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Molecular cloning and characterization of a chlorophyll degradation regulatory gene from bamboo

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Abstract

Leaf senescence constituted the final stage of leaf development and it is always accompanied by the leaf yellowing. The non-yellowing gene (*NYE1*), initially identified from *Arabidopsis* in our laboratory, is a key regulatory gene responsible for chlorophyll degradation during senescence. In this study, an orthologue of *AtNYE1* was isolated from the bamboo (*Bambusa emeiensis* cv. Viridiflavus) and tentatively named *BeNYE1*. The full length sequence of 1 386 bp contains an open reading frame of 801 bp. The protein encoded by *BeNYE1* consists of 266 amino acids. Sequence analysis revealed that BeNYE1 had high similarity with other NYE/SGR proteins from various monocotyledon species. *BeNYE1* was strongly induced by natural senescence and dark-induced senescence in bamboo. Driven by a 1.5 kb upstream fragment of *AtNYE1*, *BeNYE1* could rescue the stay-green phenotype of *nye1-1*. The constitutive over-expression of *BeNYE1* could accelerate the chlorophyll degradation. These results indicated that *BeNYE1* might play an important role in the regulation of chlorophyll degradation during leaf senescence in bamboo.

Additional key words: *Bambusa emeiensis*, leaf senescence, non-yellowing gene, stay-green phenotype.

Introduction

Leaf senescence is a final stage of leaf development (Lim *et al*. 2007). Leaf yellowing is the most visible change, which results from the chloroplast disassembly and chlorophyll (Chl) degradation (Balazadeh *et al*. 2008). For many years, Chl degradation during leaf senescence was considered a biological enigma (Hortensteiner 2009). Recently, a key regulatory gene responsible for Chl degradation during leaf senescence was identified among various species through screening for stay-green mutants which was initially termed as non-yellowing gene (*NYE1*) in *Arabidopsis* but it is now widely referred as stay-green (*SGR*; Armstead *et al*. 2006, 2007, Jiang *et al*. 2007, Park *et al*. 2007, Ren *et al*. 2007, Sato *et al*. 2007, Alos *et al*. 2008, Barry *et al*. 2008, Borovsky and Paran 2008, Ren *et al*. 2010, Hu *et al*. 2011, Wei *et al*. 2011). Over-expression of *NYE1* in *nye1-1* mutants results in the whole spectrum of leaf yellowing phenotypes, the severity of which correlates with its expression level (Ren *et al*. 2007).

There are more than 70 genera and 1 200 species of bamboo naturally distributed in Asia, Africa, South America, and Oceania (Chen *et al*. 2011). Their rapid growth, early maturation, high yields and wide application give bamboo a huge advantage and potential in the fields of architecture, papermaking, landscaping, and ecological restoration, *etc*. (Chen *et al*. 2011). So far, very limited gene resources are available from bamboo. To explore the possible genetic ways of increasing its production and improving its green period, we set out to identify the leaf senescence-related genes from bamboo. Here we report the isolation and functional characterization of a chlorophyll degradation regulatory gene, *BeNYE1*, from a bamboo species.

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Abbreviations: Chl - chlorophyll; NYE - non-yellowing; ORF - open reading frame; RACE - rapid amplification of cDNA ends; SGR - stay-green; UTR - untranslated region.
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Materials and methods

Arabidopsis thaliana L. cv. Col-0 and mutant *nye1-1* and bamboo (*Bambusa emeiensis* cv. Viridiflavus) were planted in square pots containing a peat soil + *Vermiculite* + *Perlite* mixture (3:9:0.5, v/v/v; Shanghai Institute of Landscape Science, China) with plant nutrient medium, and grown in a 16-h photoperiod (irradiance of 150 - 180 μmol m^{-2} s^{-1}), day/night temperature of 23/21 ± 1 °C, and relative humidity 70 - 75 %.

For dark treatment, the sixth leaf from 3- to 4-week-old *Arabidopsis* plants was excised and incubated on wet filter paper in total darkness at 23 °C for 4 d. For dark treatment of bamboo, the second leaf from 5- to 6-month-old plants was excised and incubated on wet filter paper in total darkness at 23 °C for 0, 6, 12, 18, 24, or 30 d.

A pair of degenerate PCR primers BeNYE1CS-F (5'-TTTGGTCCGGCGATCTTCGA-3') and BeNYE1CS-R (5'-TGAGAGGACCCCAGCACTCGAC-3') were designed for amplification of partial cDNAs of the *NYE* analogue gene from bamboo based on a multiple alignment of the full-length mRNA sequences from different plant species. Four primers 3'GSP (5'-GGACTCCGCTACTACATC TTCCGCAAG-3'), 3'NGSP (5'-TCTACTTCCACT CCAACCTCCC-3'), 5'GSP (5'-GGAGTTCCTTGCG GAAGATGTAGTAGCG-3'), and 5'NGSP (5'-TGC TTCTCCTCGTCCACCC-3') were designed for 3'-RACE and 5'-RACE cDNA synthesis using the *Smart*™ RACE cDNA amplification kit (*Clontech*, Palo Alto, USA). The clones obtained were sequenced and the overlapping region with the first clone was confirmed. After reconstruction of the open reading frame, a fragment containing the open reading frame was obtained by PCR with the primers BeNYE1FL-F (5'-GCTTTTCTT TCTTGGTCCTGAT-3') and BeNYE1FL-R (5'-GCTTTT CTTTCTTGGTCCTGAT-3') from the senescent leaf cDNA library and sequenced for further confirmation. The primers BeNYE1FL-F and BeNYE1FL-R were also used to amplify the genomic sequence of *BeNYE1*.

Total RNA was extracted using *TRIzol*® reagent (*Invitrogen*, Carlsbad, USA) according to the manufacturer's instructions. The DNase-treated RNA was reverse-transcribed using *M-MLV* reverse transcriptase (*Fermentas*, Vilnius, Lithuania). Real-time RT-PCR was performed with the *SYBR Green I* PCR kit (*Toyobo*, Osaka, Japan) on an *iCycler* according to the manufacturer's (*Bio-Rad*, Hercules, USA) suggestions with *AtACT2* or *BeACT2* as a reference. *SAG12* and a *CAB* homolog in *Bambusa emeiensis* were used as leaf senescence markers to better describe the severity of leaf senescence. The PCR thermal-cycling conditions were as follows: denaturation at 95 °C for 3 min and 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 20 s. All reactions were performed in triplicate. The threshold cycle (C_t) values of the triplicate PCRs were averaged. The relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Partial cDNA fragment of internal control *BeACT2* was obtained by PCR with degenerate primers: (forward: 5'-GAAGCACAATCCAAAAGAGGTAT-3', reverse: 5'-GAGCCTCCGATCCAGACACT-3'). The resulting fragment was cloned to pMD19-T (*Takara*, Dalian, China) and sequenced. Specific primers for real-time PCR to respective genes were as follows: *AtACT2* (forward: 5'-CGCTCTTTCTTTCCAAGCTC-3', reverse: 5'-AACAGCCCTGGGAGCATC-3'), *AtSAG12* (forward: 5'-TGGATACGGCGAATCTACTAACG-3', reverse: 5'-GCTTTCATGGCAAGACCACATAG-3'), *AtSEN1* (forward: 5'-GTCATCGGCTATTTCTCCACCT-3', reverse: 5'-GTTGTCGTTGCTTTCCTCCATC-3'), *BeACT2* (forward: 5'-TTCCTCACGCTATTCTTC GGTTGG-3', reverse: 5'-GCTCATAATCAAGGGCAAC ATAGGC-3'), *BeNYE1* (forward: 5'-CGTCCACTGCC ACATCTCCG-3', reverse: 5'-CTGAACAGGTTGCC GTCGCC-3'), *BeSAG12* (forward: 5'-CAGAGG GAGAGGAAGGTCAGCG-3', reverse: 5'-GAGAAA GCCCAGCAACTCCCG-3'), *BeCAB* (forward:5'-GGC GACTACGGGTGGGACA-3', reverse: 5'-CGCCG AACTTGACGCCATT-3').

Homology searches were performed using *BLASTN* and *BLASTP* (http://www.ncbi.nlm.nih.gov/blast/). Multiple sequence alignment of NYE/SGR proteins was performed using *GeneDoc* software. A phylogenetic tree was constructed with neighbor-joining of *MEGA 3.1* (Kumar *et al*. 2004). The genomic schematic diagram of *BeNYE1* was visualized with the *Gene Structure Display* server (http://gsds.cbi.pku.edu.cn/) (Guo *et al*. 2007).

For the complementation test, a pair of primers (forward: 5'-CAAGAATTCACCTGTTCCAAAG-3', reverse: 5'-CTCTGAGCTCTTGAAACCCA-3') was used to amplify the 1.5 kb promoter of *AtNYE1* from the wild-type *Arabidopsis*. The fragment was subcloned into the *Eco*RI and *Sac*I site of pCHF3 to replace the 35S promoter and sequenced with self-designed primers. The resulting plasmid was named P_{AtNYE1}-pCHF3. A fragment containing the *BeNYE1* ORF was amplified by PCR using two oligonucleotides, 5'-GGGTACCATGGCCGCTGC TGCTG-3' and 5'-GCTCTAGATCGGGTCGGTCA CTGC-3'. The PCR products were cloned into the pMD19-T vector and sequenced. After digestion, the released fragments were subcloned into the *Kpn*I and *Xba*I site of P_{AtNYE1}-pCHF3.

For overexpression of *BeNYE1*, the cDNA fragment containing the *BeNYE1* ORF was digested from P_{AtNYE1}-pCHF3 and subcloned into the *Kpn*I and *Xba*I site of pCHF3. The above constructs were introduced into *A. tumefaciens* strain GV3101 using the freeze-thaw method. *Arabidopsis* plants were transformed using the floral-dip method (Clough and Bent 1998). Putative transgenic plants were selected on plates supplemented with 50 mg dm^{-3} kanamycin and were further verified by PCR.

Chlorophyll content, as a physiological index, was measured to describe leaf senescence. After dark treatment, excised leaves were immediately frozen in liquid N and stored at -80 °C. Chlorophyll content was quantified by spectrophotometry according to the method of Benedetti and Arruda (2002).

Results

A cDNA of 1 386 bp in length, with an open reading frame of 801 bp, was obtained by RACE PCR from the bamboo senescent leaf cDNA library. The cDNA encoded a polypeptide of 266 amino acids, with a putative chloroplast transit peptide of 44 amino acids in the N-terminal region, predicted by online programme *ChloroP1.1* server (http://www.cbs.dtu.dk/services/ChloroP/). *BLAST* search of the *NCBI* databases showed that it belonged to the SGR gene family and shared the highest identity (82 %) with a SGR protein from *Oryza sativa* (acc. No. AAW82954.1) at the amino acid level. It was therefore tentatively named *BeNYE1*. A phylogenetic analysis of 11 SGR proteins revealed that BeNYE1 clustered with the monocotyledon SGR proteins (Fig. 1*B*). According to the PCR-amplified genomic DNA sequence, *BeNYE1* contained three exons and two introns (Fig. 1*C*).

The expression pattern of *BeNYE1* in bamboo was analyzed initially during natural senescence and dark-induced senescence. The qRT-PCR analysis showed that *BeNYE1* was not only responsive to natural senescence (Table 1), but also induced by dark treatment (Table 2). Its transcript level was correlated positively with the expression level of *BeSAG12*, a predicted homologue of *AtSAG12* (Lohman *et al*. 1994) from bamboo, but negatively correlated with the expression level of *BeCAB*, a predicted homologue of *AtCAB* (Hensel *et al*. 1993, Buchanan-Wollaston 1994) from bamboo. In addition, the transcript level of *BeNYE1* negatively correlated with the Chl content (Tables 1, 2).

Table 1. Relative expression levels of *BeNYE1*, *BeSAG12*, and *BeCAB*, and the total chlorophyll (Chl) content [mg g^{-1}(f.m.)] in the leaf tissues at different stages of natural senescence: NS - non-senescent (1 week after new leaf fully expanded), ES - early senescent (2 months after new leaf fully expanded), S - senescent (5 months after new leaf fully expanded). *BeSAG12* - homologue of a natural senescence marker gene in *Arabidopsis*, *AtSAG12*; *BeCAB* - homologue of a negative senescence marker gene in *Arabidopsis*, *AtCAB*. Values are means ± SD of 3 experiments.

	NS	ES	S
BeNYE1	1.00±0.20	7.73±0.51	8.33±0.58
BeSAG12	1.00±0.30	3.02±0.67	8.55±0.98
BeCAB	8.56±1.02	8.14±0.69	1.00±0.35
Chl	3.16±0.31	2.49±0.22	1.74±0.16

To investigate whether *BeNYE1* is a functional homologue of *AtNYE1*, the ORF of *BeNYE1*, driven by a 1.5 kb upstream fragment of *AtNYE1*, was introduced into the *nye1-1* mutant using floral-dipping method. Ten T_3 homologeous transgenic lines (3-6, 5-2, 15-3, 20-4, 21-8, 22-6, 22-7, 23-2, 24-6, and 25-7) were randomly selected for dark treatment, with Col-0 and *nye1-1* as respective controls. After dark incubation for 4 d, the leaves of these lines exhibited a variety of yellowing phenotypes (Fig. 2*A*). The transgenic lines 15-3, 20-4, 22-6, 22-7, 25-7, and Col-0 showed a rapid yellowing phenotype during dark-induced senescence (Fig. 2*A*). The transcript

Table 2. Relative expression of *BeNYE1* and the total chlorophyll (Chl) content [mg g^{-1}(f.m.)] in the bamboo leaf tissues during dark treatment lasting 30 d. Values are means ± SD of 3 experiments.

	0 d	6 d	12 d	18 d	24 d	30 d
BeNYE1	1.00±0.31	3.99±0.80	9.84±0.84	12.14±1.62	27.21±1.98	42.29±1.72
Chl	3.22±0.12	3.21±0.05	3.14±0.27	3.04±0.14	2.95±0.38	1.03±0.08

Table 3. The relative expression of *BeNYE1* and *SEN1* and the total chlorophyll (Chl) content [mg g^{-1}(f.m.)] in the leaves of the ten complemented *Arabidopsis* lines after incubation in the dark for 4 d. Values are mean ± SD of 3 experiments.

	Col-0	*nye1-1*	3-6	5-2	15-3	20-4	21-8	22-6	22-7	23-2	24-6	25-7
BeNYE1	0	0	1.00±0.07	0.43±0.12	1.97±0.14	2.28±0.28	0.44±0.10	3.09±0.29	3.20±0.15	0.08±0.02	0.14±0.08	1.90±0.18
SEN1	0.47±0.11	0.33±0.13	1.00±0.07	0.26±0.03	0.41±0.04	0.55±0.08	0.32±0.02	1.86±0.16	1.66±0.12	0.02±0.01	0.06±0.02	0.38±0.06
Chl	0.49±0.04	1.04±0.03	0.92±0.07	0.96±0.08	0.89±0.07	0.67±0.05	1.05±0.06	0.62±0.04	0.64±0.02	1.06±0.07	1.00±0.08	0.72±0.06

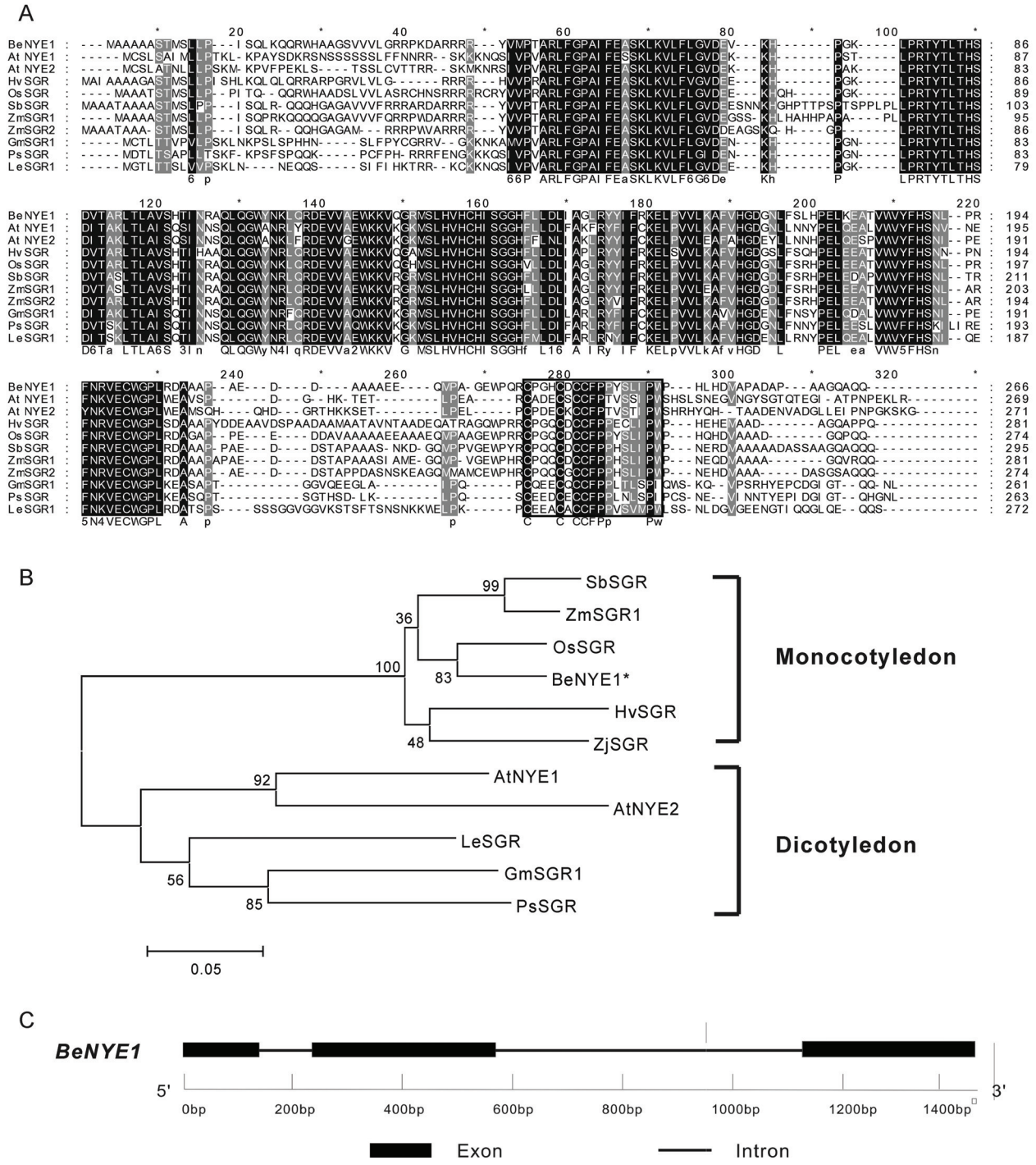

Fig. 1. Sequence and phylogenetic analysis of BeNYE1. *A* - BeNYE1 sequence analysis with *Genedoc* software. *Black box* indicate the conserved C-terminal motif (C-X3-C-X-C2-F-P-X5-P). Identical amino acids are highlighted *in black*, and residues with conservative substitutions are shaded *in gray*. *B* - Phylogenetic relationships among NYE/SGR proteins from 10 plant species. The numbers beside each node represent bootstrap values (≥ 50 %) based on 1 000 replications. The *scale bar* indicates the relative amount of change along branches. BeNYE1 is marked by an *asterisk*. *C* - Schematic diagram of the gene structure of *BeNYE1*. *Black boxes* indicate exons, and *thin black lines* indicate introns. The acc. Nos. of NYE/SGR proteins listed in *A* and *B* are as follows: AtNYE1, DQ437531; AtNYE2, NM_117261.5; GmSGR1, AY850141; PsSGR, AB303332; OsSGR, AY850134.1; ZmSGR1, AY850138.1; ZmSGR2, AY850139.1; ZjSGR1, AY850154.1; SbSGR, AY850140.1; HvSGR, AY850135.1; LeSGR1, AY850152.

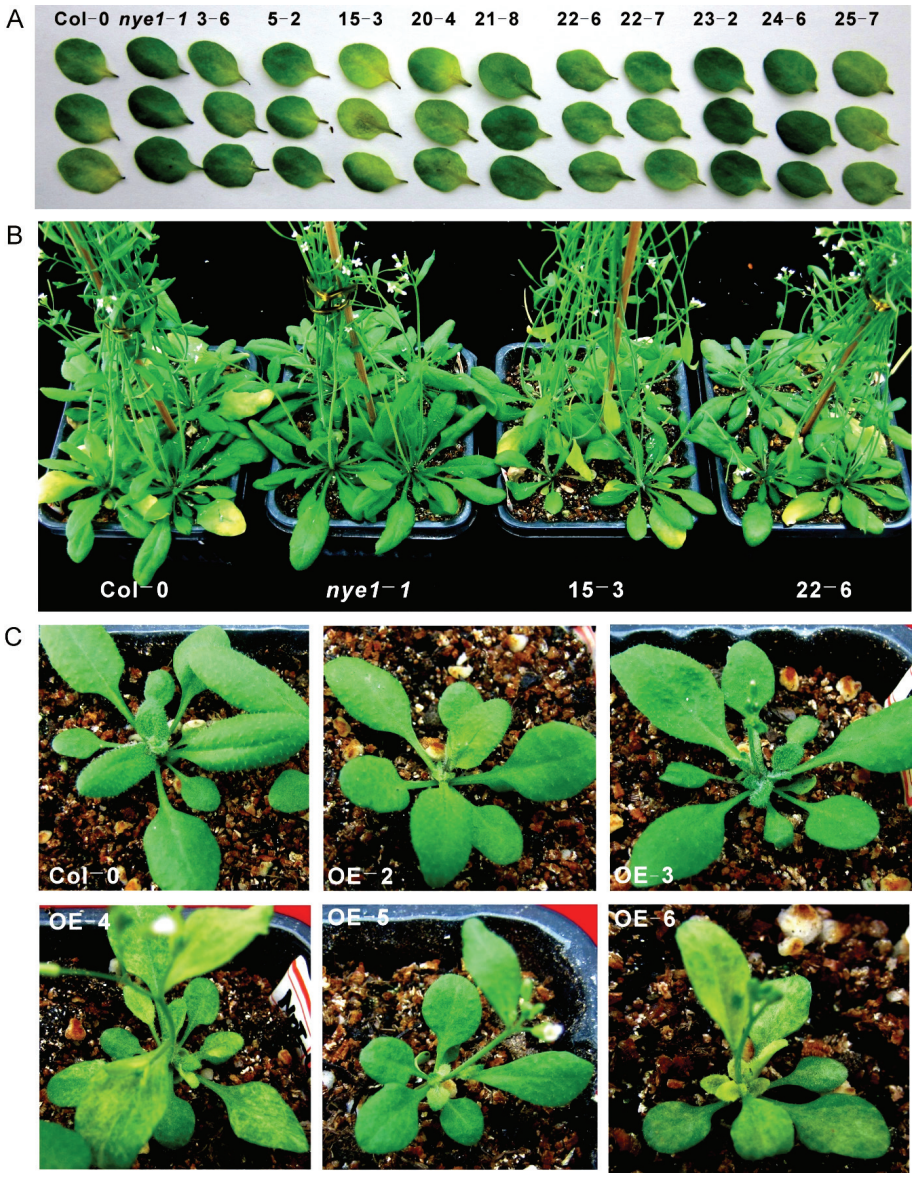

Fig. 2. Phenotype analysis of *BeNYE1* transgenic *Arabidopsis* plants. *A* - Leaves of T_3 complemented lines, Col-0, and *nye1-1* after incubation in the dark for 4 d. 3-6, 5-2, 15-3, 20-4, 21-8, 22-6, 22-7, 23-2, 24-6, and 25-7 represent ten independent lines. *B* - Phenotypes of complemented lines 15-3 and 22-6, Col-0, and *nye1-1* after growth under normal condition for 38 d. *C* - Five representative T_1 *BeNYE1* overexpressors with obvious yellowish phenotypes.

Table 4. Relative expression of *BeNYE1* in the overexpression lines. Values are means ± SD of 3 experiments.

	Col-0	OE-2	OE-3	OE-4	OE-5	OE-6
BeNYE1	0	1.00±0.28	1.10±0.30	1.61±0.37	0.99±0.31	2.73±0.74

level of *BeNYE1* in the detached leaves of the transgenic lines correlated positively with the severity of the yellowing phenotype as well as the *SEN1* expression level (Table 3). After 38-d growth under normal conditions, two complemented lines, 15-3 and 22-6, showed similar senescence phenotypes to that of Col-0 (Fig. 2*B*).

To further functionally characterize *BeNYE1 in planta*, the ORF of *BeNYE1*, driven by the 35S promoter, was introduced into Col-0. After 8-d growth on MS medium, Col-0 and the putative transgenic lines were transplanted into pots. The putative *BeNYE1* overexpressors displayed a variety of de-greening phenotypes after 18-d growth under normal conditions (Fig. 2*C*). Real-time PCR analysis revealed that the severity of the de-greening phenotype correlated well with the relative expression level of *BeNYE1* (Table 4).

Discussion

Bamboos are important part of forest resources. As the bamboo flowering period is long, flowering time is difficult to predict and the whole plant usually died after flowering. It is very difficult to use the conventional cross breeding. The transgenic technology has now become the most promising approach to the genetic improvement of bamboo, however, very limited genetic resources are currently available . In this report, a new senescence-induced gene regulating Chl degradation, *BeNYE1*, was isolated from *Bambusa emeiensis* cv. Viridiflavus.

Previous work revealed that the transcript level of SGR/NYE genes correlated well with the severity of leaf senescence (Alos *et al*. 2008, Armstead *et al*. 2006, 2007, Jiang *et al*. 2007, Park *et al*. 2007, Ren *et al*. 2007, 2010, Barry *et al*. 2008, Borovsky and Paran 2008, Wei *et al*. 2011). The similar expression pattern was observed in this work. *BeNYE1* was not only significantly responsive to natural senescence, but also strongly induced by dark treatment (Tables 2, 3). Its rapid response to senescence signals as well as the sustained high transcription level during senescence made *BeNYE1* an ideal senescence marker in bamboo.

A complementation test was carried out to confirm the function of *BeNYE1*. Driven by a 1.5 kb upstream fragment of *AtNYE1*, *BeNYE1* could restore the stay-green phenotype of *nye1-1* demonstrating that *BeNYE1* is a functional homologue of *AtNYE1*. Besides, the constitutive over-expression of *BeNYE1* in *Arobidopsis* could accelerate the Chl degradation resulting in the leaf yellowing phenotypes (Fig. 2*C*). In addition, it seemed that *BeNYE1* could initiate Chl degradation pathway independent of the leaf age (Fig. 2*C*). These results collectively indicated that *BeNYE1* might play an important role in the regulation of Chl degradation during leaf senescence in bamboo.

Recently, the successful establishment of the regeneration systems of several bamboos including *Bambusa emeiensis* by our laboratory opens up opportunities for incorporating molecular techniques into their improvement programs. The identification of *BeNYE1* in this study brings up a possibility for the enlargement of green period by genetic engineering.

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