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Overexpression of *StNF-YB3.1* reduces photosynthetic capacity and tuber production, and promotes ABA-mediated stomatal closure in potato (*Solanum tuberosum* L.)

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ABSTRACT

Nuclear factor Y (NF-Y) is one of the most ubiquitous transcription factors (TFs), comprising NF-YA, NF-YB and NF-YC subunits, and has been identified and reported in various aspects of development for plants and animals. In this work, *StNF-YB3.1*, a putative potato NF-YB subunit encoding gene, was isolated from *Solanum tuberosum* by rapid amplification of cDNA ends (RACE). Overexpression of *StNF-YB3.1* in potato (cv. Atlantic) resulted in accelerated onset of flowering, and significant increase in leaf chlorophyll content in field trials. However, transgenic potato plants overexpressing *StNF-YB3.1* (OEYB3.1) showed significant decreases in photosynthetic rate and stomatal conductance both at tuber initiation and bulking stages. OEYB3.1 lines were associated with significantly fewer tuber numbers and yield reduction. Guard cell size and stomatal density were not changed in OEYB3.1 plants, whereas ABA-mediated stomatal closure was accelerated compared to that of wild type plants because of the up-regulation of genes for ABA signaling, such as *StCPK10-like*, *StSnRK2.6/OST1-like*, *StSnRK2.7-like* and *StSLAC1-like*. We speculate that the acceleration of stomatal closure was a possible reason for the significantly decreased stomatal conductance and photosynthetic rate.

1. Introduction

Nuclear factor Y (NF-Y) transcription factors (TFs) are found in higher eukaryotes and belong to the CCAAT-binding factor (CBF) family [1]. NF-Y TFs play important roles in the regulation of diverse genes in both animal [2] and plant species [3]. NF-Y is a complex protein composed of three subunits: NF-YA (CBF-B), NF-YB (CBF-A) and NF-YC (CBF-C), and all are required for binding to the CCAAT box in target gene promoters to control gene expression [4,5]. Each subunit of the NF-Y complex is encoded by a single gene in yeast and mammalian species in contrast to plants, where each subunit is encoded by multi-member families. The duplicated genes encoding for each subunit have undergone different evolutionary patterns that has resulted in diversified functions in different plant species [6,7].

NF-Y genes have been most extensively researched in *Arabidopsis thaliana*, with a total of 36 *NF-Y* genes (10 *NF-YA*, 13 *NF-YB* and 13 *NF-YC*) having been identified in the *Arabidopsis* genome [8–11]. Various numbers of *NF-Y* genes have been documented in several plant species [6]. The functional redundancies of the *NF-Y* genes have made it challenging to specifically dissect the roles of individual genes. Several *NF-YA* genes in *Arabidopsis*, including *AtNF-YA1*, *3*, *5*, *6*, *8*, and *9*, play redundant roles in male gametogenesis, embryogenesis and seed development [12,13]. Several members of the *AtNF-YB* genes also play a role in embryogenesis and seed development, including *AtNF-YB9*, also known as *LEAFY COTYLEDON 1 (LEC1)* [14,15], and *AtNF-YB6* (*LEC1-like*, *L1L)* [16]. The non-LEC1 members of the NF-YB family have been found to be associated with stress tolerance, chloroplast development and photosynthesis [6]. Diverse functions of *NF-YC* genes were reported in different plant species [6]. The association between NF-YC members with flowering regulators was demonstrated in both *Arabidopsis* [17,18] and tomato [19].

AtNF-YB3 and AtNF-YB2 are the most similar NF-YB members in *Arabidopsis*, sharing 68% amino acid sequences [9,20]. Both AtNF-YB3 and AtNF-YB2 were demonstrated to be essential for the normal induction of flowering by long-days. AtNF-YB2/YB3 forms a trimer complex with AtNF-YC3/YC4/YC9 and AtNF-YA1/A2. This complex could interact with CONSTANS and RGA (REPRESSOR OF ga1-3), activate the expression of *FT* and *SOC1* and initiate floral growth [17,20–25]. Overexpression of *HvNF-YB1*, a barley homologue of *AtNF-YB3*, promoted early flowering in *Arabidopsis*. In addition, analysis of

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barley single-nucleotide polymorphism (SNP) data revealed a significant association of *HvNF-YB3* (a homologue of *AtNF-YB3*) with heading date [26]. These results suggested that NF-YB2 and NF-YB3 have a conserved main function for controlling flowering in plants.

Potato has become the world's third most important food crop, next to rice and wheat [27]. Potato production has been rapidly increasing in the past 40 years, mainly in developing countries [28,29] because potato can be grown in marginal lands. However, potato yields in the developing world average around 10–15 tones per hectare, less than half the average yields achieved by farmers in Western Europe and North America [30]. Modern breeding has made only limited contributions to potato yields in developing countries since modern cultivars showed similar yield potential compared to cultivars developed more than 100 years ago [31]. Potato is an autotetraploid with a highly heterogeneous genome [32]. Thus, it is especially challenging to identify and characterize key genes associated with yield potential, such as those related to flowering, photosynthesis, and tolerance to environmental stresses. However, many such genes have been identified in model plant species and other crops. We are interested in *NF-Y* genes because of the extensive research in other plant species and their roles in various biological functions, including flowering, photosynthesis, yield and response to environmental stresses. The *NF-YB3* ortholog in rice, *OsNF-YB11/HAP3H*, has been demonstrated to promote flowering and heading, and ultimately enhancing yield [33–37]. *TaNF-YB3*, the wheat homologue of *NF-YB3*, was demonstrated to enhance photosynthesis in wheat [38]. In this study, we identified 20 genes encoding NF-YB protein in the potato PGSC database, a putative potato *NF-YB* gene (PGSC0003DMT400010977) was closest to *AtNF-YB3*, *OsNF-YB11/HAP3H* and *TaNF-YB3*, and named as *StNF-YB3.1*. Here we report that the transgenic potato plants overexpressing *StNF-YB3.1* under the constitutive Cauliflower mosaic virus (CaMV) 35S promoter showed early flowering, higher chlorophyll contents, significant reduced photosynthetic capacity, few tuber numbers and less yield compared to the wild type.

2. Materials and methods

2.1. Plant materials and growth condition

Potato (*Solanum tuberosum L.* cv. Atlantic) was used as wild type (WT). Potato seedlings were grown in vitro on MS (Murashige and Skoog) agar medium (with 3% sucrose, 0.6% agar, pH 5.7 ± 0.05), in a growth chamber maintained at 16 h 24 °C/8 h 16 °C light/dark regime, under 50% relative humidity.

Mini-tubers were obtained from both WT and transgenic potato plants in the net house, and prepared for function analysis of *StNF-YB3.1*.

2.2. Identification of potato NF-YB family members

According to the updated classification of plant NF-Y members [3], ten *Arabidopsis thaliana NF-YB* genes obtained from The Arabidopsis Information Resource database (TAIR), were used to search the Potato Genomics Sequence Consortium (PGSC) database. Both BLASTn and BLASTp were used to identify the putative *NF-YB* genes in the potato genome. The full-length sequences of *NF-YB* genes in other species were downloaded from corresponding databases (**Supplementary** Table 1).

2.3. Phylogenetic and gene structure analysis

Alignment was performed by ClustalW and plotted with Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Sequence identity was analyzed by MegAlign in DNASTAR (Lasergene7.1, USA). Phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA, version 7.0.14) software using neighbor-joining via a bootstrap analysis with 1000 replicates. Conserved consensus sequences were created using Weblog [39]. The Extron/intron structure diagrams were constructed using the online Gene Structure Display Server (GSDS) bioinformatic tools (http://gsds.cbi.pku.edu.cn/). The putative *cis*-acting CCAAT motif in the promoter regions was predicted by using the program PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html).

2.4. cDNA cloning

The predicted sequence of *StNF-YB3.1* (PGSC0003DMT400010977) was obtained from PGSC database. Total RNA was extracted from potato leaves using RNAiso Plus Total RNA TRIZOL reagent (Takara Bio, Japan) and treated with RNase-free DNase I (Invitrogen, USA) to remove any contamination with genomic DNA. cDNA synthesis and rapid amplification of 3′ and 5′ cDNA ends (3′RACE and 5′RACE) were performed according to protocol supplied by 5′RACE and 3′RACE System for Rapid Amplification of cDNA End kit (Invitrogen) combined with the specific primers (**Supplementary** Table 2). PCR reaction conditions were as follows: 1 min at 94 °C, 35 cycles consisting of 30 s at 94 °C, 30 s at 65 °C, 60 s at 72 °C, and a final extension step of 5 min at 72 °C.

2.5. Vector construction and generation of transgenic plants

The full-length coding sequence of *StNFYB3.1* was amplified from cv. Atlantic using RT-PCR, cloned into the pENTR directional TOPO vector, and then transferred into the expression binary destination vector pK7GW2.0 by LR reaction (Invitrogen, USA) under the control of the CaMV 35S promoter [40]. The recombinant plasmid was introduced into *Agrobacterium tumefaciens* LBA4404, and transgenic potato plants were obtained by the internode transformation method [41]. The transgenic plants were screened using PCR with primers specific to the *nptII* gene (**Supplementary** Table 2). Amplified fragments were separated on a 1% (w/v) agarose gel. The relative transcript level of *StNF-YB3.1* in all transgenic potato lines was detected by RT-PCR and quantitative real time PCR (qRT-PCR).

2.6. Quantitative real time PCR (qRT-PCR)

RNA samples (1 μg) were reverse transcribed with PrimerScript™ RT reagent kit (Takara Bio) following the manufacturer's instructions. qRT-PCR was performed on a Quantstudio™ 7 Real Time PCR system (Applied Biosystems, USA), using SYBR Premix Ex Taq™ II (Takara Bio) with gene-specific primers (**Supplementary** Table 2). QuantiMix SYBR (Takara Bio) was used for 20 μl PCR reactions as follows: 30 s at 95 °C, and 40 cycles of 5 s at 95 °C, 31 s at 60 °C. The potato *Actin-97* gene (PGSC0003DMT400071331) was used as reference. The relative expression level of each gene was analyzed by the $2^{-\Delta\Delta Ct}$ method [42].

2.7. Field evaluation

Field trials were laid out as randomized block designs with three replicated blocks in 2015, in Huhhot (N40°46′42″, E111°43′34″), Inner Mongolia. Each block contained WT and seven transgenic lines with 10 mini-tubers per plot.

Monitoring of emergence and flowering was carried out every week since the first seedling and flower appeared. Recording the days after planting (DAP) to reach an overall emergence rate of 80 % (Emergence/DAP), and days after planting (DAP) to reach an overall flowering of 80 % (Flowering/DAP). The days after emergence (DAE) to reach an overall flowering of 80 % (Flowering/DAE) was calculated as following:

Flowering/DAE = Flowering/DAP − Emergence/DAP

Plant height was measured weekly following 14 days after emergence. Tuber number and yield per hill were assessed at harvest (fifteen