Pak. J. Bot., 50(1): 29-34, 2018.

CLONING AND FUNCTIONAL ANALYSIS OF LOW NITROGEN TOLERANCE RELATED GENE *CsMYB21* IN CUCUMBER

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Abstract

To investigate the molecular mechanism of low nitrogen (N) tolerance in cucumber, a low N tolerance related gene, named *CsMYB21* was cloned in the present study. qRT-PCR showed that *CsMYB21* expression was significantly induced under low N condition. Phylogenetic analysis showed that the homology of the gene with the melon was the highest. Functional analysis results showed that root lengths and fresh weights of Col-0 plants were significantly reduced under low N condition, while reduction ratio of root lengths and fresh weights in transgenic line harboring *35S::CsMYB21* was less than Col-0, which revealed that transgenic *Arabidopsis* plants showed higher capacity for low N tolerance than Col-0. These results revealed that the cucumber *CsMYB21* could improve low N tolerance in transgenic *Arabidopsis* plants.

Key words: Cucumber, *CsMYB21*, *Arabidopsis*, Low N tolerance.

Introduction

When plants are subjected to abiotic stresses, the physiological, biochemical and molecular mechanisms of plants can be regulated, which can quickly repair the damage caused by the stress and adapt to the changing environment. Transcription factors play an important role in the process of adaptation to the environment (Sha *et al*., 2015). MYBs are one of the largest and most diverse transcription factors, regulating many processes, such as plant secondary metabolism, responses to hormones and environmental factors, cell differentiation, organ formation, leaf morphogenesis and disease resistance (Urao *et al*., 1993; Sugimoto *et al*., 2000; Chen *et al*., 2002; Singh *et al*., 2002; Vailleau *et al*., 2002; Abe *et al*., 2003; Vannini *et al*., 2004; Liu *et al*., 2012). According to domain and the number of sequences, the MYB transcription factor is divided into four types: (1) 1R-MYB; (2) R2R3-MYB; (3) 3R-MYB; (4) 4R-MYB, R1/R2 (Rosinski & Atchley, 1998; Dubos *et al*., 2010). Although there is a lot of knowledge about MYB transcription factors, its relationship with low nitrogen (N) tolerance is still unknown.

N is the major component of nucleotides and proteins. It is not only an important part of many important structures, such as genetic and metabolic complexes (Frink *et al*., 1999; Crawford & Forde, 2002; Garnett *et al*., 2009), but also the main component of chlorophyll and energy conversion complex (Hao, 2013), therefore N plays an important role in the vast majority of plant growth and development process. *CsNRT1.7* was isolated from cucumber and it was found that it was involved in N recycling during cucumber senescence (Wu *et al*., 2014). Low N could cause ABA accumulation in cucumber plants, ABA accumulation could promote chlorophyll synthesis and inhibit its degradation (Oka *et al*., 2012). Transcriptome analysis of cucumber seedlings under N deficiency showed that some potential N regulation pathways, such as anthocyanin accumulation, chlorophyll decline and cell wall remodeling, were involved in N deficiency response in cucumber (Zhao *et al*., 2015). These studies laid an important theoretical foundation for understanding the physiological and molecular mechanisms of low N tolerance in cucumber.

To understand the relationship between N remobilization during reproductive stage and low N tolerance in cucumber, source leaf of cucumber plants during reproductive stage was analyzed for transcriptome profile analysis in our previous study (Fan, 2016). A candidate gene, *CsMYB21*, had been selected as a low N related gene in cucumber. The function of *CsMYB21* was analyzed in this study. Root length and fresh weight measurement results showed that *35S::CsMYB21* transgenic line had higher capacity for low N tolerance than the wild type. The study showed that *CsMYB21* can improve the capacity for low N in plants.

Materials and Methods

Plant materials, growth conditions and treatments: For low N experiments, the seeds of cucumber D0328 were first sown in a tray containing vermiculite supplied with 3 mM KNO_3 or 14 mM KNO_3. After three weeks, individual plants were transferred to pots (diameter of 30 cm and height of 50 cm) when three to four leaves emerged , 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, supplied with 3 mM KNO_3 or 14 mM KNO_3. Fifth leaves of seedlings were harvested at 37 d after transferred to pot (Mariko *et al*., 2012). Wild type *Arabidopsis* plants (*Arabidopsis thaliana*; Col-0) and transgenic line were grown in Illumination incubator (HPG-280HX, HDL) under 16 h day (22°C)/8 h night (18°C) and 75% relative humidity. Plants were treated with 10 mM NO_3^- (high N) and 1 mM NO_3^- (low N) conditions. Plants were supplied with high N nutrient containing 1 mM KH_2PO_4, 0.5 mM $MgSO_4·7H_2O$, 0.5 mM $CaCl_2$, 1 mM $CaCl_2$, 10 mM KNO_3, 50 μM NaFe-EDTA, 12 μM $MnCl_2$, 1.0 μM $ZnCl_2$, 1.0 μM $CuCl_2$, 50

μM H_3BO_3, 30 nM $(NH_4)_6Mo_7O_{24}·4H_2O$, and 130 nM $CoCl_2·6H_2O$. For plants grown under low N (1 mM) condition, NO_3^- was supplied as 0.5 mM $CaCl_2$, 1 mM KNO_3 and 9 mMKCl (Fujiwara *et al*., 1992).

Quantitative real-time PCR (qRT-PCR): Fifth leaves of D0328 plants grown for 37 d under 3 and 14 mM NO_3^- conditions in pots were used for RNA extraction and cDNA synthesis (Wu *et al*., 2010). qRT-PCR was performed using an optical 96-well plate with qRT-PCR gene specific primers as follows: forward, 5'-TTGCCGATTACTT CTCCACC-3' and reverse, 5'-GGGTTATTTGTGAA GAAGTACACG-3'. qRT-PCR was performed at an initial denaturation step at 95°C for 1 min followed by 40 cycles at 95°C for 15 s, annealing for 15 s, and 72°C for 45 s by using an iQ5 multicolor real time PCR system (Bio-RAD, USA). Relative quantitation of gene expression was calculated and normalized to cucumber Elongation Factor 1α (CsEF1α, forward: 5'-CCTTGGTGTCAA GCAGATGA-3'; reverse: 5'-TGAAGACACCTCCTT GATGATTT-3'). Three biological and three technical replicates were conducted in qRT-PCR.

Cloning and bioinformatics analysis of *CsMYB21*: The coding sequence of *CsMYB21* gene was obtained by searching the gene ID 'Csa1M042350.1' in the Cucurbit Genomics Database (http://www.icugi.org/cgi-bin/ICuGI/index.cgi), and primers (*CsMYB21*-S: 5'-ATGTCTAGTT CATCTCCAAAAAGCT-3' and *CsMYB21*-A: 5'-TTAAAATTGCCATAATTCATCCAC-3') were designed based on the gene sequences using Primer Premier 5.0. The amino acid sequence of CsMYB21 was input to an online BLAST at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) and the amino acid sequence of other MYB homologs were aligned using the DNAMAN 8 software. The phylogenetic tree was conducted with the MEGA6.0 software by Neighbor-Joining method.

Generation of transgenic lines harboring 35S::*CsMYB21* plasmid: In order to generate *CsMYB21* over expressing transgenic lines, cDNA of *CsMYB21* was cloned into the pBI121 vector at the *Xba*I/ *Bam H*Isites. The plasmid was then transformed into Agrobacterium strain LBA4404. Col-0 plants were transformed by using the floral dip method (Bechtold, 1993). Homozygous transgenic lines were then selected and used in this study for further analysis.

Root length and fresh weight measurement: Col-0 and transgenic line plants were grown under both low N and high N conditions at 22°C in a photoperiod of 16 h light/8 h dark for 10 d. The software ImageJ (http://rsb.info.nih.gov/ij/) was used for root length measurement. Fresh weight was measured by using analytical balance.Data from three biological replicates were used to caculate the mean and standard deviation in a data processing system based on Tukey's test ($p<0.05$), at least 30 seedlings were analyzed per replicate(Xie *et al*., 2016).

Results

Expression analysis of low N tolerance related gene *CsMYB21* in cucumber: The expression profile of low N tolerance related genes were analyzed through Solexa sequencing in our previous studies (Fan, 2016). *CsMYB21* (Csa1M042350.1) has been selected as one of the low N tolerance genes in cucumber with a significant up-regulation by low N conditions. In order to identify further the relations between *CsMYB21* and low N tolerance, expression analysis of *CsMYB21* was analyzed. Results showed that *CsMYB21* expression was increased under low N condition in D0328, which was consistent with the data of Solexa sequencing (Fig. 1). The results showed that *CsMYB21* was induced by low N in cucumber.

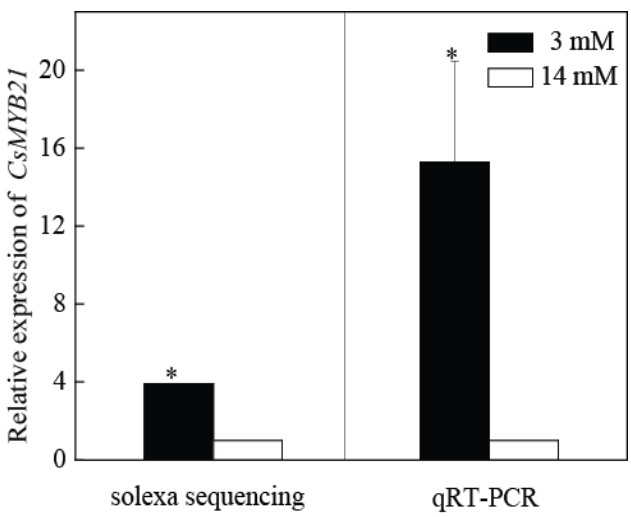

Fig. 1. Expression levels of *CsMYB21* in cucumber under low N condition. Plants were grown for 37 d under high N (14 mM NO_3^-) and low N (3 mM NO_3^-) conditions. The mRNA levels of *CsMYB2*1 was normalized to *CsEF1α*. The data are expressed as means ± SE (n = 4). Asterisks represent significant differences between the low and high N samples (*$P < 0.05$, Student's t-test).

Cloning and sequence analysis of *CsMYB21* in cucumber: The cDNA sequence of *CsMYB21* was amplified from cucumber cultivar D0328. *CsMYB21* cDNA was 756 bp in length encoding 251 amino acids (Fig. 2a). To investigate the evolutionary relationships among *CsMYB21* and 12 other plant MYB proteins, the phylogenetic tree was constructed by using MEGA6.0. The results showed that *CsMYB21* and *Cucumismelo* formed a cluster (Fig. 2b).

***CsMYB21* enhanced the capacity for low N tolerance of *Arabidopsis* plants:** Three transgenic lines (OE-1、OE-2、OE-3) and Col-0 were planted in low N (1 mM NO_3^-) and normal N (10 mM NO_3^-) conditions. As shown in Fig 3, root length of Col-0 was 3.75 ± 0.06 cm under normal N conditions, the low N treatment makes the root length of Col-0 reduced significantly by 45.1% as compared with 2.02 ± 0.14 cm; Compared with the normal N condition, the root length of transgenic plants under low N treatment was changed. Under normal N conditions, root length of three transgenic lines were 4.45 ± 0.01 cm, 4.43 ± 0.15 cm

and 4.47 ± 0.15 cm, while their root length were 3.26 ± 0.15 cm, 3.18 ± 0.07 cm, and 3.17 ± 0.06 cm under low N condition. The low N treatment makes the Col-0 root length reduced significantly by 26.75%, 28.16% and 29.00% (Fig. 3b). In addition, either in normal or low N conditions, the root length of the transgenic plants were greater than the Col-0, the root lengths of three transgenic lines were 1.87, 1.18, 1.19 times and 1.61, 1.57, 1.57 times of Col-0 under both low and normal N conditions.

Under normal N conditions, fresh weight of Col-0 was 13.33 ± 1.63 mg, the low N treatment makes Col-0 fresh weight reduced significantly by 45.53 % as compared with 6.70 ± 0.56 mg; Compared with the normal N condition, the fresh weight of transgenic plants under low N treatment was changed. Under normal N conditions, fresh weight of three transgenic lines were 17.70 ± 0.46 mg, 17.90 ± 1.87 mg and 20.67 ± 1.00 mg, while their fresh weight were 13.47 ± 1.17 mg, 15.07 ± 1.15 mg and 15.17 ± 1.84 mg under low N conditions. The low N treatment makes the Col-0 fresh weight reduced significantly by 23.99%, 16.50% and 26.22% (Fig. 3c). In addition, either in normal or low N conditions, transgenic plants had a greater fresh weight than Col-0, the fresh weight of three transgenic lines were 1.33, 1.34, 1.55 times and 2.01, 2.45, 2.26 times of Col-0 under both low and high N conditions. These results indicated that the overexpression of *CsMYB21* could enhance the capacity for low N tolerance in *Arabidopsis*.

(a)

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1     ATGTCTAGTTCATCTCCAAAAAGCTTCAGCAGCTGCAGTGAAATTGAAGATGACAATAGC
1      M  S  S  S  S  P  K  S  F  S  S  C  S  E  I  E  D  D  N  S
61    AGCAATGGCAATGAACTCCGCAGAGGCCCATGGACTCTTGAAGAAGACAATCTTCTTATC
21     S  N  G  N  E  L  R  R  G  P  W  T  L  E  E  D  N  L  L  I
121   CATTCCATTTCTACTCACGGCGAAGGCCGTTGGAATCTCCTCGCCAAACGCTCTGGATTA
41     H  S  I  S  T  H  G  E  G  R  W  N  L  L  A  K  R  S  G  L
181   CGAAGAACAGGGAAGAGCTGCAGATTGAGATGGCTTAATTACCTTAAACCAGATGTCAAG
61     R  R  T  G  K  S  C  R  L  R  W  L  N  Y  L  K  P  D  V  K
241   AGAGGAAATTTGTCTGCTCAAGAACAACTCTTGATTCTTGATCTCCATTCTAAATGGGGC
81     R  G  N  L  S  A  Q  E  Q  L  L  I  L  D  L  H  S  K  W  G
301   AACAGTGAGGCATTTCAGCAGATCATTCGATCCTATTGGATGCCAAGATTACTTCAAAAA
101    N  S  E  A  F  Q  Q  I  I  R  S  Y  W  M  P  R  L  L  Q  K
361   ATCAACCAATCCCCGCCGTCGGAATTGCCGATTACTTCTCCACCGGAGATGGCATCCCAG
121    I  N  Q  S  P  P  S  E  L  P  I  T  S  P  P  E  M  A  S  Q
421   ACTTCGTTTGGTTTTGAGGCTCCGACGACAGCGGTGACGCAACCACCGGCGATGACGCCT
141    T  S  F  G  F  E  A  P  T  T  A  V  T  Q  P  P  A  M  T  P
481   CTGCAAATCGGTGGAGATTTGATGGGAAGTTCGTGTACTTCTTCACAAATAACCCAAATT
161    L  Q  I  G  G  D  L  M  G  S  S  C  T  S  S  Q  I  T  Q  I
541   TCTTCCTACCAGAATTACGAGAATTACGCCCCGTTTGTGAAAGATTATTGTCACGACGGT
181    S  S  Y  Q  N  Y  E  N  Y  A  P  F  V  K  D  Y  C  H  D  G
601   GGCCATGGCGGAGGAGAGATGCTGAACTGGACGACGACTGCGGTGGCTGGAGACTCCGGT
201    G  H  G  G  G  E  M  L  N  W  T  T  T  A  V  A  G  D  S  G
661   TATCCGGTTTCTCATTGCCACGTGGCAGAAAATAATTGGATGGACGGCGATTTTACCGGT
221    Y  P  V  S  H  C  H  V  A  E  N  N  W  M  D  G  D  F  T  G
721   TATATGTCCAACGTGGATGAATTATGGCAATTTTAA
241    Y  M  S  N  V  D  E  L  W  Q  F  *
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(b)

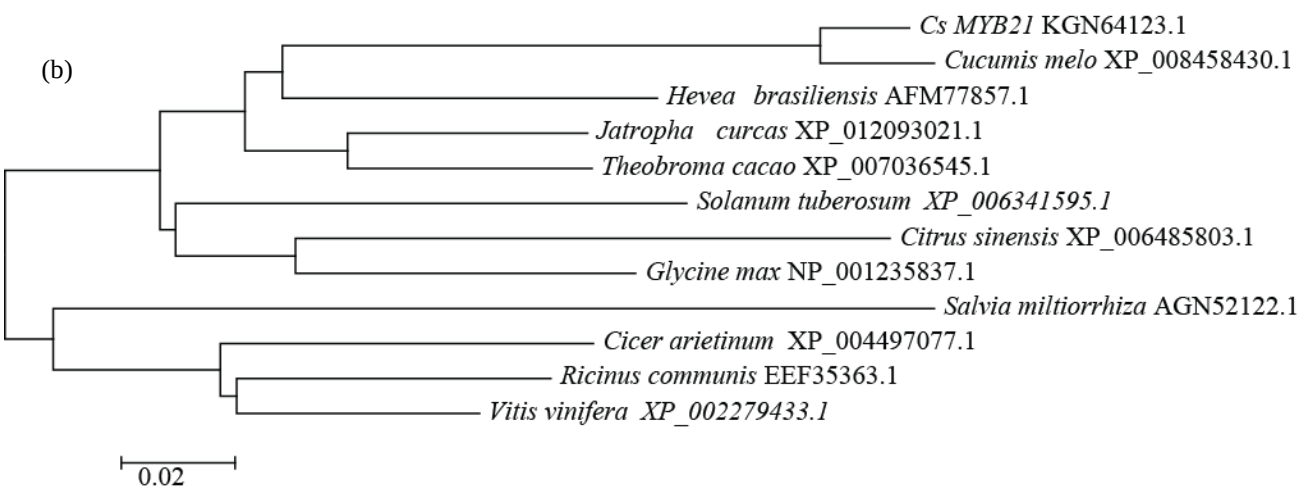

Fig. 2. Sequence and phylogenetic analysis of CsMYB21 in cucumber. (a) Nucleotide sequence and deduced amino acid sequence of *CsMYB21*. (b) Phylogenetic tree of CsMYB21 protein with its homologous proteins. The branch lengths are proportional to distance.

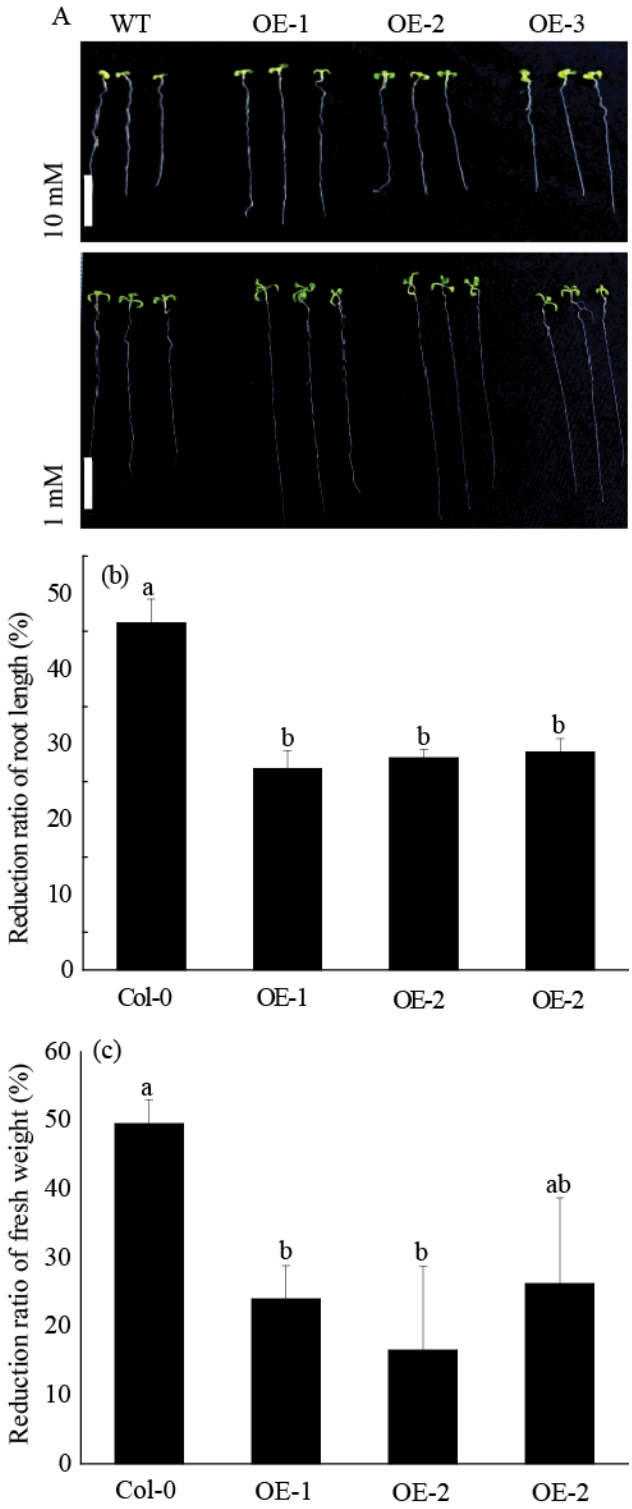

Fig. 3. Phenotypes of transgenic *Arabidopsis* plants harboring *35S::CsMYB21* in response to N conditions. (a) Col-0 and transgenic plants harboring *35S::CsMYB21* grown in 10 mM NO_3^-) and 1 mM NO_3^- conditions for 7 d. bar = 1 cm. (b) Reduction ratio of root length of Col-0 and transgenic plants harboring *35S::CsMYB21*. The primary root lengths of 10-d-old seedlings grown in 10 mM NO_3^- and 1 mM NO_3^- conditions were expressed as means ± SE (n = 10). (c) Reduction ratio of fresh weight of Col-0 and transgenic plants harboring *35S::CsMYB21*. The fresh weights of 10-d-old seedlings grown in 10 mM NO_3^- and 1 mM NO_3^- conditions were expressed as means ± SE (n = 10). Letters represent significant differences at the 0.05 level based on Tukey's test.

Discussion

Results of the present study showed that transgenic line harboring *35S::CsMYB21* had higher capacity for low N tolerance than Col-0. Through this study, we have helped people understanding a new function of the *CsMYB21* that can effectively improve the capacity for low N tolerance of plants.

Previous studies showed that MYB transcription factor is related to the flavonoid. *AtMYB12* is a transcription factor that activates the biosynthesis of flavonoids in *Arabidopsis* (Lännenpää, 2014). The expression of the transcription factor in a variety of plants can cause the increase of the content of flavonoid compounds by transgenic technology (Lännenpää, 2014). The ectopic expression of *AtMYB12* in tobacco promoted the expression of key enzymes in tobacco, and promoted the biosynthesis of flavonoids (Pandey *et al*., 2012). The expression of *AtMYB111* in tobacco transgenic plants promoted the expression of the gene in the pathway of benzene propane, and promoted the improvement of the content of flavonoids (Pandey *et al*., 2014). The expression of *LjMYB101* and *LjMYB102* increased in a large number of N deficiency conditions (Miyake *et al*., 2003). The relationship between the flavonoid pathway and N deficiency has been investigated. For instance, regulation and production of the flavonoid pathway in *Arabidopsis* was up-regulated by N limitation (Olsen *et al*., 2009). It was found that tomato leaf could accumulate flavonol, anthocyanin, petunidin, and flavonol conjugate quercetin-3-O-glucoside under N stress (Bongue-Bartelsman & Phillips, 1995; Stewart *et al*., 2001). Transcription factor, *MYB21*, which regulated the flavonol synthesis, can also be slightly regulated by N deficiency (Lea *et al*., 2007). The results of the present study revealed that transgenic Arabidopsis lines harboring *35S::CsMYB21* showed less reduction of root lengths and fresh weights than Col-o under low N treatment (Fig. 3). Considering the up-regulation of *CsMYB21*unser low N tolerance and the existing knowledge of MYB transcription factor related to flavonoids biosynthesis and low N tolerance, it was proposed that *CsMYB21* might improve the capacity for low N tolerance of plants through the regulation of flavonoids biosynthesis. Although we still need more additional data to confirm such hypothesis, the results of the present study helped people understanding further the molecular function of *CsMYB21* and provided candidate strategy for low N tolerance control of plants.

Acknowledgements

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); 'Young Talents' Project of Northeast Agricultural University (14QC07);

‘Academic backbone’ Project of Northeast Agricultural University (16XG05); Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (Northeast Region), Ministry of Agriculture/Northeast Agricultural University (neauhc201601).

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(Received for publication 6 January 2017)