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ORIGINAL ARTICLE

The *Arabidopsis* beta-carotene hydroxylase gene promoter for a strong constitutive expression of transgene

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Abstract To efficiently express a gene of interest in transgenic plants, the choice of promoter is a crucial factor as it directly affects the expression of the transgene that will yield the desired phenotype. The *Arabidopsis* β-carotene hydroxylase 1 gene (*AtBch1*) shows constitutive and ubiquitous expression and was thus selected as one of best candidates for constitutive promoter analysis by both in silico northern blotting and semi-quantitative RT-PCR analysis. To investigate *AtBch1* promoter activity, the 1,981-bp 5′-upstream region of this gene was fused with β-glucuronidase (GUS) and transformed into *Arabidopsis*. Through the molecular characterization of transgenic leaf tissues, the *AtBch1* promoter generated strong activity that drives 1.8- and 2-fold higher GUS expression than the cauliflower mosaic virus 35S (35S) promoter at the transcriptional and translational levels, respectively. Furthermore, the GUS enzyme activity driven by the *AtBch1* promoter was 2.8-fold higher than that produced by the 35S promoter. By histochemical GUS staining, the ubiquitous expression of the *AtBch1* promoter was observed in all tissues of *Arabidopsis*. Semi-quantitative RT-PCR analysis with different tissues further showed that this promoter serves as a strong constitutive driver of transgene expression in dicot plants.

Keywords β-Carotene hydroxylase · Constitutive · Promoter · Transgenesis · Transit peptide · Ubiquitous

Introduction

The choice of promoter has a potent influence on the eventual regulation of the foreign genes expressed in transgenic plants and should therefore be carefully considered in advance with regard to subsequent functional analysis of the target gene and production of the genetically modified (GM) crop. Gene promoters are generally classified into three groups based on their activity in plant cells: i.e., constitutive, tissue-specific, and inducible (Carolina 2003). A constitutive promoter is preferentially used for functional studies requiring whole plant expression of the transgene and not when specific expression in diverse tissues or inducible conditions is required.

At present, the most commonly used constitutive promoter for plant biotechnology is the cauliflower mosaic virus 35S (35S) promoter and its derivatives (Odell et al. 1985; Omirulleh et al. 1993). The strongest known constitutive promoter in plants is a super-promoter that consists of a trimer of the *Agrobacterium* octopine synthase transcriptional activating element (*ocs* activator) linked to the mannopine synthase 2′ (*mas2′*) activator/promoter region. This promoter has been shown to express β-glucuronidase (GUS) at 2- to 20-fold higher levels than the "enhanced" double Cauliflower Mosaic Virus (CaMV) 35S promoter (Ni et al. 1995). However, the use of promoters derived from plant pathogens like viruses and *Agrobacterium* spp. can result in less acceptance of the GM crop by consumers (Ahmad et al. 2009).

Constitutive promoters derived from plant sources have been differentially developed to optimize gene expression

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according to whether the plant is a monocot or dicot and not to promote public acceptance. Well-characterized constitutive promoters such as the rice *Act1* promoter and maize *Ubi1* promoter originate from monocot plants (McElroy et al. 1991; Katiyar-Agarwal et al. 2002), and their relative activities tend to be lower in dicot plants (Assem et al. 2002). Several constitutive promoters have been isolated from dicot plants, including the *Arabidopsis* ubiquitin and actin2 promoters, tobacco *iCUP* promoter, and sweet potato *AGP1* promoter (Callis et al. 1990; An et al. 1996; Malik et al. 2002; Kwak et al. 2007; Ahmad et al. 2009). At present, however, none is widely used, and this has resulted in a shortage of suitable promoters to drive the constitutive and ubiquitous expression of transgenes in dicot plants.

Since the *Arabidopsis* whole genome sequence was annotated in 2000 (Arabidopsis Genome Initiative 2000), several in silico searches of *Arabidopsis* databases, including those containing microarray data, have been become available from public sources (Joy 2002). A microarray database such as AtGenExpress (http://www.arabidopsis.org/info/expression/AtGenExpress.jsp) contains the expression profiles of whole sets of genes involved in a particular metabolic pathway, such as the carotenoids at different developmental stages in various tissues. It would be useful to screen candidate genes to identify constitutive or tissue-specific promoters that could be utilized in dicot transgenesis.

Carotenoids which are essential as secondary antenna components of the reaction center complexes in photosynthesis are pigments synthesized by the plastidic methylerythritol phosphate (MEP) pathway that forms part of the isoprenoid biosynthetic system in plants (Ha et al. 2003; DellaPenna and Pogson 2006). The genes involved in the carotenoid metabolic pathway are differentially expressed during development in diverse plant tissues (Ha et al. 2007; Clotault et al. 2008). Hence, these genes are strong candidates for promoter analysis due to their diverse expression patterns.

Within the *Arabidopsis* whole genome, more than 29 carotenogenic genes have been identified, including 5 MEP pathway genes that serve isopentenyl diphosphate (IPP) and its allyl isomer dimethylallyl diphosphate (DMAPP) as carotenoid precursors, 4 geranylgeranyl pyrophosphate synthase (GGPS) genes, 11 carotenoid biosynthetic genes encoding phytoene synthase, phytoene desaturase, ζ-carotene desaturase, carotenoid isomerase, lycopene-β-cyclase, lycopene-ε-cyclase, β-carotene hydroxylase 1, β-carotene hydroxylase 2, ε-carotene hydroxylase, zeaxanthin epoxidase, violaxanthin de-epoxidase, and 9 carotenoid cleavage dioxygenase (CCD) genes (DellaPenna and Pogson 2006). All the corresponding expression patterns in silico can be sourced from the AtGenExpress database and then compared on the basis of levels and localization.

In our present study, we selected the *Arabidopsis* β-carotene hydroxylase 1 gene (*AtBch1*), which is involved in the conversion of zeaxanthin from β-carotene through a β-cryptoxanthin intermediate, as a candidate for promoter analysis. We thus tested the *AtBch1* promoter for its ability to drive constitutive and ubiquitous expression. The expression of endogenous *AtBch1* in the whole *Arabidopsis* plant was confirmed by semi-quantitative RT-PCR in addition to in silico northern data from the online public sources. The strength of activity and the spatial expression profile of the *AtBch1* promoter were characterized in comparison with those of the *35S* promoter using *Arabidopsis* transgenic analyses at a molecular level to evaluate its usefulness as a strong constitutive promoter.

Materials and methods

RT-PCR analysis

Total RNAs were isolated from various tissues of *Arabidopsis* using Plant RNA Purification Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA from each tissue (1 μg) was simultaneously synthesized to first-strand cDNA and amplified using an mRNA Selective PCR Kit (Takara, Tokyo, Japan). Semi-quantitative PCR was performed for 25 cycles using the gene specific primer sets for *AtBch1* (5′-GATTCTCTCCGTCTCTTCG-3′/5′-AATGAAAGAGGGACTTACG-3′), *Gus* (5′-ACCTGCGTCAATGTAATGTTCTGC-3′/5′-CTCCCTGCTGCGGTTTTTCA-3′), and *AtEF1α* (5′-GTTCACATTAACATTGTGGTCATT-3′/5′-CAGGTACCAGTGATCATGTTCTTG-3′) with expected products of 560, 478, and 305 bp, respectively.

Vector construction and *Arabidopsis* transformation

A 781-bp region of the dual 35S promoter was amplified by PCR with the primer set 35SP-B1/35SP-B2 (5′-AAAAAGCAGGCTAGAGATAGATTTGTA-3′/5′-AGAAAGCTGGGTATGGTGGAGCACGA-3′) from the pCAMBIA 2301 vector (Cambia, Canberra, Australia). A putative promoter region of 1,981 bp 5′-upstream of the *AtBch1* gene (At4g25700) was amplified with the primer set AtBch1P-B1/AtBch1P-B2 (5′-AAAAAGCAGGCTGCCTTAATCTTGTCTGC-3′/5′-AGAAAGCTGGGTCCTAATGGAAGGAGGAG-3′) from *Arabidopsis* genomic DNA. To attach a 153-bp transit peptide (TP) as a following sequence to the putative *AtBch1* promoter, a reverse primer AtBch1P-TP-B2 (5′-AGAAAGCTGGGTTCGACGACGTAACAGA-3′) was additionally designed and used with the AtBch1P-B1 primer to amplify a 2,134-bp fragment.

Each PCR product was incorporated into the Gateway® destination vector pBGWFS7 (VIB-Ghent University, Ghent, Belgium) through several gateway cloning steps as previously described (Chung et al. 2008). Both constructs were transformed into the *Arabidopsis* ecotype Columbia (Col-0) after mediation by the *Agrobacterium* strain GV3101.

Western blot analysis

For protein gel-blot analysis, protein extracts from transgenic and non-transgenic wild-type *Arabidopsis* leaves was separated on a 12% SDS/polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Oh et al. 2008). The immunoreactive proteins was detected using a primary polyclonal antibody raised against recombinant GUS protein (Fitzgerald, Concord, MA, USA) and an anti-rabbit IgG (Fc) alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI, USA).

Histochemical and fluorometric GUS assay

To perform a GUS histochemical assay, several *Arabidopsis* tissues from transgenic and non-transgenic plants were stained with 1% 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) solution, and observed and image-captured as previously described (Chung et al. 2008). For a 4-methyl umbelliferyl β-D-glucuronide (MUG) assay of GUS activity, various tissues of transgenic and non-transgenic *Arabidopsis* plants were ground in extraction buffer (50 mM sodium phosphate buffer pH 7.0, 10 mM dithiothreitol, 1 mM disodium EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100). The protein concentration of the supernatants was measured using a Qubit fluorometer (Invitrogen). The GUS enzyme activity levels of the supernatants were assayed with buffer containing 1 mM MUG as the fluorescent substrate and using the FluorAce™ β-glucuronidase Reporter Assay Kit (Bio-Rad, Hercules, CA, USA). Values were calculated from triplicate experiments with each independent transgenic line.

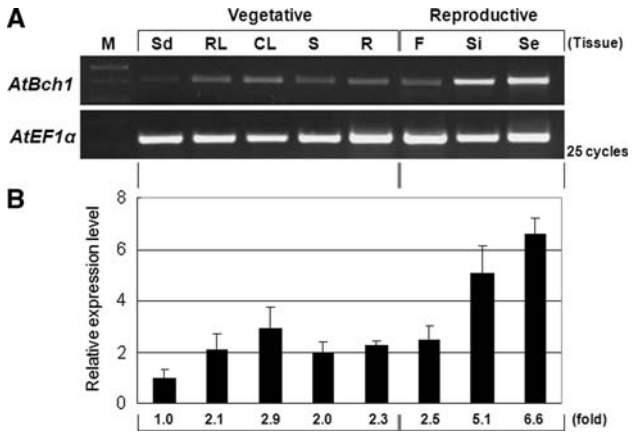

Fig. 1 Spatial gene expression pattern of *AtBch1* in various tissues of wild-type *Arabidopsis*. **a** Semi-quantitative RT-PCR analysis of *AtBch1* expression in seedling (*Sd*), rosette leaf (*RL*), cauline leaf (*CL*), stem (*S*), root (*R*), flower (*F*), silique (*Si*), and seed (*Se*) of wild-type *Arabidopsis*. *Arabidopsis* elongation factor 1-α (*AtEF1α*) was used as a normalization control. **b** Relative expression levels measured using the Quantity One program (version 4.4.1; Bio-Rad). *Error bars* show the standard deviations (SD) of three replicates

Results and discussion

Endogenous expression of the *AtBch1* gene

Using the information deposited at the AtGenExpress microarray database (http://www.arabidopsis.org/info/expression/AtGenExpress.jsp) during development in different tissues of *Arabidopsis*, the expression profiles of whole gene sets involved in a carotenoid metabolic

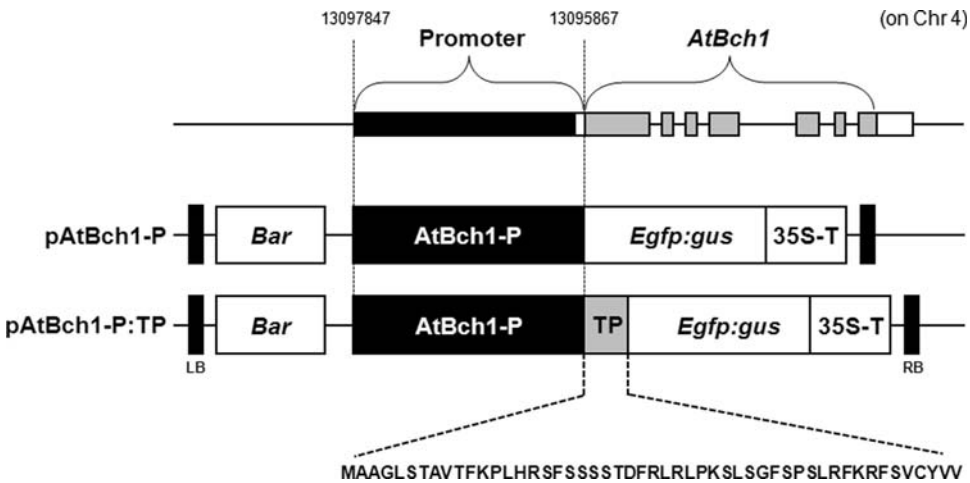

Fig. 2 Schematic representation of the binary vectors used for *Arabidopsis* transformation. DNA fragments including AtBch1-P and AtBch1-P:TP extracted from *Arabidopsis* chromosome 4 were introduced into pBGWFS7 by Gateway cloning. *AtBch1-P* *AtBch1* promoter, *Bar* bialaphos resistant gene, *Egfp-gus* fusion gene of the enhanced green fluorescent protein gene and β-glucuronidase genes, *35S-T* cauliflower mosaic virus 35S terminator, *TP* transit peptide, *LB* left border, *RB* right border

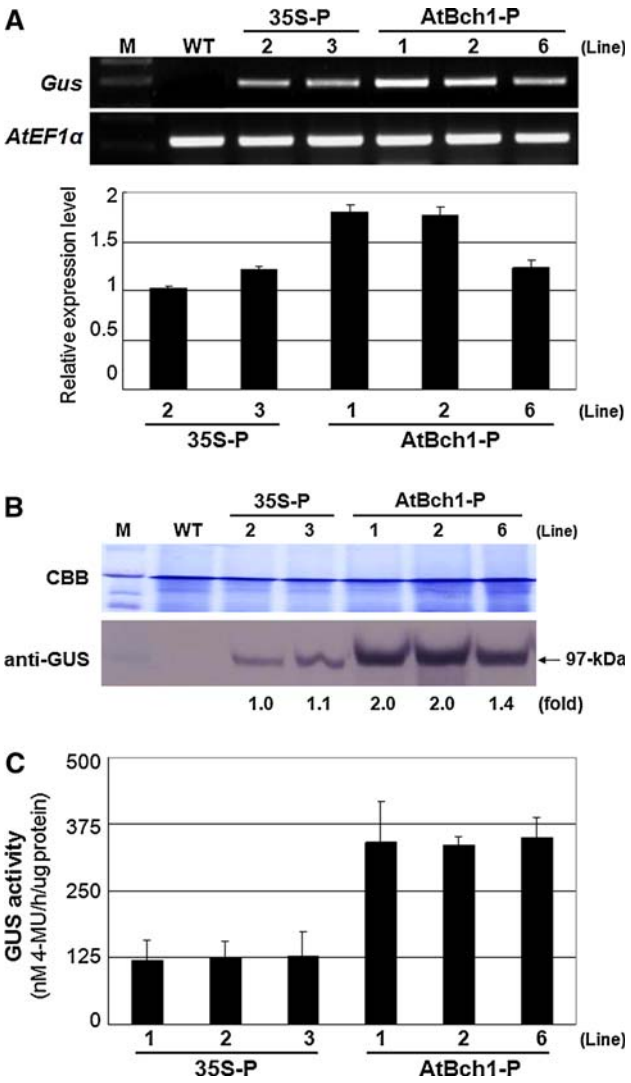

Fig. 3 Comparison of *35S* and *AtBch1* promoter strength in the leaf tissues of independent transgenic *Arabidopsis* plants. **a** Semi-quantitative RT-PCR analysis with *Gus* specific primers. **b** Western-blot analysis with anti-GUS antibodies. *CBB* Coomassie brilliant blue R-250 dye. The relative gene expression levels and fold expression changes in **a** and **b** were calculated using Quantity One (version 4.4.1; Bio-Rad). **c** Enzymatic values for the GUS activity levels quantified using a fluorometric MUG assay. *Error bars* show the standard deviations (SD) of three replicates for **a** and **c**

pathway were analyzed to screen constitutive and tissue-specific promoter candidates for transgenic plant production. Among the 29 carotenogenic genes within the *Arabidopsis* genome, the promoter of the *AtBch1* gene was considered to be a strong candidate for use in transgenic applications as it drives high and even levels of expression in all tissues including cotyledon, leaf, stem, root, flower, silique, and seed (Electronic supplementary material, ESM, Table S1). The presence of *AtBch1* mRNA transcripts has been confirmed previously in the leaf, flower, stem, root, and silique tissues of *Arabidopsis* using a TaqMan RT-PCR

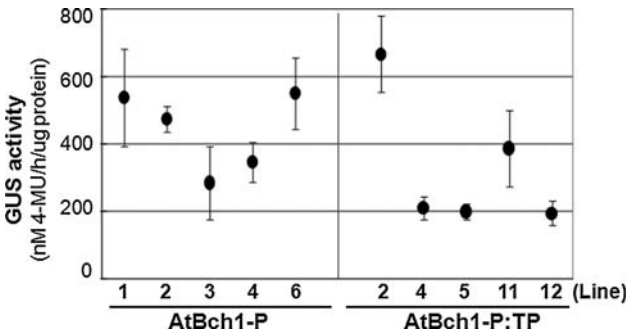

Fig. 4 Activity comparison of the *AtBch1* promoter toward transgenes with or without a transit peptide. GUS enzyme activities in the leaf tissues of five independent *AtBch1-P:Gus* and *AtBch1-P:TP:Gus* transgenic *Arabidopsis* lines were compared by MUG assay

method (Tian and DellaPenna 2001). Moreover, no significant changes in *AtBch1* expression are evident by AtGenExpress microarray after hormone, abiotic or biotic treatments (ESM Table S2) which is a crucial consideration for the use of a constitutive promoter in plant biotechnology.

Endogenous *AtBch1* expression was further confirmed using semi-quantitative RT-PCR analysis and transcripts of this gene were detectable in all *Arabidopsis* tissues including seedling, rosette leaf, cauline leaf, stem, root, flower, silique, and seed (Fig. 1). In particular, *AtBch1* mRNAs were more abundant in the reproductive organs of the silique and seed than in vegetative organs of the seedling, leaf, stem, or root. This is consistent with the AtGenExpress microarray data (ESM Table S1). *AtBch1* was thus selected for promoter activity analysis in transgenic plants to evaluate whether its 5′-upstream region has potential as constitutive promoter for future use with this technology.

Strong activity of the *AtBch1* promoter in transgenic *Arabidopsis*

The nucleotide sequence of a 2-kb *AtBch1* 5′-flanking region (Genbank NC_003075.4) was analyzed using PLACE, a database of plant *cis*-acting regulatory DNA elements (http://www.dna.affrc.go.jp, Higo et al. 1999). We identified several basal regulatory elements including a TATA box (TATAAAT) at position −29 and a CAAT box (CAAT) at position −779. To then analyze *AtBch1* gene promoter activity, a 1,981-bp 5′-upstream region (AtBch1-P) was isolated from *Arabidopsis* leaf genomic DNA by PCR and introduced into a promoter-less pBGWFS7 vector (Fig. 2). As a reference control of promoter strength, the 35S promoter (35S-P) was also linked to the GUS reporter gene (*Gus*) using the same pBGWFS7 vector. Through *Agrobacterium*-mediated *Arabidopsis* transformation, 20 and 12 independent transgenic lines for

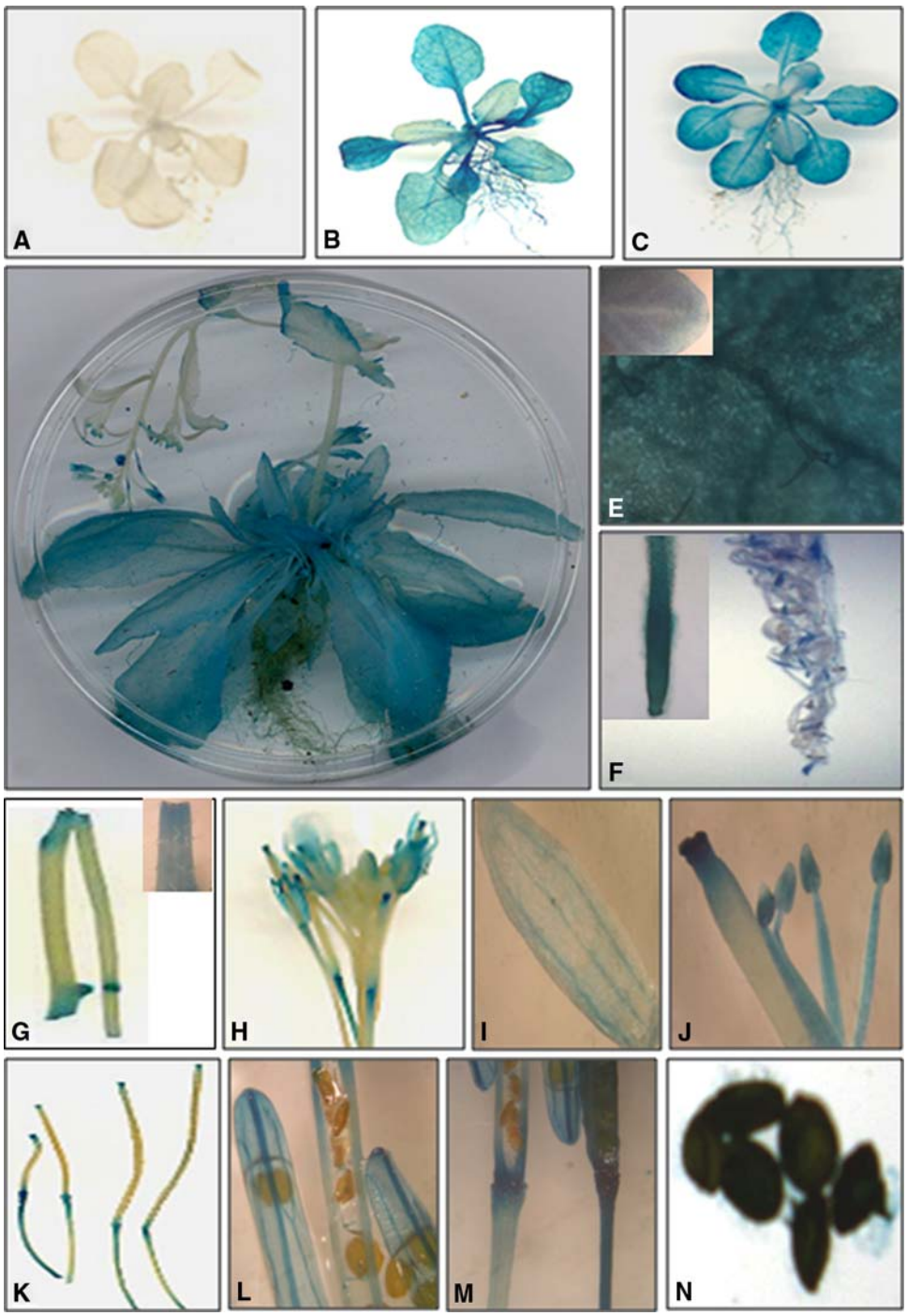

Fig. 5 Histochemical staining of GUS driven by the *AtBch1* promoter in various tissues of transgenic *Arabidopsis*. **a** Three-week-old wild-type *Arabidopsis* seedling; **b** 3-week-old dual *35S* promoter transgenic *Arabidopsis* seedling; **c** 3-week-old *AtBch1* promoter transgenic *Arabidopsis* seedling; **d** whole body of 5-week-old *AtBch1* promoter transgenic *Arabidopsis* plant, and also the rosette leaf (**e**), root (**f**), stem (**g**), flower (**h**), sepal (**i**), pistil and stamen (**j**), and young silique (**k**); **l** and **m** show the apex and lower part of silique of 6-week-old *AtBch1* promoter transgenic *Arabidopsis* plants, respectively; **n** mature seed harvested at 7 weeks after flowering

35S-P:Gus and *AtBch1-P:Gus* were obtained by 0.3% Basta® selection, respectively (data not shown). Among these, two and three representative plant lines showing average GUS enzyme activity for *35S-P:Gus* and *AtBch1-P:Gus* were selected by MUG assay with 4-week-old leaf tissues for further promoter analysis.

Stepwise analyses of transgenic leaf tissues using semi-quantitative RT-PCR, western blotting, and fluorometric GUS enzyme assays were performed to compare the activity of the *35S* and *AtBch1* promoters at the transcriptional, translational, and enzymatic activity levels, respectively. Semi-quantitative RT-PCR analysis of *Gus* revealed that the *AtBch1* promoter drives 1.8-fold higher expression than the *35S* promoter (Fig. 3a). Consistently, western blot analysis to detect an EGFP-GUS fusion reporter product of 97-kDa with an anti-GUS antibody showed that the *AtBch1* promoter induces up to a 1.4- to 2-fold higher accumulation of GUS protein than the *35S* promoter (Fig. 3b). Additionally, by

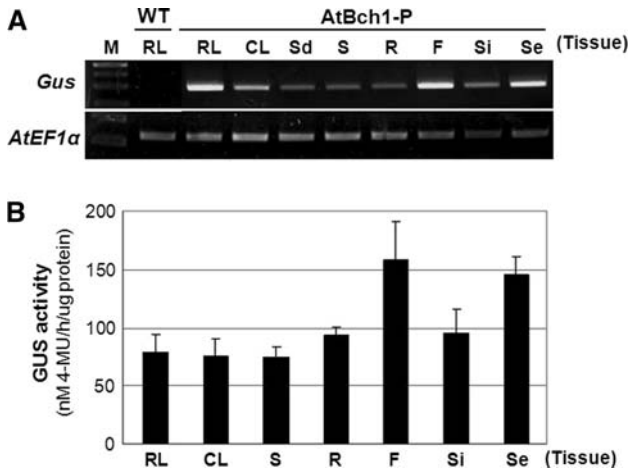

Fig. 6 Activity of the *AtBch1* promoter in driving GUS expression in different tissue types. **a** Detection of *Gus* transcripts by semi-quantitative RT-PCR. **b** Enzyme activity of GUS measured by MUG assay. Both experiments were performed in triplicate with the same tissue samples from a rosette leaf (*RL*), cauline leaf (*CL*), seedling (*Sd*), stem (*S*), root (*R*), flower (*F*), silique (*Si*), and seed (*Se*) of a 4-week-old *AtBch1* promoter transgenic plant

MUG fluorometric assay, we found that the GUS enzyme activity driven by the *AtBch1* promoter was 2.8-fold higher than that produced by the *35S* promoter (Fig. 3c). These results together clearly indicate that the *AtBch1* promoter is stronger than the *35S* promoter at all levels (transcription, translation, and enzyme activity) in transgenic *Arabidopsis*.

Activity of the *AtBch1* promoter with or without a transit peptide sequence

Protein targeting to subcellular compartments such as chloroplasts, the endoplasmic reticulum, and protein storage vacuoles is one of methods used to regulate transgene expression levels for plant biotechnology (Streatfield 2007). In particular, analyses to evaluate whether an increase in exogenous protein expression could be achieved by chloroplast targeting have been previously undertaken through the generation of transgenic plants (Kwak et al. 2007; Kim et al. 2009). Most of the enzymes that are involved in carotenoid biosynthesis in chloroplasts are expected to possess a transit peptide (TP) within their amino acid sequence (Tan et al. 2003; Hsieh et al. 2008).

The N-terminal 51 amino acids of *AtBch1* are predicted as a TP by the ChloroP 1.1 program (http://www.cbs.dtu.dk/services/ChloroP/). To examine whether this putative TP exerts any influence upon *AtBch1* promoter activity, a 2,134-bp fragment (AtBch1-P:TP) including the 1,981-bp *AtBch1* promoter and 153-bp region encoding the TP was isolated from *Arabidopsis* leaf genomic DNA by PCR and fused with the *Gus* gene using the promoter-less pBGWFS7 vector for *Arabidopsis* transformation (Fig. 2).

Among the 16 independent *AtBch1-P:TP:Gus* transgenic lines we isolated from this transformation, five representative lines that showed average GUS enzyme activity levels were selected for comparison with the *AtBch1-P:Gus* transgenic lines. Semi-quantitative RT-PCR analysis of 4-week-old leaf tissues showed equivalent levels of gene expression between *AtBch1-P:Gus* and *AtBch1-P:TP:Gus* (data not shown). The fluorometric GUS activities for *AtBch1-P:TP:Gus* transgenic leaves were also found to be in a similar range to those of *AtBch1-P:Gus* plants (Fig. 4). These data suggest that the presence of a TP sequence in a transgene has no enhancing effect upon the activity of the *AtBch1* promoter.

Constitutive activity of the *AtBch1* promoter in transgenic *Arabidopsis*

To visualize the whole plant body expression of GUS driven by the *AtBch1* promoter, histochemical GUS staining of different tissues of *Arabidopsis* was performed. In comparison with GUS-negative wild-type Col-0, both *35S-P:Gus* and *AtBch1-P:Gus* transgenic plants showed strong GUS expression as 3-week-old seedlings (Fig. 5a–c). GUS staining was also detected throughout the whole body of adult *AtBch1-P:Gus* transgenic plants (Fig. 5d). Moreover, individual tissues including the leaf, root, stem, flower, silique, and seed of *AtBch1-P:Gus* transgenic *Arabidopsis* all showed blue GUS signals, further demonstrating the constitutive and ubiquitous activity of the *AtBch1* promoter (Fig. 5e–n).

To compare the activity of the *AtBch1* promoter in driving *Gus* gene expression between different plant tissues, semi-quantitative RT-PCR and fluorometric GUS activity assays were performed with the same samples of various tissues from *AtBch1-P:Gus* transgenic *Arabidopsis*. *Gus* transcripts were detectable in all tissues of this transgenic plant with moderate differences in the expression levels (Fig. 6a). These mRNAs were found to be more abundant in the reproductive organs of the flower, silique, and seed than in other tissues, which is very consistent with the pattern of endogenous *AtBch1* gene expression (Fig. 1). In addition, the GUS enzyme activities driven by the *AtBch1* promoter were measured at equivalent levels among all of the tissues examined except the flower and seed (Fig. 6b). Hence, the data shown in Figs. 5 and 6 together indicate that the *AtBch1* promoter drives gene expression in all *Arabidopsis* tissue types from the transcriptional to enzyme activity levels.

In conclusion, our current findings reveal that the *AtBch1* promoter is a strong and constitutive promoter that can drive transgene expression in dicot plants such as *Arabidopsis* in a manner that is comparable to the 35S promoter.

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