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ORIGINAL PAPER

Expression of An Antisense *Brassica oleracea GIGANTEA* (*BoGI*) Gene in Transgenic Broccoli Causes Delayed Flowering, Leaf Senescence, and Post-Harvest Yellowing Retardation

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Abstract Broccoli (*Brassica oleracea* L. var. *italica*) is an important vegetable crop all over the world. However, rapid post-harvest senescence in harvested floral heads reduces its value. Mutation in *GIGANTEA* (*GI*) caused delay of flowering and increased tolerance level to H_2O_2-induced oxidative stress in Arabidopsis. *BoGI*, a *GI* orthologue, was isolated and characterized from *B. oleracea*. *BoGI* mRNA is expressed throughout development and can be detected in leaves, stem, root, and flowers. Further analysis indicated that the expression of *BoGI* is modulated by the circadian clock. To investigate the senescence flowering-associated mechanism regulated by *BoGI* gene and the agricultural application of *BoGI* in controlling flowering time and floret yellowing for *B. oleracea*, constructs containing antisense cDNA of *BoGI* driven by 35S or a flower-specific *AP1* promoter were transformed into *B. oleracea* and the transgenic plants were generated. The flowering time and the senescence of the detached leaves were significantly delayed in transgenic 35S::*BoGI* antisense plants. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis showed that clear reduction of *BoGI* expression was observed in these 35S::*BoGI* antisense plants compared to that in wild-type plants. Furthermore, post-harvest yellow and flower senescence was delayed in AP1::*BoGI* antisense plants. These findings indicate that *BoGI* could be involved in regulation of flowering time, leaf, floret, and flower senescence in broccoli.

Keywords *Agrobacterium*-mediated transformation · Antisense *GIGANTEA* gene · *Brassica oleracea* L. var. *italica* · Delayed flowering · Post-harvest senescence

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Introduction

The transition from vegetative rosette leaf to reproductive inflorescence development has been extensively studied in *Arabidopsis*. This process involves regulation by multiple environmental and endogenous inputs and corresponding genes which have been identified and characterized (Koornneef et al. 1991; Amasino 1996; Levy and Dean 1998; Reeves and Coupland 2000; Araki 2001; Simpson and Dean 2002). Flowering genes such as *FLOWERING LOCUS T* (*FT*) has been thought to be a floral integrator gene, *CONSTANS* (*CO*), and *GIGANTEA* (*GI*) were involved in the photoperiod flowering pathway, whereas *FCA*, *LUMINIDEPENDENS* (*LD*), and *FLOWERING LOCUS D* (*FLD*) have been thought to function in the autonomous flowering pathway to promote flowering (Levy and Dean 1998; Chou and Yang 1998; Reeves and Coupland 2000; Araki 2001; Simpson and Dean 2002). The Arabidopsis *GIGANTEA* (*GI*) gene is an earliest acting conserved gene encoding a protein of 1173 amino acids (Koornneef et al. 1991; Fowler et al. 1999; Park et al. 1999; Mizoguchi et al. 2005). GI protein has no sequence similarity to any known proteins in the database (Park et al. 1999; Fowler et al. 1999) and appears to be absent from genomes of *Chlamydomonas* (microorganism) and animals (Mittag et al. 2005). The *GI* was originally discovered as a late-flowering mutant (Rédei 1962; Koornneef et al. 1991; Araki and Komeda 1993) and contains novel nuclear localization signals (NLSs) (Huq et al. 2000). *GI* positively regulates the

expression of flowering time genes and acts upstream of *CO* and *FT* (Kardailsky et al. 1999; Suárez-López et al. 2001).

In *Arabidopsis*, *gi* mutants flowered later and produced more leaves than wild-type plants in long-day (LD) conditions, but not in short-day (SD) conditions (Koornneef et al. 1991). Ectopic expression of *Arabidopsis* antisense *GI* gene fragment also caused late flowering in transgenic radish (Curtis et al. 2002). This data indicated that downregulation of the *GI* gene could delay bolting in a heterologous plant. *Arabidopsis GI* gene is also important in controlling circadian rhythms (Park et al. 1999; Fowler et al. 1999; Yanovsky and Kay 2002; Edwards et al. 2005; Gould et al. 2006; Kim et al. 2007; Sawa et al. 2007) and phytochrome signaling (Huq et al. 2000). In wild-type *Arabidopsis*, *GI* expression is regulated by circadian clock with the highest expression at 8 to 12 h of the light period and the lowest expression at dawn (Park et al. 1999; Fowler et al. 1999). *GI* has been thought to have a role in early phytochrome signaling by transcriptional regulation of *CCA1* and *LHY* since the expression patterns of these genes are altered in *gi* mutants (Park et al. 1999; Fowler et al. 1999).

In addition to regulate flowering, it has been shown that *GI* might also play a role in response to oxidative stress, cold responses, and salt tolerance (Huq et al. 2000; Cao et al. 2005, 2006, 2007; Kim et al. 2013). Mutation in *GI* significantly increased tolerance level to paraquat or H_2O_2-induced oxidative stress (Kurepa et al. 1998). *GI* has been thought to play a complex role in regulating plant development in diverse ways. It is interesting to explore whether mutation in *GI* can also cause tolerance to other biotic and abiotic stresses in plants and to investigate the possible mechanism of *GI* regulation.

Broccoli, *Brassica oleracea* L. var. *italica*, is a floral vegetable that is consumed worldwide. It contains rich amount of vitamins A and C and isothiocyanine, especially glucoraphanin and its derivative sulforaphane. Broccoli is used for the prevention of breast cancer (Fahey et al. 1997, 2002). Increasing demand for broccoli will necessitate improvement of its agronomic characteristics, such as disease resistance and environmental tolerance. In recent years, several reports have pointed out the merits and biological impacts of transformation technologies (Potrykus 1991; Walden and Wingender 1995; Christou 1996; Birch 1997). Indeed, the genetics of broccoli were recently improved via transgenic breeding (Chen et al. 2008). Antisense approaches regulate fruit ripening, increase floral longevity, and slow post-harvest yellowing have been reported (Smith et al. 1988; Oeller et al. 1991; Gray et al. 1992; Ayub et al. 1996; Aida et al. 1998; Henzi et al. 1999; Giovannoni 2004; Higgins et al. 2006). Broccoli has been transformed with antisenescence genes such as isopentenyl transferase (ipt) and broccoli mutated ethylene response sensor gene (boers) to slow post-harvest yellowing, and transgenic lines with these genes have been developed (Chen et al. 2001, 2004). In this study, we describe that *BoGI*, the *GI* orthologue from broccoli, was involved in leaf senescence, flowering time, post-harvest yellowing and flower senescence in broccoli.

Materials and Methods

Plant Materials

Broccoli (*B. oleracea* var. *italica* cv. Green King) seeds were obtained from the Know-You Seed Company (Kaohsiung, Taiwan). The seeds were surface-sterilized for 2 min with 70 % EtOH, then 20 min for 1 % sodium hypochlorite, followed by five rinses with sterile distilled water. Seeds were then germinated under aseptic conditions on a hormone-free germination medium in Magenta boxes at 25 °C under 16-h light/8-h dark photoperiod with light intensity of 35 μmol m^{-2} s^{-1}. This germination medium containing half strength MS salts (Murashige and Skoog 1962) plus B_5 (Gamborg et al. 1968) vitamins (MSB_5) supplemented with 15 g l^{-1} sucrose and 8.0 g l^{-1} agar buffered to pH 5.7. Explants of cotyledon, hypocotyl, and epicotyl from 7-day-old seedlings were used for transformation.

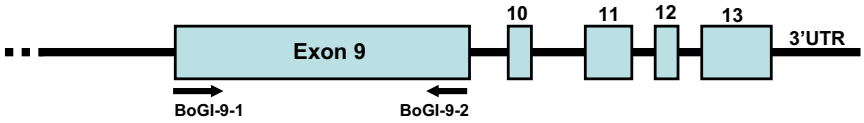
Determination of Antibiotic Sensitivity

A dose-response assay was conducted to determine the optimal concentrations of kanamycin and carbenicillin in the selection medium. Different concentrations of kanamycin (0, 25, 50, 75, and 100 mg l^{-1}) were added to the callus induction medium (MSB_5, 30 g l^{-1} sucrose supplemented with 2.0 μM benzylaminopurine (BAP), 0.5 μM naphthaleneacetic acid (NAA), and 8.0 g l^{-1} agar). As a control, uninfected explants were cultured on selection medium. Explants were cultured on each selection medium for 5 weeks at 25 °C under 16/8-h photoperiod. This experiment was performed with three replications. After 3 weeks, the number of explants producing callus was calculated. In addition, bacterial cells of *A. tumefaciens* were cultured on MSB_5 medium containing different concentrations of carbenicillin (100–400 mg l^{-1}) to determine the appropriate concentration for inhibiting bacterial growth.

The Cloning of *BoGI* and the Construction of *BoGI* Antisense Constructs

A 2.4-kb cDNA fragment containing the sequence from exon 9 to 3′-UTR of *BoGI* (Fig. 1a) was cloned from broccoli (*B. oleracea* var. *italica* cv. Green King) using polymerase chain reaction (PCR) strategy. The 1.7 kb Arabidopsis *AP1* promoter fragment was obtained by PCR using primers AP1p 5′ (5′-GTCTTCAAGGCCACAAGCTTAG-3′) and AP1p 3′ (5′-CTCTAAAGGATCCAAACAAAACAAA-3′). The primers contained the generated *Hind*III (5′-AAGCTT-3′,

a



b

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AtGI  MASSSSSERW  IDGLQFSSLL  WPPPRDPQQH  KDQVVAYVEY  FGQFT--SEQFP DDIAELVRHQ  YPSTEKRLLD  DVLAMFVLHH   PEHGHAVILP  IISCLIDGSL
BoGI
OzGI  --M.A.N.K.  .........F  ....Q.S..K  QA..L.....  .....AD..... E...Q.IQSC  ...K....V.  E.........  .......VH.  .L.RI...T.

AtGI  VYSKEAHPFA  SFISLVCPSS  ENDYSEQWAL  ACGEILRILT  HYNRPIYKTE  QQNGDTERNC  LSKATTSGSP  TSEPKAGSP-T  QHERKPLRPL  SPWISDILLA
BoGI
OzGI  S.DRNGF..M  .....FSHT.  .KE.......  .......V..  ........VD  H.HSEA.CSS  T.DQAS.CES  MEKRAN...RN  EPD.......  ....T.....

AtGI  APLGIRSDYF  RWCSGVMGKY  AA-GELKPPTI  ASRGSGKHPQ  LMPSTPRWAV  ANGAGVILSV  CDDEVARYET  ATLTAVAVPA  LLLPPPTTSL  DEHLVAGLPA
BoGI
OzGI  ..........  .-.G......  ..GGELK...T  Y.........  ..........  ..........  ..E.......  .N...A....  ........P.  .........P

AtGI  LEPYARLFHR  YYAIATPSAT  QRLLLGLLEA  PPSWAPDALD  AAVQLVELLR  AAEDYASGVR  LPRNWMHLHF  LRAIGIAMSM  RAGVAADAAA  ALLFRILSQP
BoGI
OzGI  .........-  ..........  ....F.....  ..........  ..........  .....D..M.  ..K.......  .....T....  ...I...TS.  ..........

AtGI  ALLFPPLSQV  EGVEIQHAPI  GGYSSNYRKQ  ----------  ------IEVP  AAEATIEAT   AQGIASMLCA  HGPEVEWRIC  TIWEAAYGLI  PLNSSAVDLP
BoGI                                                  ....       .........  ..........  ..........  ..........  ......I...
OzGI  T......RHA  ....LH.E.L  ...V.S.KR.  LHIDLYFHMC  SFNCTKL...  .S....D..  ..........  ...D......  .........L  ..S.......

AtGI  EIIVATPLQP  PILSWNLYIP  LLKVLEYLPR  GSPSEACLMK  IFVATVETIL  SRTFPPE-SS  RELTRKARSS  FTTRSATKNL  AMSELRAMVH  ALFLESCAGV
BoGI  ..........  ..........  ..........  ..........  ..........  .......T.I  ----....A.  LA...S....  ..A.......  ..........
OzGI  ..V..A....  .T...S..L.  ....F.....  .........R  .......A..  R.......-T.  -.QS..P..Q  ------S...  .VA...T...  S..V....SM

AtGI  ELASRLLFVV  LTVCVSHEAQ  SSGSKRPRSE  YASTTENIEA  NQPVSNNQTA  N-----RKSR  NVKGQGPVAA  FDSYVLAAVC  ALACEVQLYP  MISGGGNFS-
BoGI  .IP.......  ..........  ......R...  D.TAE.----  ..D---...S  .-----....  ..........  ..........  ..........  .........-
OzGI  D.........  .......Q.L  PG..------  --TGSD.HSS  EEVTNDSRLT  .-----GRN.  CK.R.....T  ..........  ..S.....F.  F..KN..H.-

AtGI  --NSAVAGTI  TKPVKINGSS  KEYGAGIDSA  ISHTRRILAI  LEALFSLKPS  SVGTPWSYSS  SEIVAAAMVA  AHISELFRRS  KALTHALSGL  MRCKWDKEIH
BoGI  --.....A..  ..S.......  ......V...  .K....V...  ..........  .....R....  ..........  ..........  ..........  ..........
OzGI  --.LKDSIK.  VI.G.TT.I.  N.LHNS.S..  .L......G.  ..........  ....S.....  N.........  ..V.....R.  RPCLN...A.  KQ....A..S

AtGI  KRASSLYNLI  DVHSKVVASI  VDKAEPLEAY  LKNTPVQKDS  VTCLNWKQEN  TCASTTCFDT  A-VTSASRTE  MNPRG-NHKY  AR----HSDE  GSG-RPS-EK
BoGI  ..........  ..........  ..........  ...A......  LA......Q.  .SS.AGG.G.  .A...T..N.  .A...G....  ..----....  ...S.S.S..
OzGI  T.........  .L.G.T.T..  .N.......H  .TL...K..E  PP-IEEKNI.  SSDGGALEKK  D-ASRSH.KN  GFA.P-LL.C  .EDVILNG.V  A.----..-G.

AtGI  GIKDFLLDAS  DLANFLTAD-  RLAGFYCGTQ  KLLRSVLAEK  PELSFSVVSL  LWHKLIAAPE  IQPTAESTSA  QQGWRQVVDA  LCNVVSATPA  KAAAAVVLQA
BoGI  ....L.....  .........D  ......R...  V....I....  ..........  .......S..  ..........  ..........  ..........  ..........
OzGI  A.ASLQVE..  .......M.-  .HG.-.R.S.  T......S..  Q..C......  ..Q.......  M.MS......  H....K....  ..DI...S.T  ..S..I....

AtGI  ERELQPWIAK  DDEEGQKMWK  INQRIVKVLV  ELMRNHDRPE  SLVILASASD  LLLRATDGML  VDGEACTLPQ  LELLEATARA  IQPVLAWGPS  GLAVVDGLSN
BoGI  ..........  ..........  .........M  ..........  ..........  ..........  ..........  ..........  ..S.......  ..........
OzGI  .KD......R  ...Q.....R  V......LIA  .......S..  A.........  ..........  ..........  .....V....  VHLI.E..D.  .VS.......

AtGI  LLKCRLPATI  RCLSHPSAHV  RALSTSVLRD  IMNQSSIPI-  KVTPKLP---  TTEKNGMNSP  SYRFFNAASI  DWKADIQNCL  NWEAHSLLST  TMPTQFLDTA
BoGI  ..........  ......N...  ..........  .....G.TTS  .A...P.PTI  ......TD..  .........A.  .......K..  ..........  ..........
OzGI  ......ST..  ..........  ....M.....  .L.SGQ--.-  NSSKLIQ---  GEHR..IQ..  T.QCLA.SII  N.Q..VER.I  E.....RRA.  GLTLA..TA.

AtGI  ARELGCTISL  -SQ          1173
BoGI  .........M  S..          745
OzGI  .K....PLTC  ---          1160
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Fig. 1 Sequence information for the *BoGI* protein. **a** The genomic structure of cloned partial *BoGI*. This 2.4-kb cDNA fragment of partial *BoGI* contained the sequence from entire exon 9 to 13 and the 3′-UTR of *BoGI*. A 1.5-kb antisense DNA fragment encoding entire exon 9 was obtained by PCR using primers BoGI-9-1 and BoGI-9-2. **b** Sequence comparison of *BoGI* (*B. oleracea*), *AtGI* (*Arabidopsis thaliana*), and *OzGI* (rice). The 2.4-kb cDNA fragment of partial *BoGI* encodes 745 amino acids which showed 91 and 63 % sequence identity to *AtGI* and *OzGI*, respectively. The *boxed regions* represent nuclear localization signals (NLSs) sequence. The *underlined regions* represent putative transmembrane domains. Amino acid residues identical to *AtGI* are indicated as *dots*. To improve the alignment, *dashes* were introduced into the sequence

underlined) or *Bam*HI recognition site (5′-GGATCC-3′, underlined) to facilitate the cloning of this cDNA into PBI121 in which 35S promoter was replaced. A 1.5-kb antisense DNA fragment encoding entire exon 9 was obtained by PCR using primers BoGI-9-1 (5′-AGGATCCGCACTATATG GG-3′) and BoGI-9-2 (5′-GGATCCCAGGAGAAGCGATC-3′). Both specific 5′ and 3′ primers contained the generated *Bam*HI recognition site (5′-GGATCC-3′, underlined) to facilitate the cloning of this cDNA. This antisense fragment was cloned into binary vector PBI121 under the control of cauliflower mosaic virus (CaMV) 35S promoter (35S::*BoGI*-anti) or flower specific *AP1* promoter (AP1::*BoGI*-anti).

Plant Transformation

The *Agrobacterium tumefaciens* strain LBA 4404 harboring the binary vector pBI121, with *35S*::*BoGI*-anti or *AP1*::*BoGI*-anti, was used for broccoli transformation. Hypocotyl explants were cultured on callus induction medium for pre-cultivation (0–4 days). The explants were dipped into an overnight suspension of *Agrobacterium*. The bacterial concentration was controlled at 0.8–1.0 OD600 and diluted to tenfold. After overnight of co-incubation, explants were transferred in medium containing half MSB_5 salts, 20 g l^{-1} sucrose, and 8.0 g l^{-1} agar at 25 °C under a 16/8-h (light/dark) photoperiod with light intensity of 45 μmol m^{-2} s^{-1} for 2 days. Explants were washed under shaking in half MSB_5 liquid medium containing 300 mg l^{-1} carbenicillin for 2 days with medium replaced daily. After this washing, explants were transferred to selection medium containing MSB_5, 2.0 μM BAP, 0.5 μM NAA, 300 mg l^{-1} carbenicillin, and 75 mg l^{-1} kanamycin for callus induction. After 3 weeks of culture, well-developed callus was produced from the cut ends of the explants and transferred to MSB_5 medium with 30 g l^{-1} sucrose, 8.0 g l^{-1} agar, and 2.0 μM BAP for induction of transgenic shoots. After 4 weeks of culture, multiple shoots developing from the callus were separated and transferred to rooting medium containing half MSB_5 media supplemented with 20 g l^{-1} sucrose, 2.0 g l^{-1} gelrite, 1.0 μM IBA, and 75 mg l^{-1} kanamycin. The rooted plantlets were washed in sterile distilled water to remove traces of medium and then transferred to plastic pots (5 cm diameter) containing sterile soil, sand, and vermiculite mixture (3:1:1) for 2–3 weeks under 35 μmol m^{-2} s^{-1} with 16/8-h photoperiod before transfer to 30 cm pots. The hardened plants were grown to maturity to collect seeds from the T_0 plants and raised T_1 plants which were also observed under greenhouse condition. The plants (transgenic and non-transgenic) were separately allowed to undergo self-pollination and seeds were collected.

Polymerase Chain Reaction and Reverse Transcriptase Polymerase Chain Reaction Analysis

Total DNA or RNA was isolated from leaves of different putative transgenic plants and non-transformed plants using the Trizol method according to the instructions of the manufacturer. For cDNA synthesis, total RNA (1 μg) was reverse-transcribed in a 20-μl reaction mixture using the BcaBEST™ RNA PCR system (TaKaRa Shuzo Co., Shiga, Japan). A 5.0-μl of cDNA sample from RT reaction was used for PCR. Primers specific for *BoGI*: BoGI-9-1 (5′-AGGATCCGCACTATATGGG-3′) and BoGI-9-2 (5′-GGATCCCAGGAGAAGCGATC-3′); BoGIin-1 (5′-AACCGTAAAAGTAGGAACGTCAAGG-3′) and BoGIin-2 (5′-AGCAGATCTTTGATACCCTTCTCTG-3′). Primers specific for detection of the presence of *BoGI* antisense fragment from genomic DNA of 35S::*BoGI* antisense and AP1::*BoGI* antisense plants: BoGI-9-2 (in BoGI) (5′-GGATCCCAGGAGAAGCGATC-3′) and GUS-2 (in T-DNA) (5′-AGTTCAGTTCGTTGTTCACACA-3′). The fragment was amplified under the following conditions of initial hot start at 95 °C for 5 min, then 35 cycles of denaturation (95 °C; 1 min), annealing (56 °C; 1 min), and extension (72 °C; 1), followed by a final extension of 20 min at 72 °C. The reverse transcriptase polymerase chain reaction (RT-PCR) products were separated on a 1 % agarose gel by electrophoresis and photographed using a Kodak EDAS 290 Electrophoresis documentation system.

Phenotypic Analysis of Leaves, Floral Heads, and Flowers

Leaves from lower parts of mature plants without any symptom of yellow were detached, and floret heads were harvested 7–15 days after the beginning of bolting. The detached leaves and a branch from each floret head were placed in a plastic container, covered with a transparent plastic film, and exposed under light (14 h light/10 h dark) at 25 °C in the controlled room or an incubator for 12 days. Flower senescence analyses were observed under greenhouse condition.

Inheritance Analysis

Seeds (25 seeds from each plant) collected from five transgenic plants (T_0) were sterilized and germinated on MSB_5 basal media supplemented with 75 mg l^{-1} kanamycin. The plates were incubated under the conditions described for in vitro culture. After 1 week, kanamycin sensitive seedlings germinated but bleached quickly, whereas resistant seedlings were green and formed true leaves and roots. The transgenic T_1 plants were analyzed for the presence of the transgenic *BoGI* gene by the PCR assay.

Statistical Analysis

All the experiments were set up in a completely randomized design, and experiments were performed in triplicates and each experiment was repeated twice. The data was expressed as means±standard error. One-way ANOVA analysis followed by Duncan's test was used to determine significant ($P<0.05$) differences. All the statistical analyses were done by using SPSS software version 9.0 (SPSS Inc. USA).

Results

Isolation of *BoGI* cDNA from *B. oleracea*

To investigate the function of *GI* gene in broccoli, the identification and functional analysis of broccoli *GI* homologues is necessary. For this purpose, a partial sequence of broccoli *GI* homologue of (*BoGI*) (Fig. 1a) was cloned and characterized.

This 2.4 kb cDNA fragment contained the sequence from exon 9 to 3′-UTR of *BoGI* and encodes 745 amino acids (Fig. 1b). The *BoGI* showed 91 and 63 % sequence identity to Arabidopsis *AtGI* and rice *OzGI*, respectively (Fig. 1b). Similar to GI, a region contained four nuclear localization signal (NLS) sites was also identified for *BoGI* (Fig. 1b).

Gene Expression of *BoGI*

To further explore the gene expression patterns, the detection of *BoGI* expression is necessary. As shown in Fig. 2a, *BoGI* mRNA is expressed throughout development and can be detected in leaves, stem, root, and flowers. In addition to high sequence homology to *GI* in *Arabidopsis*, the expression of *BoGI* was also regulated by light. The expression level of *BoGI* mRNA varied under LD conditions, with the level highest at the 8 to 12 h of the light period (Fig. 2b, c) similar to that observed for *GI* in Arabidopsis (Fowler et al. 1999). This result suggested that *GI* homologue of broccoli was regulated by mechanisms similar to that in Arabidopsis.

Genetic Transformation of Broccoli

Hypocotyl has a better survival rate than cotyledon or epicotyl (Table 1). Similar results were reported for *B. oleracea* by Chen et al. (2001). After 4 weeks of culture, 90 % callus induction was attained in explants cultured on callus induction medium but lacking kanamycin (Tables 1 and 2). On medium containing kanamycin, the percentage of explants with callus (36 %) was obtained at 25 mg l^{-1}. At 75 mg l^{-1}, 100 % of explants bleached and died (Table 2). To analyze the effect of *35S*::*BoGI* antisense and *AP1*::*BoGI* antisense in *B. oleracea*, *Agrobacterium*-mediated genetic transformation was performed. In broccoli, the reported kanamycin concentrations employed for *nptII* gene selection were 50 mg l^{-1} (De Block et al. 1989) and 25 mg l^{-1} (Metz et al. 1995). Chen et al. (2001) reported that 75 mg l^{-1} kanamycin for selection, over 60 % of survival plantlets was positive for both the *nptII* and the *ipt* genes. We have used 75 mg l^{-1} kanamycin for the transformation experiments.

The effect of pre-culture on the frequency of transformation in broccoli was examined using hypocotyl explants cultured on MSB_5 medium supplemented with 2.0 μM BAP and 0.5 μM NAA for 3 days. Similar reports have also addressed the adaptation of pre-culture for the success of transformation (McHughen et al. 1989; Sangwan et al. 1992; Metz et al. 1995; Villemont et al. 1997), and it was shown that putative transformation competent cells could be increased by pretreatment (Sangwan et al. 1992). After 2 days of co-cultivation with *35S*::*BoGI* antisense, the explants were transferred onto the callus induction medium consisted of MSB_5 containing 2.0 μM BAP, 0.5 μM NAA, 300 mg l^{-1} carbenicillin, and 75 mg l^{-1} kanamycin. This was in accordance with other

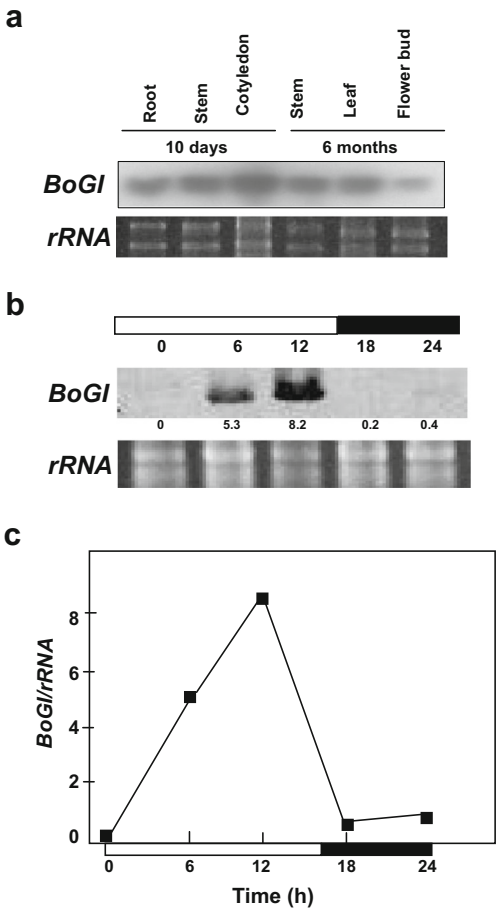

Fig. 2 Detection of expression of *BoGI* by RT-PCR analysis. **a** Total RNA isolated at 8 h of the light period from root, stem, cotyledon of a 10-day-old seedlings, and stem, leaf, and flower bud of a 6-month-old *B. oleracea* var. *italica* were used as templates to detect the expression of *BoGI*. rRNA stained in an EtBr gel was used to show the amount of RNA used for each RT-PCR reactions. **b** To examine whether the expression of *BoGI* was influenced by light, total RNA was isolated and analyzed from leaf of 25-day-old *B. oleracea* var. *italica* plants every 6 h. Sample collection started at the beginning of the light period (time 0) and continued every 6 h for 24 h in LD conditions. Each experiment was repeated three times with similar results. rRNA stained in an EtBr gel was used to show the amount of RNA used for each RT-PCR reactions. The related strength of the *BoGI* expression for each transgenic plant were listed below. **c** A statistical result from **b** which clearly showed that the expression level of *BoGI* mRNA varied under LD conditions, with the level highest at the 8 to 12 h of the light period and lowest at dawn

Brassica species (Metz et al. 1995; Paul and Sikdar 1999; Cao et al. 2008). Callus initiation was observed in selective medium within 4 weeks of culture, while non-transformed control explants on selective medium turned yellow and did not produce calli. Transgenic callus were transferred to MSB_5 containing 2.0 μM BAP for induction of transgenic shoots. Shoots that survived this selection stage were transgenic and were transferred to rooting medium containing half MSB_5 media supplemented with 20 g l^{-1} sucrose, 2.0 g l^{-1} gelrite, 1.0 μM IBA, and 75 mg l^{-1} kanamycin. The rooted plantlets were transferred to pots, acclimated for 2 weeks in the culture room,

Table 1 Effect of growth regulators on callus induction, shoot formation and root induction from different seedling explants of *Brassica oleracea* L. var. *italica*

Growth regulators (μM)		Percent of callus per explant			No. of shoots per calli			Percent of root induction		
		Hypocotyl	Epicotyl	Cotyledon	Hypocotyl	Epicotyl	Cotyledon	Hypocotyl	Epicotyl	Cotyledon
BAP	NAA									
1.0	0.5	58.76±0.23^{b}	39.50±0.50^{b}	34.33±0.66^{b}						
2.0	0.5	90.66±0.33^{a}	70.66±0.88^{a}	60.66±0.66^{a}						
3.0	0.5	57.83±0.16^{c}	35.33±0.33^{c}	31.33±0.33^{c}						
1.0					15.33±0.86^{b}	10.33±0.33^{b}	8.06±0.24^{b}			
2.0					35.66±0.66^{a}	21.33±0.66^{a}	17.33±0.33^{a}			
3.0					16.60±0.40^{b}	7.60±0.40^{c}	6.16±0.44^{c}			
IBA										
0.5								68.00±1.00^{b}	55.50±0.28^{b}	47.00±0.57^{b}
1.0								86.66±0.66^{a}	71.06±0.63^{a}	62.00±0.28^{a}
1.5								64.50±0.50^{c}	49.33±0.88^{c}	43.03±0.57^{c}

Data represents mean values±SE of three replicates; each experiment was repeated twice. Mean separations within column by Duncan's multiple range test at $P<0.05$ level are indicated by different letters. Mean values of three independent experiments (±) with standard errors

and were moved to the greenhouse; similar to that for the non-transgenic rooted plants generated from the tissue culture. Progeny analysis for most transgenic T_0 plants in 75 mg l^{-1} kanamycin medium showed a 3:1 ratio for kanamycin resistant/susceptible phenotype, indicating a likely single gene insertion.

Delay of Flowering and Leaf Senescence was Observed in *35S*::*BoGI* Antisense of *B. oleracea*

The construct contained antisense of *BoGI*, a *GI* homologue of *B. oleracea*, driven by the CaMV 35S promoter was transformed into broccoli, and ten transgenic plants (T_1) were obtained and analyzed. These *35S*::*BoGI* antisense transformants showed similar late-flowering phenotype that differed strongly from non-transformed broccoli plants. The flowering time for these *35S*::*BoGI* antisense plants was significantly delayed (Fig. 3a). Compared to non-transformed wild-type broccoli that flowered at about 100 days after sowing, *35S*::*BoGI* antisense plants flowered at about 180 days after sowing (Fig. 4a). When the detached leaves from these *35S*::*BoGI* antisense plants were examined, their senescence was significantly delayed (Fig. 3b–e). Detached leaves from non-transformed wild-type broccoli were senescent within 3 days by showing the dehydration of the leaf tissue and diminution of the chloroplasts resulting in the yellowish color of the leaves (Fig. 3c). By contrast, leaves from *35S*::*BoGI* antisense plant were not senescent even after 5 days of detachment (Fig. 3c). At 7 days after detachment, wild-type leaves were completely senescent, whereas only slight yellowing was observed in *35S*::*BoGI* antisense leaves (Fig. 3d). At 12 days after detachment, wild-type leaves were completely dry (Fig. 3e, f), whereas petiole and part of the leaf were still green in *35S*::*BoGI* antisense leaves (Fig. 3e, g). This result indicated that *BoGI* regulates flowering and leaf senescence in broccoli.

Table 2 Effect of kanamycin concentrations on the callus formation and bud induction from hypocotyl explants of *B. oleracea* L. var. *italica*

Kanamycin (mg l^{-1})	Number of explants used	Callus formation rate (%)	Average number of buds/explant
0	35	90.33±0.88^{a}	35.33±0.88^{a}
25	32	36.00±1.15^{b}	1.06±0.008^{b}
50	30	15.66±0.88^{c}	0.53±0.003^{b}
75	32	0	0
100	32	0	0

Data represents mean values±SE of three replicates; each experiment was repeated twice. Mean separations within column by Duncan's multiple range test at $P<0.05$ are indicated by different letters. Mean values of three independent experiments (±) with standard errors

The presence of the *BoGI* antisense gene in transgenic *35S*::*BoGI* antisense plants was further confirmed by the PCR assay (Fig. 5a). To explore whether the phenotype correlated to *BoGI* expression in *35S*::*BoGI* antisense plants, RT-PCR analysis was performed. As shown in Fig. 5b, a clear reduction of *BoGI* expression was observed in these *35S*::*BoGI* antisense plants compared to that in wild-type plants. This result indicated that the phenotypes generated in *35S*::*BoGI* antisense transgenic broccoli were due to the repression of the *BoGI* expression.

Delay of Floret Yellowing was Observed in *AP1*::*BoGI* Antisense of *B. oleracea*

To provide more evidence that *GI* function was related with senescence, construct containing antisense of *BoGI* driven by the flower specific *AP1* promoter was transformed into

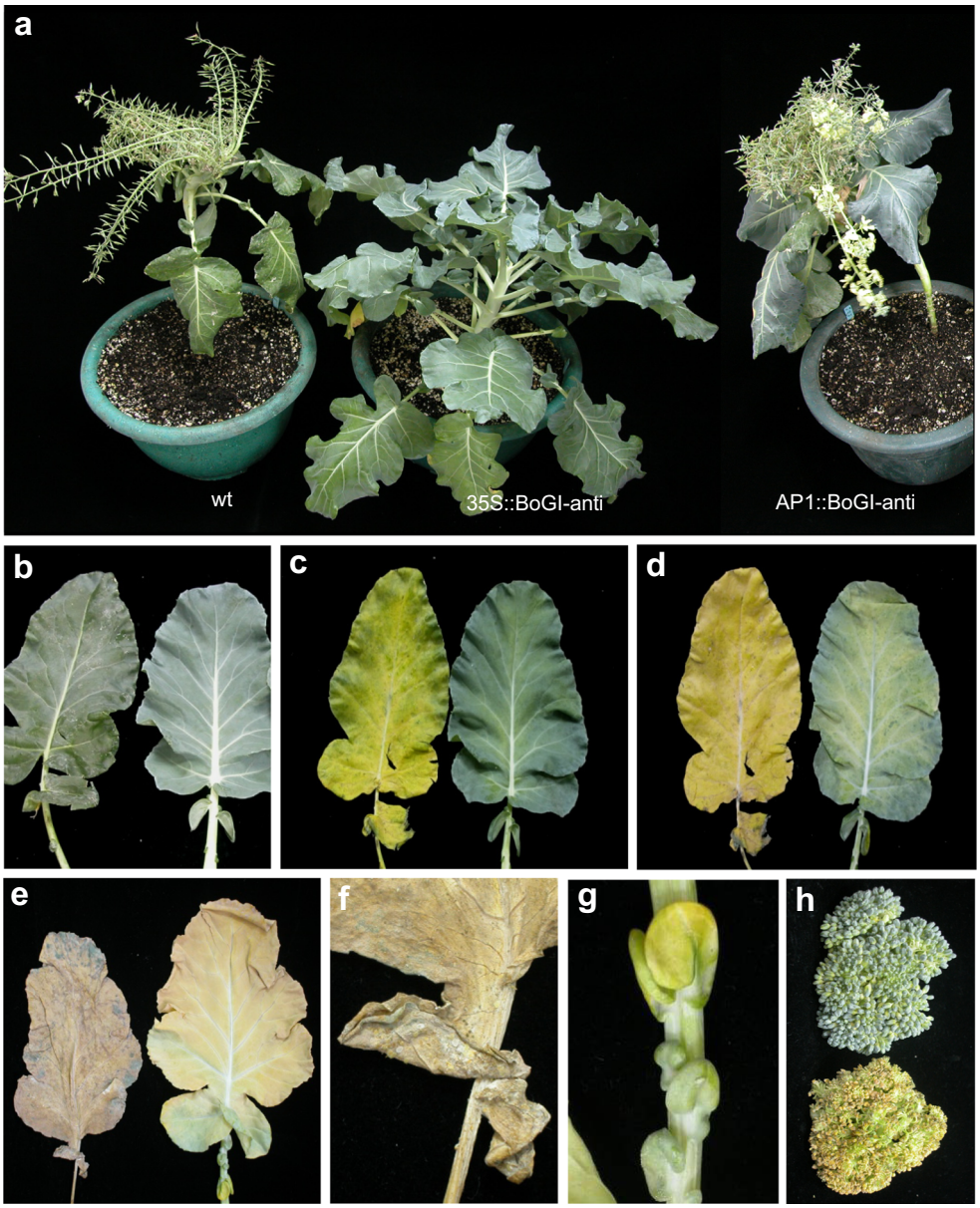

Fig. 3 Phenotypic analysis of transgenic (T_1) *B. oleracea* var. *italica* ectopically expressing antisense of *BoGI*. **a** A *35S*::*BoGI* antisense broccoli plant (*middle*) flowered significantly later than wild-type (*left*) and *AP1*::*BoGI* antisense plant (*right*). At 180 days after germination, wild-type and *AP1*::*BoGI* antisense plants already flowered and set siliques and seeds, whereas *35S*::*BoGI* antisense plant remained in vegetative stage without flowering. **b–e** Detached leaves from wild-type broccoli (*left*) were senescent within 3 days by showing the dehydration of the leaf tissue and diminution of the chloroplasts resulting in the yellowish color of the leaves. By contrast, leaves from *35S*::*BoGI* antisense plant (*right*) was not senescent after 5 days of detachment. **b** 1 day, **c** 5 days, **d** 7 days, and **e** 12 days after detachment. **f** Close-up of the wild-type leaf petiole in **e**. In this stage, leaf tissue was completely dried. **g** Close-up of the *35S*::*BoGI* antisense leaf petiole in **e**. In this stage, some leaf tissue still remained green. **h** The floral head of an *AP1*::*BoGI* antisense broccoli plant (*top*) was not senescent 7 days after detachment, whereas the floral head of a wt broccoli plant (*bottom*) was completely senescent and turned to yellow in the same stage

broccoli and ten transgenic plants (T_1) were obtained and analyzed. The flowering time for these *AP1*::*BoGI* antisense plants was similar to the non-transformed plants (Figs. 3a and 4a). When the post-harvested florets from these *AP1*::*BoGI* antisense plants were examined, their yellowing was significantly delayed (Fig. 3h). Post-harvested florets from non-transformed wild-type broccoli were yellow within 3 days. By contrast, florets from *AP1*::*BoGI* antisense plant were not yellow even after 7 days of harvested florets (Fig. 3h). At 7 days after harvested florets, wild-type florets were completely yellowing, whereas no yellowing was observed in *AP1*::*BoGI* antisense florets (Fig. 3h). When the flower senescence time was measured in plants from flowering until fading, the flower senescence for these *AP1*::*BoGI* antisense plants was also significantly delayed (Fig. 4b). Compared to non-transformed wild-type broccoli flowers that were senescent at about 15 days after flowering, *AP1*::*BoGI* antisense flowers faded at about 25–30 days after flowering (Fig. 4b). This result indicated that *BoGI* also regulates floret and flower senescence in broccoli. The presence of the *BoGI* antisense gene in transgenic *AP1*::*BoGI* antisense plants was further confirmed by the PCR assay (Fig. 5c).

Discussion

In an attempt to investigate the roles for the *GI* gene in regulating plant development beside flowering, *GI* orthologue *BoGI* was cloned and characterized from *B. oleracea*. *BoGI* contains several characteristics that were conserved among *GI*

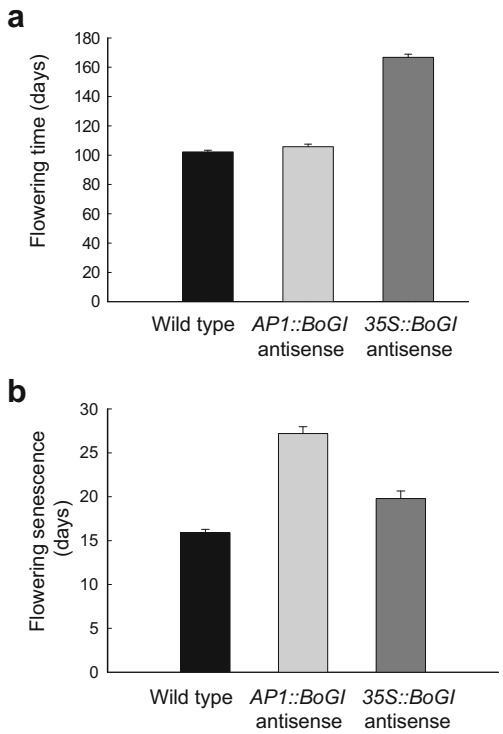

Fig. 4 Comparison of flowering time in non-transgenic and *BoGI* antisense transgenic plants (T_1) of *B. oleracea* var. *italica*. **a** Flowering time of wild type, *AP1*::*BoGI* antisense, and *35S*::*BoGI* antisense transgenic broccoli under long-day condition. The genotype tested is shown along the horizontal axis and the average from 10 (T_1) anti-*BoGI* transformants (*AP1*:: and *35S*::), and non-transgenic plants were compared. *Vertical bar* represented SD. **b** Comparison of flower senescence time in non-transgenic and transgenic plants of *AP1*::*BoGI* antisense and *35S*::*BoGI* antisense. The data measured survival days of 10 mature flowers from flowering until fading. *Vertical bar* represented SD

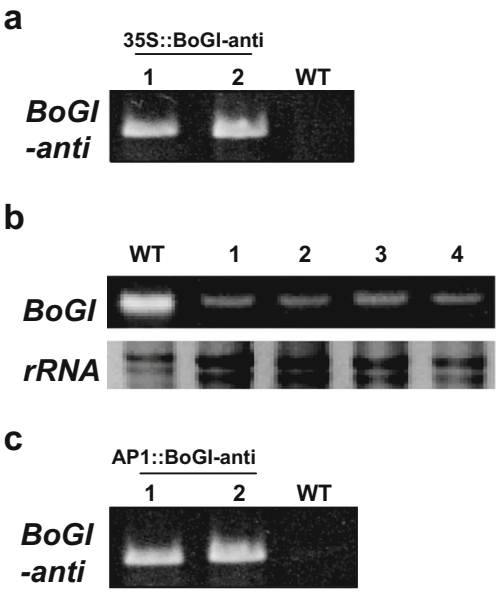

Fig. 5 PCR and RT-PCR assay for transgenic (T_1) *B. oleracea* var. *italica* ectopically expressing antisense of *BoGI*. **a** Detection of the presence of the DNA fragment for *BoGI* antisense gene in two *35S*::*BoGI* antisense transgenic (T_1) *Brassica* plants (*1–2*) by PCR. A 1.5-kb fragment was amplified in these two transgenic plants, whereas no band can be amplified in wild-type (*WT*) plant. **b** Detection of the *BoGI* expression in four *35S*::*BoGI* antisense transgenic (T_1) *Brassica* plants (*1–4*) and in a non-transgenic wild-type (*WT*) plant through RT-PCR analysis. rRNA stained in an EtBr gel was used to show the amount of RNA used for each RT-PCR reactions. **c** Detection of the presence of the DNA fragment for *BoGI* antisense gene in two *AP1*::*BoGI* antisense transgenic (T_1) *Brassica* plants (*1–2*) by PCR. A 1.5-kb fragment was amplified in these two transgenic plants, whereas no band can be amplified in wild-type (*WT*) plant

orthologues. For example, all four putative NLS regions identified in GI protein (Fowler et al. 1999; Huq et al. 2000) were found in the BoGI proteins (Fig. 1b). Furthermore, similar to *GI* (Park et al. 1999; Fowler et al. 1999), *BoGI* mRNA is also regulated by circadian clock with the level highest at 8 to 12 h of the light period and lowest at dawn (Fig. 2b, c). Based on the sequence, structure, and expression similarity, it is clear that *BoGI* is *B. oleracea GI* orthologue.

Leaf senescence exhibits chloroplast degradation and declined photosynthetic activity that finally results in cell death, which could be regarded as a form of programmed cell death (Noodén and Leopold 1978; Gan and Amasino 1997; Lim et al. 2003; van Doorn and Woltering 2004). The terminal phase of leaf senescence was indicated by loss of antioxidant capacity and increased release of reactive oxygen species (ROS) in plants (Leshem 1988; Buchanan-Wollaston 1997; Zimmermann and Zentgraf 2005). Since *gi* mutants significantly increased tolerance level to H_2O_2-induced oxidative stress (Kurepa et al. 1998), the senescence of the leaf was therefore analyzed in antisense *BoGI* plants of *B. oleracea* in this study. Ectopic expression of *GI* homologue *BoGI* antisense did not only cause the late flowering but also the delay of leaf senescence phenotypes in transgenic broccoli. Therefore, in addition to promote flowering time, the ability for *GI* homologues to regulate leaf senescence in plants was confirmed. Since we only tested the flowering time and leaf senescence under long-day conditions (14 h light/10 h dark), whether the delay of the flowering time and leaf senescence in *35S*::*BoGI* antisense transgenic broccoli was similar under short-day conditions remains to be investigated. Interestingly, the correlation between flowering time and leaf senescence has been reported. For example, the development of early flowering and leaf senescence under long-day condition was observed in *vtcl* and *vtc2* mutants (Barth et al. 2006). Delay of flowering and senescence was also reported in *HDA6* mutant of *axe1-5* (Wu et al. 2008). These data further support the idea that a complex mechanism regulating both flowering and leaf senescence.

More recently, researchers have been studying early gene changes in an attempt to understand the regulation of post-harvest senescence (Coupe et al. 2003; Eason et al. 2005; Gapper et al. 2005a, b; Page et al. 2001; Pogson et al. 1995;

Pramanik et al. 2005). External application of cytokinins is known to retard yellowing in broccoli (Rushing 1990; Clarke et al. 1994; Tian et al. 1995). Rushing (1990) reported that post-harvest broccoli florets deteriorated rapidly and became unmarketable in 3 to 4 days, while florets treated with benzyladenine or zeatin lasted yellow retardation for 5 to 6 days. Harvested broccoli deteriorates quickly due to relatively high respiration and susceptibility to wilting when stored at room temperature (Gillies and Toivonen 1995). Wang (1977) studied the length of broccoli shelf life and observed that florets showed significant yellowing after 2–3 days at 20 °C and essentially complete yellowing in 4 days. The yellowing is caused by sepal chlorophyll degradation, but the pedicel and stem did not turn yellow after harvest (Corcuff et al. 1996). In this study, we found that the floret of *AP1*::*BoGI* antisense transgenic broccoli did not turn yellow even after 7 days of being harvested. By contrast, wild-type florets were completely yellowing at 7 days after being harvested. Furthermore, the flower senescence of the mature flowers in these *AP1*::*BoGI* antisense plants was significantly delayed compared to non-transformed wild-type and *35S*::*BoGI* antisense broccoli flowers. *35S*::*BoGI* antisense flowers faded at about 20 days after flowering, slightly later than that in non-transformed wild-type plant (about 15 days) (Fig. 4b). Thus, we have demonstrated that the expression of an anti-*GI* homologue gene fragment in broccoli could cause effects in delaying flowering time, leaf senescence, and post-harvest yellow retardation.

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References

Aida R, Yoshida T, Ichimura K, Goto R, Shibata M (1998) Extension of flower longevity in transgenic torenia plants incorporating ACC oxidase transgene. Plant Sci 138:91–101

Amasino RM (1996) Control of flowering time in plants. Curr Opin Genet Dev 6:480–487

Araki T (2001) Transition from vegetative to reproductive phase. Curr Opin Plant Biol 4:63–68

Araki T, Komeda Y (1993) Analysis of the role of the late flowering locus, *GI*, in the flowering of *Arabidopsis thaliana*. Plant J 3:231–239

Ayub R, Guis M, Amor MB, Gillot L, Roustan JP, Latche A, Bouzayen M, Pech JC (1996) Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits. Nat Biotechnol 14:862–866

Barth C, Tullio MD, Conklin PL (2006) The role of ascorbic acid in the control of flowering time and the onset of senescence. J Exp Bot 57:1657–1665

Birch RG (1997) Plant transformation: problems and strategies for practical application. Annu Rev Plant Physiol Plant Mol Biol 48:297–326

Buchanan-Wollaston V (1997) The molecular biology of leaf senescence. J Exp Bot 48:181–199

Cao SQ, Ye M, Jiang ST (2005) Involvement of *GIGANTEA* gene in the regulation of the cold stress response in *Arabidopsis*. Plant Cell Rep 24:683–690

Cao SQ, Jiang ST, Zhang RX (2006) The role of *GIGANTEA* gene in mediating the oxidative stress response and in *Arabidopsis*. Plant Growth Regul 48:261–270

Cao SQ, Song YQ, Su L (2007) Freezing sensitivity in the *gigantean* mutant of *Arabidopsis* is associated with sugar deficiency. Biol Plant 51:359–362

Cao J, Shelton AM, Earle ED (2008) Sequential transformation to pyramid two *Bt* genes in vegetable Indian mustard (*Brassica juncea* L.) and its potential for control of diamondback moth larvae. Plant Cell Rep 27:479–487

Chen LFO, Hwang JY, Charng YY, Sun CW, Yang SF (2001) Transformation of broccoli (*Brassica oleracea* var. italica) with isopentenyltransferase gene via *Agrobacterium tumefaciens* for postharvest yellowing retardation. Mol Breed 7:243–257

Chen LFO, Huang JY, Wang YH, Chen YT, Shaw JF (2004) Ethylene insensitivity and postharvest yellowing retardation in mutant ethylene response sensor (boers) gene transformed broccoli (*Brassica oleracea* var. italica.). Mol Breed 14:199–213

Chen LFO, Lin CH, Kelkar SM, Chang YM, Shaw JF (2008) Transgenic broccoli (*Brassica oleracea* var. *italica*) with antisense chlorophyllase (*BoCLH1*) delays postharvest yellowing. Plant Sci 174:25–31

Chou ML, Yang CH (1998) *FLD* interacts with genes that affect different developmental phase transitions to regulate *Arabidopsis* shoot development. Plant J 15:231–242

Christou P (1996) Transformation technology. Trends Biotechnol 1:423–431

Clarke SF, Jameson PE, Downs C (1994) The influence of 6-benzylaminopurine on post-harvest senescence of floral tissues of broccoli *Brassica oleracea* var. *italica*. Plant Growth Regul 14:21–27

Corcuff R, Arul J, Hamza F, Castaigne F, Makhlouf J (1996) Storage of broccoli florets in ethanol vapor enriched atmosphere. Postharvest Biol Technol 7:219–229

Coupe SA, Sinclair BK, Watson LM, Heyes JA, Eason JR (2003) Identification of dehydration-responsive cysteine proteases during post-harvest senescence of broccoli florets. J Exp Bot 54:1045–1056

Curtis IS, Nam HG, Yun JY, Seo KH (2002) Expression of an antisense *GIGANTEA* (*GI*) gene fragment in transgenic radish causes delayed bolting and flowering. Transgenic Res 11:249–256

De Block M, De Brouwer D, Tenning P (1989) Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression bar and new genes in transgenic plants. Plant Physiol 91:694–701

Eason JR, Ryan DJ, Watson LM, Hedderley D, Christey MC, Braun RH, Coupe SA (2005) Suppression of the cysteine protease, aleurain, delays floret senescence in *Brassica oleracea*. Plant Mol Biol 57:645–657

Edwards KD, Lynn JR, Gyula P, Nagy F, Millar AJ (2005) Natural allelic variation in the temperature compensation mechanisms of the *Arabidopsis thaliana* circadian clock. Genetics 170:387–400

Fahey JW, Zhang Y, Talalay P (1997) Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. Proc Natl Acad Sci U S A 94:10367–10372

Fahey JW, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, Talalay P, Lozniewski A (2002) Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of *Helicobacter pylori* and prevents benzo[a]pyreneinduced stomach tumors. Proc Natl Acad Sci U S A 99:7610–7615

Fowler S, Lee K, Onouchi H, Samach A, Richardson K, Morris B, Coupland G, Putterill J (1999) *GIGANTEA*: a circadian clock controlled gene that regulates photoperiodic flowering in *Arabidopsis*

and encodes a protein with several possible membrane-spanning domains. EMBO J 18:4679–4688

Gamborg O, Miller R, Ojima K (1968) Nutrients requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gan S, Amasino RM (1997) Making sense of senescence. Molecular genetic regulation and manipulation of leaf senescence. Plant Physiol 113:313–319

Gapper NE, Coupe CA, McKenzie MJ, Sinclair BK, Lill RE, Jameson PE (2005a) Regulation of harvest-induced senescence in broccoli (*Brassica oleracea* var. *italica*) by cytokinin, ethylene, and sucrose. J Plant Growth Regul 24:153–165

Gapper NE, Coupe SA, McKenzie MJ, Scott RW, Christey MC, Lill RE, McManus MT, Jameson PE (2005b) Senescence-associated down-regulation of 1-aminocyclopropane-1-carboxylate (ACC) oxidase delays harvest-induced senescence in broccoli. Funct Plant Biol 32:891–901

Gillies SL, Toivonen PMA (1995) Cooling method influences the post-harvest quality of broccoli. HortSci 30:313–315

Giovannoni JJ (2004) Genetic regulation of fruit development and ripening. Plant Cell 16:170–180

Gould PD, Locke JCW, Larue C, Southern MM, Davis SJ, Hanano S, Moyle R, Milich R, Putterill J, Millar AJ, Hall A (2006) The molecular basis of temperature compensation in the *Arabidopsis* circadian clock. Plant Cell 18:1177–1187

Gray J, Picton S, Shabbeer J, Schuch W, Frierson D (1992) Molecular biology of fruit ripening and its manipulation with antisense genes. Plant Mol Biol 19:69–87

Henzi MX, Christey MC, McNeil DL, Davies KM (1999) *Agrobacterium rhizogenes*-mediated transformation of broccoli (*Brassica oleracea* L. var. *italica*) with an antisense 1-aminocyclopropane-1-carboxylic acid oxidase gene. Plant Sci 143:55–62

Higgins JD, Newbury HJ, Barbara DJ, Muthumeenakshi S, Puddephat IJ (2006) The production of marker-free genetically engineered broccoli with sense and antisense ACC synthase 1 and ACC oxidase 1 and 2 to extend shelf-life. Mol Breed 17:7–20

Huq E, Tepperman JM, Quail PH (2000) GIGANTEA is a nuclear protein involved in phytochrome signaling in *Arabidopsis*. Proc Natl Acad Sci U S A 97:9789–9794

Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, Chory J, Harrison MJ, Weigel D (1999) Activation tagging of the floral inducer *FT*. Science 286:1962–1965

Kim WY, Fujiwara S, Suh SS, Kim J, Kim Y, Han L, David K, Putterill J, Nam HG, Somers DE (2007) ZEITLUPE is a circadian photoreceptor stabilized by *GIGANTEA* in blue light. Nature 449:356–360

Kim WY, Ali Z, Park HJ, Park SJ, Cha JY, Perez-Hormaeche J, Quintero FJ, Shin G, Kim MR, Qiang Z, Ning L, Park HC, Lee SY, Bressan RA, Pardo JM, Bohnert HJ, Yun DJ (2013) Release of SOS2 kinase from sequestration with GIGANTEA determines salt tolerance in Arabidopsis. Nat Commun 4:1352–1364

Koornneef M, Hanhart CJ, van der Veen JH (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. Mol Gen Genet 229:57–66

Kurepa J, Smalle J, Van Montagu M, Inze D (1998) Effects of sucrose supply on growth and paraquat tolerance of the late-flowering *gi- 3* mutant. Plant Growth Regul 26:91–96

Leshem YY (1988) Plant senescence processes and free radicals. Free Rad Biol Med 5:39–49

Levy YY, Dean C (1998) The transition to flowering. Plant Cell 10:1973–1990

Lim PO, Woo HR, Nam HG (2003) Molecular genetics of leaf senescence in Arabidopsis. Trends Plant Sci 8:272–278

McHughen A, Jordan M, Feist G (1989) A preculture period prior to *Agrobacterium* inoculation increase production of transgenic plants. J Plant Physiol 135:245–248

Metz TD, Dixit R, Earle ED (1995) *Agrobacterium tumefaciens*-mediated transformation of broccoli (*Brassica oleracea* var. italica) and cabbage (*B. oleracea* var. capitata). Plant Cell Rep 15:287–292

Mittag M, Kiaulehn S, Johnson CH (2005) The circadian clock in *Chlamydomonas reinhardtii*. What is it for? What is it similar to? Plant Physiol 137:399–409

Mizoguchi T, Wright L, Fujiwara S, Cremer F, Lee K, Onouchi H, Mouradov A, Fowler S, Kamada H, Putterill J, Coupland G (2005) Distinct roles of *GIGANTEA* in promoting flowering and regulating circadian rhythms in *Arabidopsis*. Plant Cell 17:2255–2270

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Noodén LD, Leopold AC (1978) Phytohormones and the endogenous regulation of senescence and abscission. In: Letham DS, Goodwin PB, Higgins TJV (eds) Phytohormones and related compounds: A comprehensive treatise, vol II. Elsevier, Amsterdam, pp 329–369

Oeller PA, Ming-Wong L, Taylor L, Pike DA, Theologis A (1991) Reversible inhibition of fruit senescence by antisense RNA. Science 254:437–439

Page T, Griffiths G, Buchanan-Wollaston V (2001) Molecular and biochemical characterization of postharvest senescence in broccoli. Plant Physiol 125:718–727

Park DH, Somers DE, Kim YS, Choy YH, Lim HK, Soh MS, Kim HJ, Kay SA, Nam HG (1999) Control of circadian rhythms and photoperiodic flowering by the *Arabidopsis GIGANTEA* gene. Science 285:1579–1582

Paul S, Sikdar SR (1999) Expression of *npt* II marker and *gus* reporter genes and their inheritance in subsequent generations of transgenic *Brassica* developed through *Agrobacterium* mediated gene transfer. Curr Sci 76:1569–1573

Pogson BJ, Downs CG, Davies KM (1995) Differential expression of two 1-aminocyclopropane-1-carboxylic acid oxidase genes in broccoli after harvest. Plant Physiol 108:651–657

Potrykus I (1991) Gene transfer to plants: assessment of published approaches and results. Annu Rev Plant Physiol Plant Mol Biol 432: 205–225

Pramanik BK, Matsui T, Suzuki H, Kosugi Y (2005) A sucrose synthase gene from broccoli: cDNA cloning sequencing and its expression during storage. Biotechnology 4:288–295

Rédei GP (1962) Supervital mutants of Arabidopsis. Genetics 47:443–460

Reeves PH, Coupland G (2000) Response of plant development to environment: control of flowering by daylength and temperature. Curr Opin Plant Biol 3:37–42

Rushing JW (1990) Cytokinins affect respiration, ethylene production, and chlorophyll retention of packaged broccoli florets. HortSci 25:88–90

Sangwan RS, Bourgeois Y, Brown S, Vasseur G, Sangwan-Norreel B (1992) Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation in *Arabidopsis thaliana*. Planta 188:439–456

Sawa M, Nusinow DA, Kay SA, Imaizumi T (2007) FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. Science 318:261–265

Simpson GG, Dean C (2002) *Arabidopsis*, the Rosetta stone of flowering time? Science 296:285–289

Smith CJS, Watson CF, Ray J, Bird CR, Morris PC, Schuch W, Grierson D (1988) Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. Nature 334:724–726

Suárez-López P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G (2001) *CONSTANS* mediates between the circadian clock and the control of flowering in Arabidopsis. Nature 410: 1116–1120

Tian MS, Davies L, Downs CG, Liu XF, Lill RE (1995) Effects of floret maturity, cytokinin and ethylene on broccoli yellowing after harvest. Postharvest Biol Tech 6:29–40

van Doorn WG, Woltering EJ (2004) Senescence and programmed cell death: substance or semantics? J Exp Bot 55:2147–2153

Villemont E, Dubois F, Sangwan RS, Vasseur G, Bourgeois Y, Sangwan-Norreel B (1997) Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of *Petunia*: evidence of an S-phase control mechanism for T-DNA transfer. Planta 201:160–172

Walden R, Wingender R (1995) Gene-transfer and plantregeneration techniques. Trends Biotechnol 13:324–331

Wang CY (1977) Effect of aminoethoxy analog of rhizobitoxine and sodium benzoate on senescence of broccoli. HortSci 12:54–56

Wu K, Zhang L, Zhou C, Yu CW, Chaikam V (2008) HDA6 is required for jasmonate response, senescence and flowering in Arabidopsis. J Exp Bot 59:225–234

Yanovsky MJ, Kay SA (2002) Molecular basis of seasonal time measurement in Arabidopsis. Nature 419:308–312

Zimmermann P, Zentgraf U (2005) The correlation between oxidative stress and leaf senescence during plant development. Cell Mol Biol Lett 10:515–553