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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*]

[1]*Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (Northeast Region), Ministry of Agriculture, Northeast Agricultural University, 59 Mucai Street, Harbin 150030, China;*
[2]*Horticulture College, Northeast Agricultural University, 59 Mucai Street, Harbin 150030, China*
[3]*These 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 (NO_3^-) 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 (NO_3^-) 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 NO_3^- fertilizer conditions in a cucumber cultivation environment is important for the accurate and timely input of NO_3^- fertilizer without wastage. In this process, the rapid and accurate detection of the NO_3^- 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 NO_3^- 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 NO_3^-) containing 1.512 mM $NaH_2PO_4{\cdot}2H_2O$, 0.257 mM $Na_2HPO_4{\cdot}12H_2O$, 1.5 mM $MgSO_4{\cdot}7H_2O$, 4 mM $Ca(NO_3)_2{\cdot}4H_2O$, 6 mM KNO_3, 8.6 µM $C_{10}H_{12}FeN_2NaO_8{\cdot}3H_2O$, 10.3 µM $MnSO_4$, 1 µM $CuSO_4{\cdot}5H_2O$, 30 µM H_3BO_3, 24 nM $Na_6Mo_7O_{24}{\cdot}$ $4H_2O$, and 130 nM $CoCl_2{\cdot}6H_2O$ (Fujiwara *et al.*, 1992). For plants grown under low-N conditions (3 mM NO_3^-), NO_3^-

was supplied as 1 mM KNO_3 and 1 mM $Ca(NO_3)_2·4H_2O$. 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 NO_3^-), 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/(m^2·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 N_2, 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 NO_3^-). 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 log_2. 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 H_2O_2 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')
CsZHD5	Csa1G043040	ACAGAGAATGCCTTCGGAAC	AGGTTGGCTGGAGAAGAAGA
CsGolS1	Csa3G680120	CTCCTCCTACTCCATTTGCC	CCACTTTGACGCGATTAAGA
CsFTSH6	Csa5G636530	TCTGTTGAGCTCCACTGGTC	TACGACAATCGACTCGAAGC
CsPR1	Csa1G420360	AGAAATACGCAAAGGATGGG	AGGGTCGTTCACCCAGATAG
CsCHS	Csa3G600020	CGTTGTACGAGTTGGTTTGG	AGTCCCACTTCCCTCAAATG
CsGRP5	Csa7G336450	CCAAATTGTTCATGGCTTTG	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_2fold 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 *CsPR1* (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.

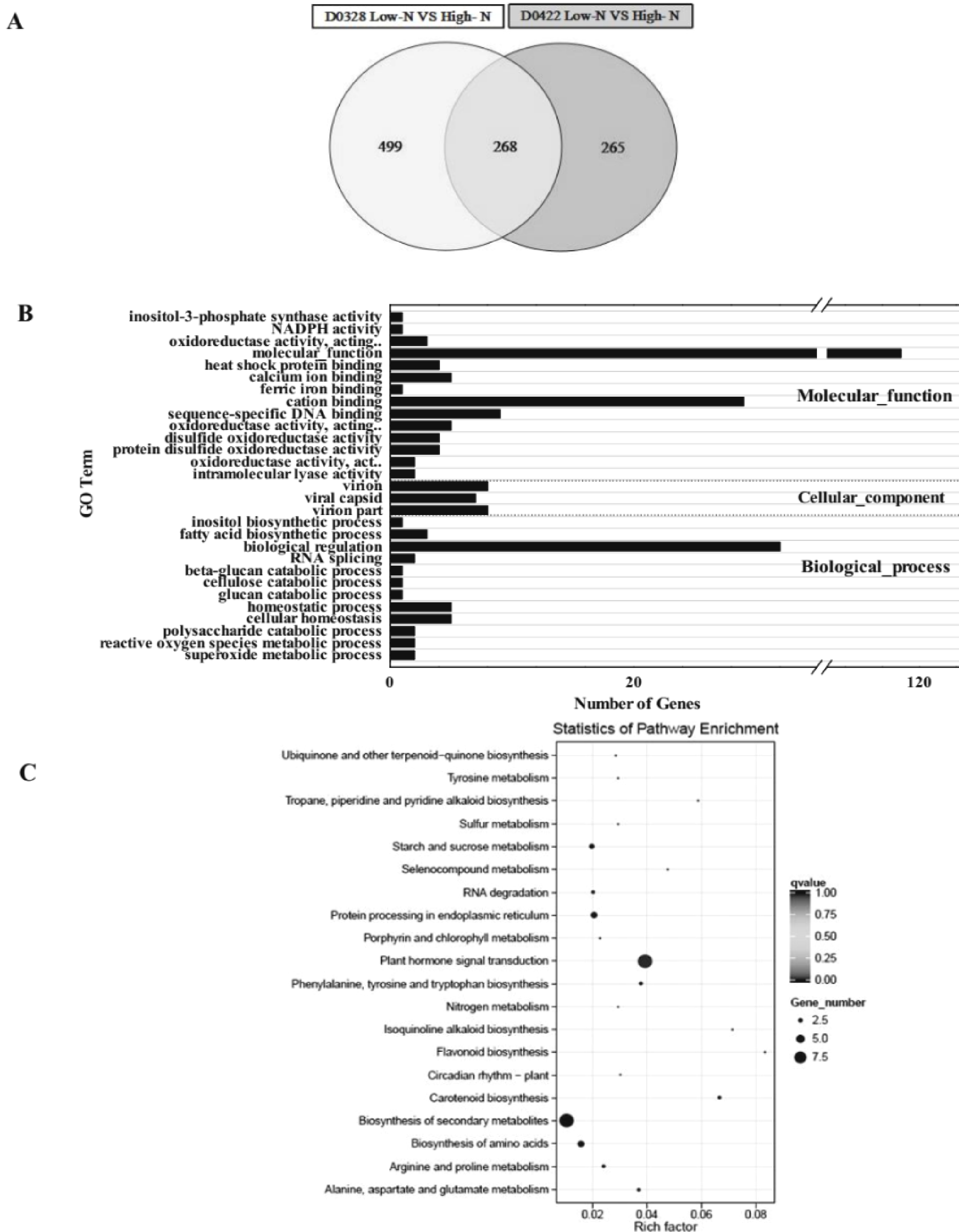

Fig. 1. Transcriptome profile analysis of low-N tolerance related genes in both D0328 and D0422 by Solexa sequencing. (A) Candidate genes identified from D0328 and D0422 under low- and high-N conditions by Solexa sequencing. Source leaves of cucumber plants grown for 63 d under low- and high-N condition 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. Log_2. 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.

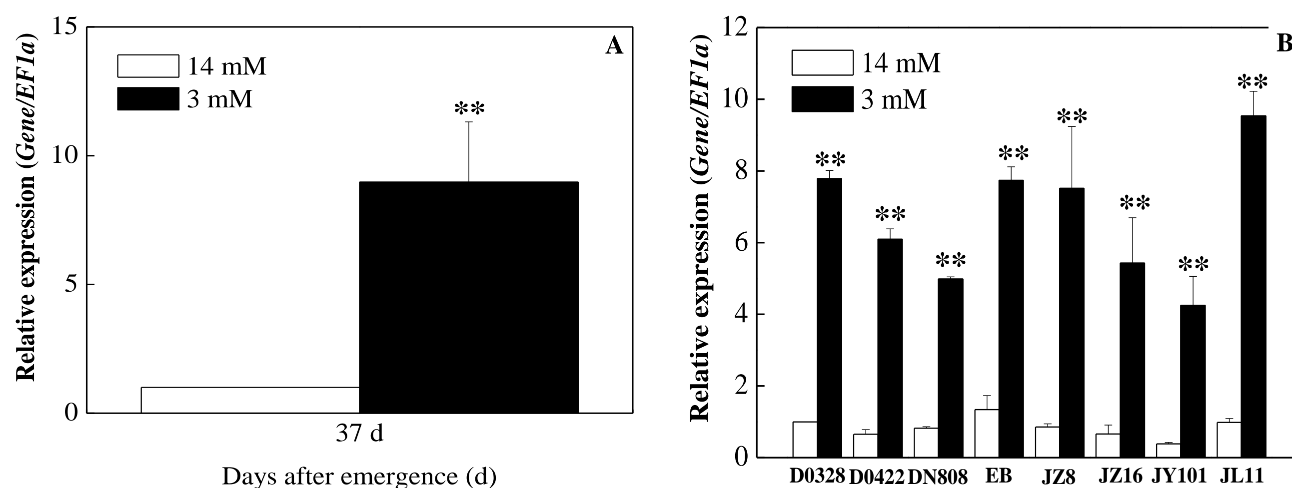

Fig. 2. Expression patterns of *CsCHS* by RT-qPCR. (A) Relative expression of *CsCHS* in different cucumber cultivars. Eight cucumber plants were grown for 63 d under low (3 mM NO_3^-) and high N (14 mM NO_3^-) conditions; (B) Expression patterns of *CsCHS* in different N concentrations at vegetative stage. D0328 plants were grown for 37 d under low- and high-N conditions. The mRNA levels were normalized to the level of *EF1α* 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 (*Lycopersiconesculentum* 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.

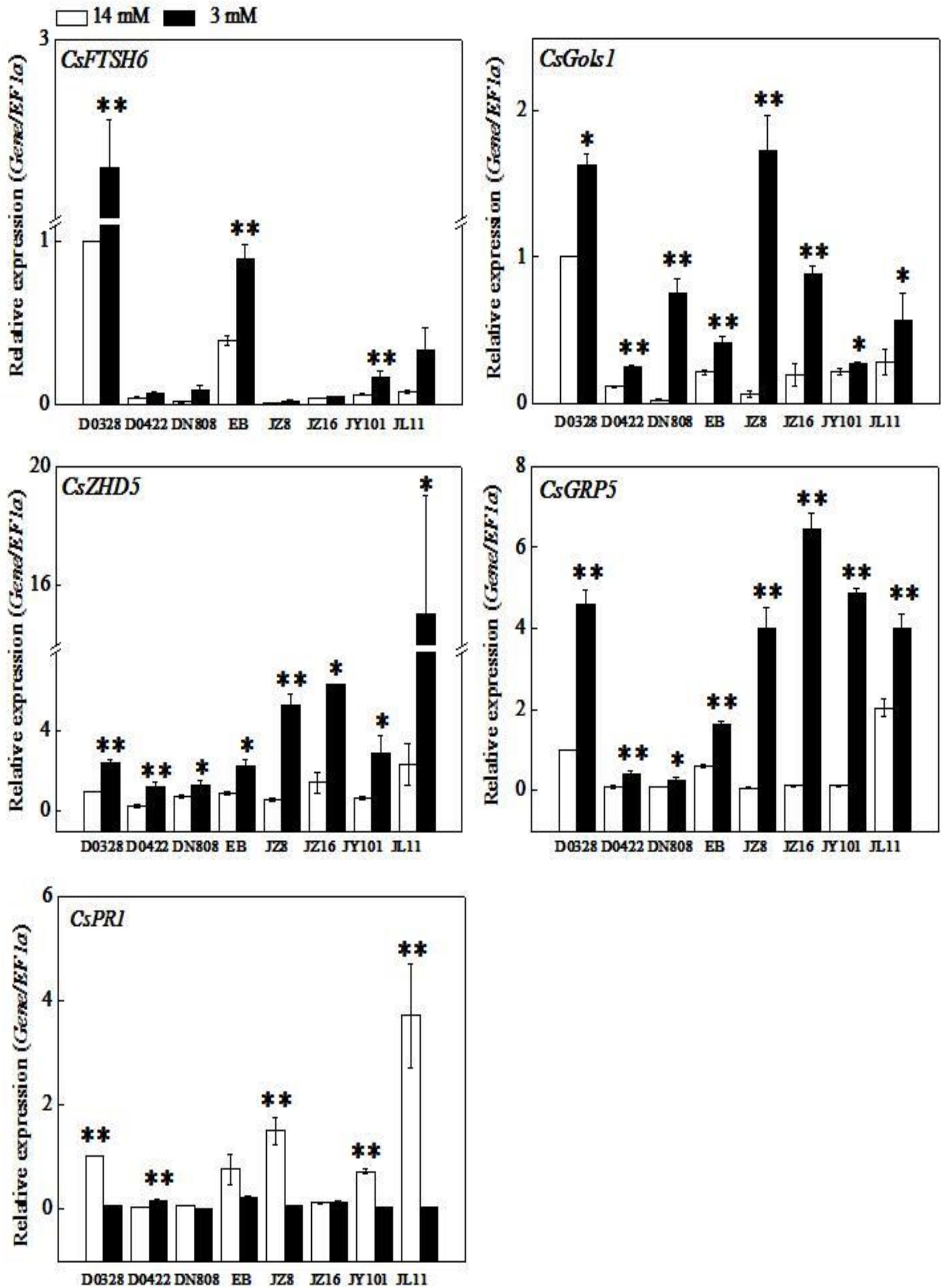

Fig. 3. Expression patterns of other candidate genesby RT-qPCR. Plants were grown for 63 d under low-N (3 mM NO_3^-) and high-N (14 mM NO_3^-) conditions. Total RNA was prepared from the source leaves of plants. The mRNA levels were normalized to the level of *EF1α* and expressed as a ratio relative to the value at 14 mM NO_3^-. * 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.

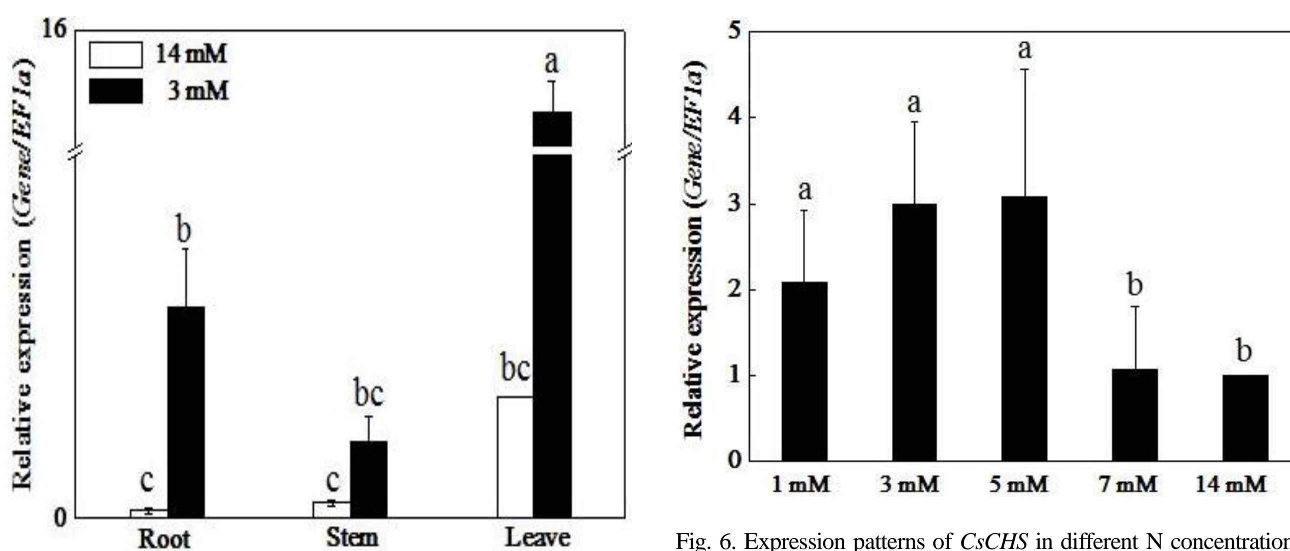

Fig. 5. Expression patterns of *CsCHS* in different tissues. D0328 plants were grown for 63 d under low (3 mM NO_3^-) and high N (14 mM NO_3^-) conditions. Different letters represent significant differences at the 0.05 level on the basis of Tukey's test.

Fig. 6. Expression patterns of *CsCHS* in different N concentration. D0328 plants were grown for 63 d under 1, 3, 5, 7, 14 mM NO_3^- 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 NO_3^-. Different letters represent significant differences at the 0.05 level on the basis of Tukey's test.

Acknowledgments

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