biochem.*, 33(5): 539-546.
- Chen, Q.S., K.K. Yi, G. Huang, X.B. Wang, F.Y. Liu, Y.R. Wu and P. Wu. 2003. Cloning and expression pattern analysis of nitrogen-starvation-induced genes in rice. *Acta. Bot. Sin.*, 40(1): 1-38.
- Dao, T.T.H., H.J.M. Linthorst and R. Verpoorte. 2011. Chalcone synthase and its functions in plant resistance. *Phytochem. Rev.*, 10(3): 397-412.
- de Oliveira, D.E., J. Seurinck, D. Inzé, M. Van Montagu and J. Botterman. 1990. Differential expression of five *Arabidopsis* genes encoding glycine-rich proteins. *Plant Cell*, 2(5): 427-436.
- Dixon, R.A. and N.L. Paiva. 1995. Stress-induced phenylpropanoid metabolism. *Plant Cell*, 7(7): 1085-1097.
- Feinbaum, R.L. and F.M. Ausubel. 1988. Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. *Mol. Cell. Biol.*, 8(5): 1985-1992.
- Fujiwara, T., M.Y. Hirai, M. Chino, Y. Komeda and S. Naito. 1992. Effects of sulfur nutrition on expression of the soybean seed storage protein genes in transgenic petunia. *Plant Physiol.*, 99(1): 263-268.
- Hong, S.Y., O.K. Kim, S.G. Kim, M.S. Yang and C.M. Park. 2010. Nuclear import and DNA binding of the *ZHD5* transcription factor is modulated by a competitive peptide inhibitor in *Arabidopsis*. *J. Biol. Chem.*, 286(2): 1659-1668.
- Huang, S., R. Li, Z. Zhang, L. Li, X. Gu, W. Fan, W.J. Lucas, X. Wang, B. Xie, P. Ni, Y. Ren, H. Zhu, J. Li, K. Lin, W. Jin, Z. Fei, G. Li, J. Staub, A. Kilian, E.A. van der Vossen, Y. Wu, J. Guo, J. He, Z. Jia, Y. Ren, G. Tian, Y. Lu, J. Ruan, W. Qian, M. Wang, Q. Huang, B. Li, Z. Xuan, J. Cao, Asan, Z. Wu, J. Zhang, Q. Cai, Y. Bai, B. Zhao, Y. Han, Y. Li, X. Li, S. Wang, Q. Shi, S. Liu, W.K. Cho, J.Y. Kim, Y. Xu, K. Heller-Uszynska, H. Miao, Z. Cheng, S. Zhang, J. Wu, Y. Yang, H. Kang, M. Li, H. Liang, X. Ren, Z. Shi, M. Wen, M. Jian, H. Yang, G. Zhang, Z. Yang, R. Chen, S. Liu, J. Li, L. Ma, H. Liu, Y. Zhou, J. Zhao, X. Fang, G. Li, L. Fang, Y. Li, D. Liu, H. Zheng, Y. Zhang, N. Qin, Z. Li, G. Yang, S. Yang, L. Bolund, K. Kristiansen, H. Zheng, S. Li, X. Zhang, H. Yang, J. Wang, R. Sun, B. Zhang, S. Jiang, J. Wang, Y. Du and S. Li. 2009. The genome of the cucumber, *Cucumis sativus* L. *Nat. Genet.*, 41(12): 1275-1281.
- Hunt, J.E. and D.L. McNeil. 1998. Nitrogen status affects UV-B sensitivity of cucumber. *Aust. J. Plant Physiol.*, 25(1): 79-86.
- Kamiya, T., M. Yamagami, M.Y. Hirai and T. Fujiwara. 2012. Establishment of an in planta magnesium monitoring system using *CAX3* promoter-luciferase in *Arabidopsis*. *J. Exp. Bot.*, 63(1): 355-363.
- Kaur-Sawhney, R., H.E. Flores and A.W. Galston. 1981. Polyamine oxidase in oat leaves: a cell wall-localized enzyme. *Plant Physiol.*, 68(2): 494-498.
- Leyva, A., J.A. Jarillo, J. Salinas and J.M. Martinez-Zapater. 1995. Low temperature induces the accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs of *Arabidopsis thaliana* in a light-dependent manner. *Plant Physiol.*, 108(1): 39-46.
- Li, J., T.M. Ou-Lee, R. Raba, R.G. Amundson and R.L. Last. 1993. *Arabidopsis* flavonoid mutants are hypersensitive to UV-B irradiation. *Plant Cell*, 5(2): 171-179.
- Liang, D., M. Liu, Q. Hu, M. He, X. Qi, Q. Xu, F. Zhou and X. Chen. 2015. Identification of differentially expressed genes related to aphid resistance in cucumber (*Cucumis sativus* L.). *Sci. Rep.*, 5: 9645.
- Martinez, V., J.M. Nuñez, A. Ortiz and A. Cerda. 1994. Changes in amino acid and organic acid composition in tomato and cucumber plants in relation to salinity and nitrogen nutrition. *J. Plant Nutr.*, 17(8): 1359-1368.
- Matsumoto, H. and K. Tamura. 1981. Respiratory stress in cucumber roots treated with ammonium or nitrate nitrogen. *Plant Soil*, 60(2): 195-204.
- Misyura, M., J. Colasanti and S.J. Rothstein. 2012. Physiological and genetic analysis of *Arabidopsis thaliana* anthocyanin biosynthesis mutants under chronic adverse environmental conditions. *J. Exp. Bot.*, 64(1): 229-240.
- Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.*, 7(9): 405-410.
- Nishizawa, A., Y. Yabuta, E. Yoshida, T. Maruta, K. Yoshimura and S. Shigeoka. 2006. *Arabidopsis* heat shock transcription factor A2 as a keyregulator in response to several types of environmental stress. *Plant J.*, 48: 535-547.
- Olsen, K.M., R. Slimestad, U.S. Lea, C. Brede, T. Løvdaal, P. Ruoff, M. Verheul and C. Lillo. 2009. Temperature and nitrogen effects on regulators and products of the flavonoid pathway: experimental and kinetic model studies. *Plant Cell Environ.*, 32(3): 286-299.
- Ottow, E.A., M. Brinker, T. Teichmann, E. Fritz, W. Kaiser, M. Brosche, J. Kangasjarvi, X. Jiang and A. Polle. 2005. *Populus euphratica* displays apoplastic sodium accumulation, osmotic adjustment by decreases in calcium and soluble carbohydrates, and develops leaf succulence under salt stress. *Plant Physiol.*, 139(4): 1762-1772.
- Park, J.H., M.C. Suh, T.H. Kim, M.C. Kim and S.H. Cho. 2008. Expression of glycine-rich protein genes, *AtGRP5* and *AtGRP23*, induced by the cutin monomer 16-hydroxypalmitic acid in *Arabidopsis thaliana*. *Plant Physiol. Biochem.*, 46(11): 1015-1018.
- Peoples, M.B., J.R. Freney and A.R. Mosier. 1995. Minimizing gaseous losses of nitrogen. In: *Nitrogen Fertilization in the Environment*, (Ed.): P.E. Bacon, Marcel Dekker, New York, 565-602.
- Salt, D.E., I. Baxter and B. Lahner. 2008. Ionomics and the study of the plant ionome. *Annu. Rev. Plant Biol.*, 59(59):709-733.
- Stewart, A.J., W. Chapman, G.I. Jenkins, I. Graham, T. Martin and A. Crozier. 2001. The effect of nitrogen and phosphorus deficiency on flavonol accumulation in plant tissues. *Plant Cell Environ.*, 24(11): 1189-1197.
- Taji, T., C. Ohsumi, S. Iuchi, M. Seki, M. Kasuga, M. Kobayashi, K. Yamaguchi-Shinozaki and K. Shinozaki. 2002. Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant J.*, 29(4): 417-426.
- Wang, C., J. Wang, C. Tian, L. Fan and T. Wu. 2018. Cloning and functional analysis of low nitrogen tolerance related gene *CsMYB21* in cucumber. *Pak. J. Bot.*, 50(1): 29-34.
- Winkel-Shirley, B. 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol.*, 126(2): 485-493.
- Yu, M., Z. Qin, J. Xu and X. Zhou. 2011. Screening of cucumber genotypes with low-nitrogen tolerance and its genetic analysis. *China Vegetables*, 1: 46-51. (In Chinese).
- Yu, T.C. and R.O. Sinnhuber. 1957. 2-Thiobarbituric acid method for the measurement of rancidity in fishery products: II. The quantitative determination of malonaldehyde. *Food Tech.*, 12: 9-12.
- Zhao, W., X. Yang, H. Yu, W. Jiang, N. Sun, X.R. Liu, X.L. Liu, X. Zhang, Y. Wang and X. Gu. 2015. RNA-Seq-based transcriptome profiling of early nitrogen deficiency response in cucumber seedlings provides new insight into the putative nitrogen regulatory network. *Plant Cell Physiol.*, 56(3): 455-467.