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Identification and expression pattern analysis of the glucosinolate biosynthetic gene *BoCYP83B1* from broccoli

R. XU, W. W. KONG, Y. F. PENG, K. X. ZHANG, R. LI, and J. LI*

College of Life Sciences, Northeast Agricultural University, Harbin, 150030, P.R. China

Abstract

Glucosinolates are a branch of amino acid-derived metabolites, which are specifically found in *Brassicales*. In *Arabidopsis*, tryptophan derived indolic glucosinolates are required for plant defense against a wide range of pathogens and herbivores due to their strong antimicrobial activity and potential signaling function. An important enzyme in indolic glucosinolate biosynthesis pathway is CYP83B1, which oxidizes indole-3-acetaldoxime, a precursor of indole-3-acetic acid (IAA). In this study, we reported isolation and expression characterization of a *CYP83B1* gene from *Brassica oleracea* L. var. *italica* Plenck, which we termed *BoCYP83B1*. Overexpression of *BoCYP83B1* in *Arabidopsis* resulted in an altered glucosinolate profile and early flowering phenotype. By expressing the reporter gene β-glucuronidase under the control of the *BoCYP83B1* promoter in *Arabidopsis*, we analyzed the spatial expression pattern of *BoCYP83B1* under normal growth conditions as well as in response to several hormones and stresses. The *BoCYP83B1* was primarily expressed in vascular tissue through the almost whole plant. It was strongly induced by methyl jasmonate, 1-amino-1-cyclopropanecarboxylic acid, salicylic acid (SA), gibberellin, and IAA, suggesting its involvement in complex signaling pathways. Mannitol, NaCl, UV, and Flagelin 22 significantly up-regulated *BoCYP83B1* expression, indicating its possible role in stress response. Interestingly, the response of *BoCYP83B1* to SA and NaCl showed tissue specificity. Thus, *BoCYP83B1* might have different functions in different tissues.

Additional key words: ethylene, gibberellins, indole-3-acetic acid, jasmonates, mannitol, salinity, UV radiation.

Introduction

Glucosinolates are a class of amino acid-derived specialized metabolites, which are especially found in the *Brassicaceae* family (Agerbirk and Olsen 2012). This family includes many economically important vegetables and the model plant *Arabidopsis thaliana*. According to their various precursor amino acids, glucosinolates are broadly divided into aliphatic glucosinolates (derived from Leu, Ala, Val, Ile, or Met), indolic glucosinolates (derived from Trp), and aromatic glucosinolates (derived from Phe and Tyr) (Halkier and Du 1997, Halkier and Gershenzon 2006). Once hydrolyzed by thioglucosidase, *e.g.*, myrosinase, glucosinolates release a range of hydrolysis products with diverse biological activities (Halkier and Gershenzon 2006, Textor and Gershenzon 2009). In plants, glucosinolates and their products are essential for an effective defense response to a wide range of pathogens and herbivores(Zhang *et al.* 2006, Clay *et al.* 2009, Andersson *et al.* 2015, Frerigmann *et al.* 2016, Halkier 2016). These metabolites have also high pharmaceutical values (Fahey *et al.* 1997, Rose *et al.* 2000, Fahey *et al.* 2002).

In addition to plant defense and human health protection, biosynthesis of indolic glucosinolates is related to other metabolic pathways (Bak and Feyereisen 2001, Bak *et al.* 2001, Nafisi *et al.* 2007). During biosynthesis of indolic glucosinolates, Trp is firstly converted to indole-3-acetaldoxime (IAOx) by CYP79B2/B3; then, IAOx is converted to indole-3-acetonitrile oxide in a

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Abbreviation: ABA - abscisic acid; ACC - 1-amino-1-cyclopropanecarboxylic acid; CaMV 35S - cauliflower mosaic virus promoter; CDS - coding sequence; Flg22 - Flagelin 22; GA - gibberellic acid; GUS - β-glucuronidase; IAA - indole-3-acetic acid; IAOx - indole-3-acetaldoxime; MeJA - methyl jasmonate; MS - Murashige and Skoog; NCBI - National Center for Biotechnology Information; RACE - rapid amplification of cDNA ends; RT-PCR - reverse transcription PCR; SA - salicylic acid; UTR - untranslated region.

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* Corresponding author; e-mail: lijing@neau.edu.cn

reaction catalyzed by CYP83B1 (Hull *et al.* 2000, Mikkelsen *et al.* 2000, Hansen *et al.* 2001). A series of enzymes including γ-glutamyl peptidase 1 (GGP1), superroot 1 (SUR1), UDP-glucosyl transferase 74B1 (UGT74B1), and sulfotransferase 16 (SOT16) are involved in the subsequent pathway leading to the core structure, indol-3-ylmethyl glucosinolate (Grubb *et al.* 2004, Mikkelsen *et al.* 2004, Piotrowski *et al.* 2004, Geu-Flores *et al.* 2009). The IAOx is an intermediate in the biosynthesis of indolic glucosinolates and a precursor for indole-3-acetic acid (IAA) and a phytoalexin camalexin (Nafisi *et al.* 2007, Moldrup *et al.* 2013). The *Arabidopsis* recessive *sur1* and *cyp83b1* mutants show a high-auxin phenotype, which is thought to arise due to blockage of the IAOx-glucosinolate pathway, resulting in accumulation of IAOx, which is channeled into IAA (Delarue *et al.* 1998, Barlier *et al.* 2000, Bak and Feyereisen 2001, Bak *et al.* 2001, Naur *et al.* 2003, Nafisi *et al.* 2006). Thus, IAOx constitutes a key branching point between primary and secondary metabolism. As the enzyme catalyzing the conversion of IAOx, CYP83B1 plays an important role in balancing IAOx and must be tightly regulated to maintain IAA homeostasis and control its flow into different parts.

The glucosinolate metabolic pathway and the IAOx have been well studied in the model plant *Arabidopsis* (Barlier *et al.* 2000, Bak and Feyereisen 2001, Bak *et al.* 2001, Hansen *et al.* 2001, Naur *et al.* 2003, Morant *et al.* 2010). However, in other glucosinolate-containing crops, the metabolism of IAOx and the characteristics of the gene encoding IAOx-metabolizing enzyme remain unclear.

Broccoli is a worldwide important vegetable and contains abundant glucosinolates. The biotic stress resistance and medical value of broccoli is largely dependent on glucosinolate production (Fahey *et al.* 1997, Latte *et al.* 2011, Ares *et al.* 2013).

In this article, we report the isolation and identification of a *CYP83B1* in broccoli named *BoCYP83B1*. Overexpression of *BoCYP83B1* in *Arabidopsis* resulted in an early flowering phenotype and affected the glucosinolate profile. This phenotype resembles the *Arabidopsis* overexpressing *AtCYP83B1*, which suggested the function of BoCYP83B1 is similar to its ortholog in *Arabidopsis*. Though the expression of *BoCYP83B1* partially overlapped with the one of *Arabidopsis*, it displayed a distinct pattern both in spatial distribution and in response to exogenous hormones and stress treatments. The aim of our study was to provide data concerning expression characteristics and the potential functions of BoCYP83B1, which might be helpful for better understanding indolic glucosinolate biosynthesis in *Brassica* vegetables.

Materials and methods

Plants, growth conditions, and treatments: Seeds of broccoli (*Brassica oleracea* L. var. *italica* Plenck) cv. Qingxiu were placed on moist filter paper in Petri dishes under continuous irradiance for 3 - 4 d. The germinated seedlings were transplanted to soil and grown in a greenhouse under a 16-h photoperiod, a photosynthetic photon flux density of 100 μmol m^{-2} s^{-1}, a temperature of 23 °C, and a relative humidity of 70 % for 4 weeks. *Arabidopsis thaliana* L. ecotype Columbia (Col-0) wild type and transgenic plants were grown under the same conditions.

To investigate the expression pattern of *BoCYP83B1*, seeds of $P_{BoCYP83B1}$*::GUS* transgenic *Arabidopsis* plants were allowed to germinate on a 1/2 Murashige and Skoog (MS) agar medium for 5 d. Then, the seedlings were transferred to new 1/2 MS agar media containing 25 μM methyl jasmonate (MeJA), 200 μM salicylic acid (SA), 10 μM abscisic acid (ABA), 20 μM 1-amino-1-cyclopropanecarboxylic acid (ACC, precursor of ethylene), 10 μM gibberellic acid (GA), 5 μM indole-3-acetic acid (IAA), 1 μM Flagelin (Flg) 22, 200 mM mannitol, or 150 mM NaCl. Then, the seedlings were grown for 1, 2, 3, and 4 d. For treatments by UV radiation, high temperature, and low temperature, the seedlings were exposed to UV (254 nm) for 1.5 h, 40 °C for 2 h, or 4 °C for 2 h. Wounding treatment was performed by cutting cotyledons in half prior to β-glucuronidase (GUS) detection.

Bioinformatics analysis of *BoCYP83B1*: Sequences of genes and proteins were obtained from the National Center for Biotechnology Information (*NCBI*) website (http://www.ncbi.nlm.nih.gov/). Multiple alignments and phylogenetic analysis were performed using the *MEGA 5* software. Physicochemical properties were predicted using *Protparam* (http://web.expasy.org/protparam/), transmembrane prediction was made using *TMHMM* (http://www.cbs.dtu.dk/services/TMHMM/), hydrophilicity/hydrophobicity were predicted using *ProtScale* (http://web.expasy.org/protscale/), translational modification of amino acids was predicted using the *NetPhos2.0* server (http://www.cbs.dtu.dk/services/NetPhos/), the secondary structure was predicted and analyzed using *SOPMA* (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html), functional domains were predicted by *SMART* (http://smart.embl-heidelberg.de/), and the three-dimensional structure was predicted by *Swiss-Model* (http://swissmodel.expasy.org/) (Geourjon and Deleage1995, Schultz *et al.* 1998, Blom *et al.* 1999, Chen *et al.* 2003, Gasteiger *et al.* 2003, Johnson *et al.* 2008, Biasini *et al.* 2014).

Generation of the *35S::BoCYP83B1* construct and transgenic plant: To obtain the full-length mRNA sequence of *BoCYP83B1*, rapid amplification of cDNA ends (RACE) PCR (*Takara*, Otsu Shiga, Japan) was

performed according to the manufacturer's instructions. Two pairs of primers 3' outer and 3' inner, 5' outer ' and 5' inner (primer sequences are listed in Table 1 Suppl.) were used to obtain the 3' and 5' end sequences of the *BoCYP83B1* mRNA. Based on the mRNA sequence, the coding sequence (CDS) of *BoCYP83B1* was amplified through reverse transcription PCR (RT-PCR). Total RNA was extracted using a *TRIzol* reagent (*Invitrogen*, Carlsbad, CA, USA) from four-week-old leaves of broccoli, and the first strand of cDNA was synthesized using a *PrimeScript* RT reagent kit (*Takara*). The CDS of *BoCYP83B1* was amplified with Ex Taq DNA polymerase (*Takara*) from the first-strand products using primers BoCYP83B1-F and BoCYP83B1-R (Table 1 Suppl.). The CDS of *BoCYP83B1* was cloned into pCAMBIA230035Su expression vector under control of the cauliflower mosaic virus promoter (CaMV 35S) using the *USER* cloning method described by Nour-Eldin *et al.* (2006). The recombinant vectors were introduced into the *Agrobacterium tumefaciens* L. strain LBA4404 and then transformed into *Arabidopsis* plants using the floral dip method (Clough and Bent 1998). Transgenic seeds T_0 were selected on a 1/2 MS agar medium containing 50 μg cm^{-3} kanamycin. Three independent T_3 homozygous transgenic lines were confirmed and used for the analysis.

Generation of the $P_{BoCYP83B1}$*::GUS* construct and transgenic plants: To identify the spatial expression pattern of *BoCYP83B1*, the $P_{BoCYP83B1}$*::GUS* construct was prepared, in which *GUS* expression was driven by the *BoCYP83B1* promoter. A 2kb DNA fragment containing the *BoCYP83B1* promoter was PCR-amplified from the genomic DNA with primers *BoCYP83B1*-pro-F and *BoCYP83B1*-pro-R (Table 1 Suppl.). The obtained DNA fragment was considered as the promoter of *BoCYP83B1*, and it was analyzed by *PlantCARE* (http:// bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Rombauts *et al.* 1999). The *BoCYP83B1* promoter was cloned into the pCAMBIA3300 GUS expression vector using the *USER* method (Nour-Eldin *et al.* 2006). The pCAMBIA3300 GUS was generated from the original pCAMBIA3300 vector by adding the coding sequence of *GUS* with the *USER* cloning sites (Nour-Eldin *et al.* 2006). In the resulting $P_{BoCYP83B1}$*::GUS* construct, *GUS* was driven by the *BoCYP83B1* promoter.

The construct was transformed into *Arabidopsis* using the *Agrobacterium*-mediated floral dip method (Clough and Bent 1998). Selection of transgenic plants was performed by sowing seeds directly on soil followed by spraying the herbicide *Basta* (0.2 mM) on alternate days starting one week after germination. Three independent transgenic lines were used in the subsequent analyses.

Analysis of IAA: Extraction, purification, and determination of IAA were performed by an *ELISA* technique as previously described (Yang *et al.* 2001). Content of IAA was calculated following Weiler *et al.* (1981).

Glucosinolate detection: The *35S::BoCYP83B1* and wild type plants were grown simultaneously for four weeks. Individual leaves (50 - 100 mg) and seeds (20 mg) from each plant were harvested. Glucosinolate extraction was performed as described Hansen *et al*. (2007). High performance liquid chromatography analysis was performed as described by Pang *et al*. (2009).

Detection of GUS: Histochemical detection of GUS was performed as described Jefferson *et al.* (1987). Plant material was incubated in a substrate solution containing 2mM X-gluc (5-bromo-4-chloro-3-indoxyl β-D-glucuronide cyclohexylammonium salt), 50 mM sodium phosphate buffer at pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1 % (v/v) *Triton X-100*, and 10 mM EDTA at 37 °C for 12 h followed by removal of chlorophyll by submerging the samples in 75 % (v/v) ethanol. The samples were examined and photographed using a stereomicroscope (*Nikon SMZ1270*, Tokyo, Japan). Quantitative assay of GUS activity was performed as described by Bhuria *et al.* (2016).

Real-time PCR analysis: To determine the effects of *BoCYP83B1* overexpressing on the glucosinolate metabolism pathway in *Arabidopsis*, transcription of genes encoding enzymes in aliphatic and indolic glucosinolate pathways were detected. Quantitative real-time RT-PCR was performed using *Trans Start Top Green qPCR Supermix* on an *ABI 7500* sequence detection system (*Takara*). The selected genes and primers used are listed in Table 1 Suppl. The *AtACTIN-2* gene was used as an internal control. Transcription of the genes was calculated with the $2^{-\Delta\Delta CT}$ method. To enable statistical analysis, three independent biological replicates and three technical repeats were conducted. To determine whether the responsive expression pattern of *BoCYP83B1* observed in $P_{BoCYP83B1}$*::GUS* transgenic *Arabidopsis* represents that in broccoli, expressions of *BoCYP83B1* in broccoli under the different treatments were detected as described above using primers BoCYP83B1-QRT-F and BoCYP83B1-QRT-R (Table 1 Suppl.). The *BoACTIN-2* gene in broccoli was used as an internal control.

Results and discussion

Based on the partial CDS of *CYP83B1* in broccoli, RACE was performed to amplify the full-length cDNA from the leaves of the broccoli cv. Qingxiu. A *BLAST* search (Altschul *et al.* 1990) revealed that the CDS of the

obtained gene shared a high sequence similarity with the other *Brassicaceae*, including 90 % identity with *CYP83B1* of *Arabidopsis* and 99 % identity with *B. napus* and *B. rapa*. Therefore, we named the obtained gene *BoCYP83B1* (GenBank accession no. KU559565). The full-length mRNA of *BoCYP83B1* was 1 795 bp and

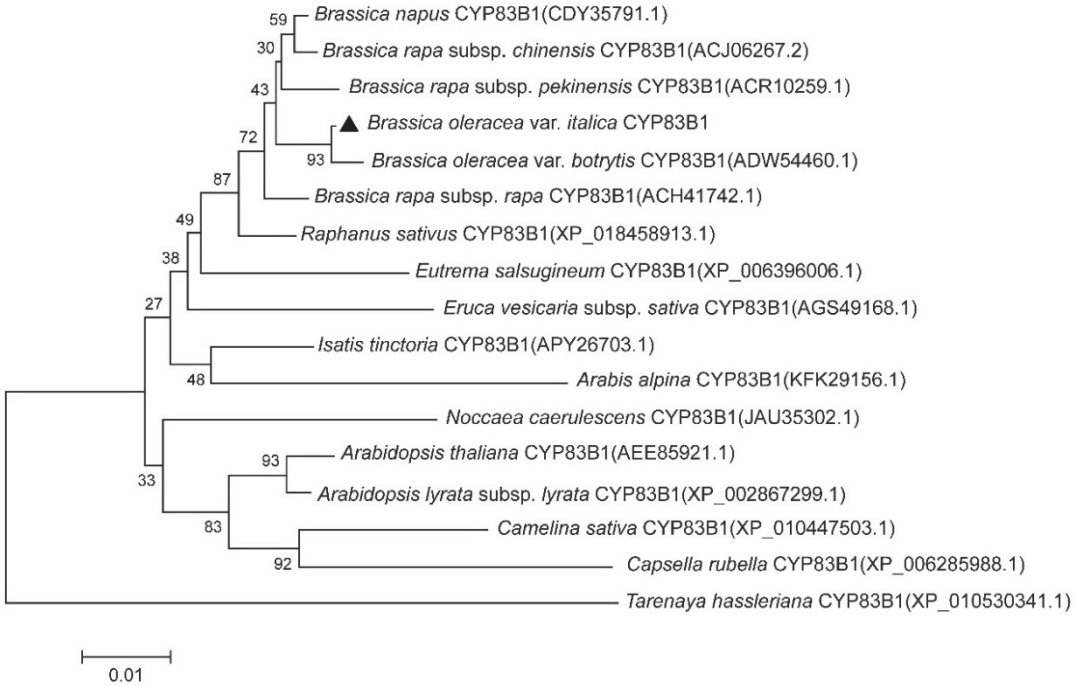

Fig. 1. Evolutionary relationships between BoCYP83B1 proteins. Phylogenetic analysis of BoCYP83B1 with its orthologs from *Brassicaceae* plants which share more than 90 % sequence similarity was performed using the neighbor-joining method in the *MEGA 5.0*. The percentage of replicate trees in which the associated proteins clustered together in the bootstrap test (1 000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

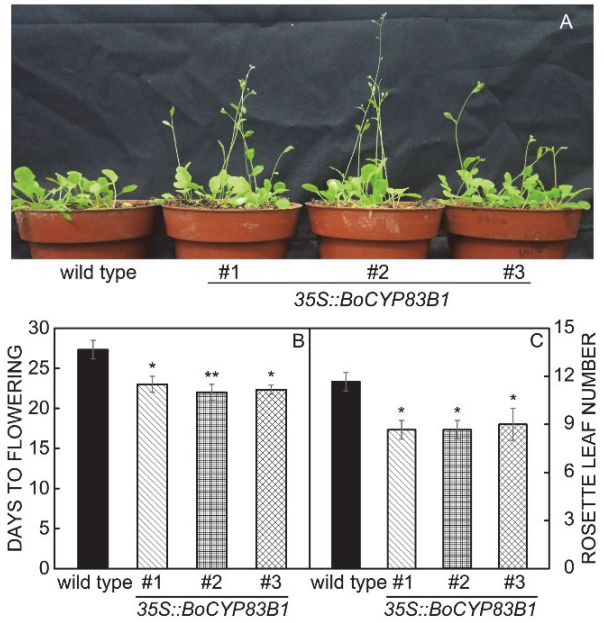

Fig. 2. The *BoCYP83B1* overexpression promotes flowering in *Arabidopsis*. *A* - Flowering phenotype of wild type and *35S::BoCYP83B1* plants under a 16-h photoperiod. *B* - Average flowering time (days after germination). *C* - The average number of rosette leaves at the time of flowering. Three independent *35S::BoCYP83B1* transgenic lines (#1, #2, and #3) were confirmed by reverse transcription PCR. All values in *B* and *C* are means ± SEs from three independent experiments (10 seedlings per experiment); * and ** indicate significant differences from the corresponding wild type at $0.01 < P < 0.05$ and $P < 0.01$, respectively (*t*-test).

contained a 23-bp 5' untranslated region (UTR), a 272-bp 3'-UTR and a 1 500-bp CDS encoding 499 amino acids. Multiple alignment of the deduced BoCYP83B1 with its orthologs from *Brassicaceae* plants indicates that BoCYP83B1 showed a high similarity with other proteins (Fig. 1 Suppl.).

To investigate evolutionary relationships, all the CYP83B1 proteins with available sequences in the *Brassicaceae* family were applied for phylogenetic analysis using CYP83B1 from *Tarenaya hassleriana* of the *Cleomaceae* family as an outgroup. As shown in Fig. 1, all the CYP83B1 proteins were tightly clustered together with the exception of CYP83B1 from *Tarenaya hassleriana*. The CYP83B1 proteins were clustered into two clades, which is consistent with the previously identified phylogenesis of *Brassicaceae* (Warwick *et al.* 2006). All CYP83B1s from the *Brassica* genus formed a subclade. The closest related protein to BoCYP83B1 was CYP83B1 in *B. oleracea* var. *botrytis*.

The physicochemical properties, hydrophilicity/ hydrophobicity and a 3D structure model of BoCYP83B1 were predicted by *Protparam*, *ProtScale*, and *Swiss-Model*, respectively (Fig. 2 Suppl. and Table 2 Suppl.). Analysis using *TMHMM*, *NetPhos2.0Server*, *SOPMA*, and *SMART* suggests that BoCYP83B1 had potentially the following characteristics: not a trans-membrane protein contained 16 phosphorylation sites, the most abundant secondary structure was an α-helix and inclusion of a typical P450 domain (Fig. 2 Suppl.).

In *35S::BoCYP83B1* transgenic *Arabidopsis* (over-expression of *BoCYP83B1* was confirmed by RT-PCR, Fig. 3 Suppl.), an early flowering phenotype was observed (Fig. 2*A*). Comparing to the wild type, *35S::BoCYP83B1* plants flowered about 5 d earlier when they had less leaves than the wild type (Fig. 2*B*,*C*). This result is consistent with a previous study in which an early flowering

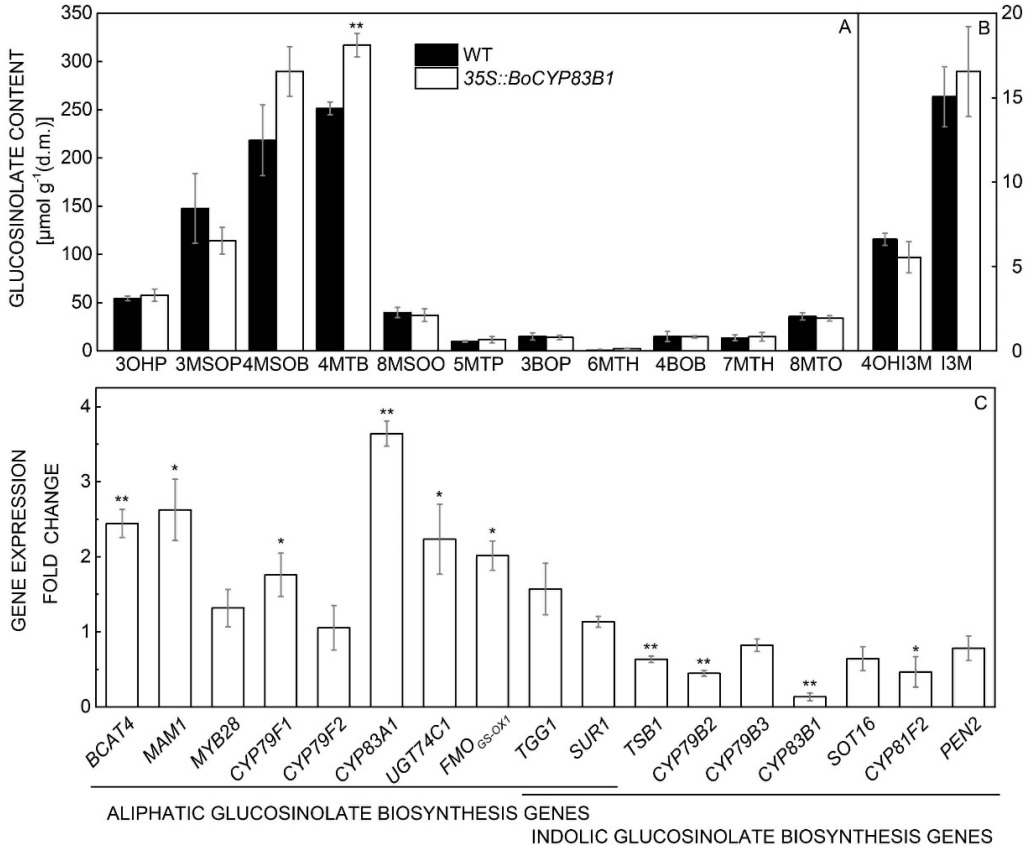

Fig. 3. Glucosinolate content analyzed by HPLC and expression of glucosinolate metabolism pathway genes in *35S::BoCYP83B1*. *A* - Aliphatic glucosinolate content in seeds. *B* - Indolic glucosinolate content in seeds. 3OHP - 3-hydroxylpropyl glucosinolate; 3BOP - 3-benzoylpropyl glucosinolate; 3MSOP - 3-methylsulphinylpropyl glucosinolate; 4BOB - 4-benzoylbutyl glucosinolate; 4MSOB - 4-methylsulphinylbutyl glucosinolate; 4MTB - 4-methylthiobutyl glucosinolate; 5MTP - 5-methylthiopentyl glucosinolate; 6MTH - 6-methylthiohexyl glucosinolate; 7MTH - 7-methylthioheptyl glucosinolate; 8MSOO - 8-methylsulphinyloctyl glucosinolate; 8MTO - 8-methylthiooctyl glucosinolate; 4OHI3M - 4-hydroxyindol-3-ylmethyl glucosinolate; I3M - indol-3-ylmethyl glucosinolate. *C* - The fold change of gene expression in seedlings of wild type and *35S::BoCYP83B1* transgenic *Arabidopsis* plants determined by real time reverse transcription PCR. For *A*, *B* and *C*, three independent transgenic lines confirmed with a high expression level of *BoCYP83B1* and a clear phenotype was used. The experiment included three biological repeats and three technical repeats; means ± SEs are shown; * and ** indicate significant differences from the corresponding wild type at $0.01 < P < 0.05$ and $P < 0.01$, respectively (*t*-test).

phenotype was observed in *Arabidopsis* overexpressing its own *CYP83B1* gene (Naur *et al.* 2003). The substrate of CYP83B1, IAOx, is also a precursor of IAA, thus, it is expected that consumption of IAOx by *BoCYP83B1* overexpression results in decrease of IAA. However, no significant alteration of IAA content was detected in *35S::BoCYP83B1* (Fig. 4 Suppl.). This indicates that the deficit of IAOx derived IAA was possibly compensated by balancing other IAA biosynthesis pathways. Thus, the early flowering phenotype was probably induced by other factors instead of IAA.

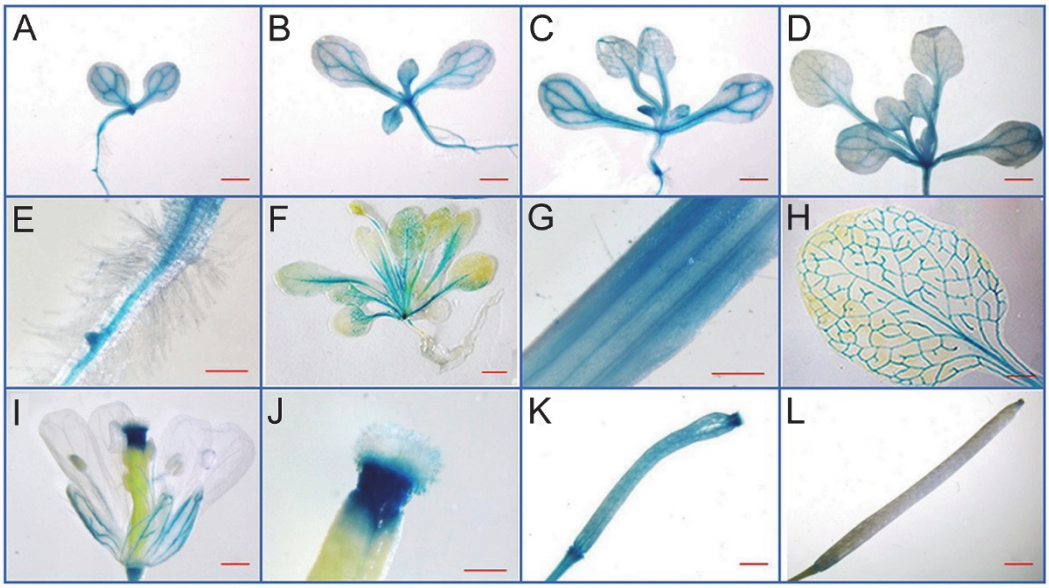

Fig. 4. The spatial pattern of *BoCYP83B1*. The *BoCYP83B1* expression was analyzed by detecting β-glucuronidase (GUS) activity in transgenic plants expressing *BoCYP83B1* promoter-directed *GUS*. *A* - cotyledon stage; *B* - two-leaf stage; *C* - four-leaf stage; *D* - six-leaf stage; *E* - root; *F* - mature plant; *G* - flower stalk; *H* - mature leaf; *I* - flower; *J* - stigma; *K* - immature silique; *L* - mature silique. *Bars* = 1 mm (*A*, *B*, *C*, *D*, *K*, *L*); 0.25 mm (*E*, *J*); 0.5 cm (*F*); 0.5 mm (*G*, *I*); 1.5 mm (*H*).

To determine the catalytic function of BoCYP83B1, glucosinolate content was detected in *35S::BoCYP83B1*. To minimize possible maternal effects, heterozygous transgenic lines with the confirmed high expression of *BoCYP83B1* and the early flowering phenotype were selected, and the glucosinolate content was detected in the leaves and seeds of the segregating progeny obtained from the heterozygous transgenic parents. No significant change in glucosinolate accumulation was detected in the leaves (Fig. 5 Suppl.). In the seeds, the content of indolic glucosinolates was also comparable to the wild type plant, whereas the content of aliphatic glucosinolates was higher in *35S::BoCYP83B1* than in the wild type. The increased content of aliphatic glucosinolates was primarily due to a significantly increased accumulation of 4-methylthiobutyl glucosinolate (Fig. 3*A*). This result was quite unexpected since AtCYP83B1 has been demonstrated to be an enzyme metabolizing IAOx to corresponding S-alkyl-thio-hydroximates, which is a part of indolic glucosinolate biosynthetic pathway (Bak *et al.* 2001). However, similar results were shown in several other reports. Naur *et al.* (2003) showed that overexpression of *AtCYP83B1* in *Arabidopsis* leads to a higher accumulation of aliphatic glucosinolates without significantly increasing content of indolic glucosinolates. Zang *et al.* (2008) overexpressed *AtCYP83B1* in Chinese cabbage, but they did not observe any alteration in content of indolic glucosinolates. One possible explanation for this discrepancy is that *BoCYP83B1* overexpression caused rebalancing the whole biosynthetic pathway. To validate this hypothesis, transcriptions of genes in the glucosinolate metabolism pathway were detected. As shown in Fig. 3*B*, in the *35S::BoCYP83B1* transgenic *Arabidopsis*, a half of the genes in the indolic glucosinolate pathway including *AtCYP83B1* were significantly down-regulated, and a half of the genes in the aliphatic pathway were up-regulated. These results explain the glucosinolate phenotype in *35S::BoCYP83B1* and indicate that IAOx catalysis is not a rate-limiting step in the indolic glucosinolate biosynthesis pathway.

Based on the facts that BoCYP83B1 shared a high sequence similarity with its ortholog AtCYP83B1, contained the same P450 function domain, and overexpression of the two *CYP83B1* genes in the transgenic plants presented a similar morphological phenotype and glucosinolate profile, we speculate that BoCYP83B1 has a similar enzyme catalytic activity as its *Arabidopsis* ortholog.

To investigate the spatial expression pattern of *BoCYP83B1*, a 2 kb fragment upstream of the *BoCYP83B1*

start codon was amplified from the genomic DNA as the promoter. The transgenic plants $P_{BoCYP83B1}$::*GUS*, which expressed *GUS* under control of the *BoCYP83B1* promoter, were generated. Promoter activity was detected by the GUS signal *in vivo*. In seedlings at cotyledon, two-leaf, four-leaf, and six-leaf stages, the GUS signals were detected in the vascular tissue of radicles, hypocotyls, cotyledons, leaves, and roots (Fig. 4*A*,*B*,*C*,*D*). In the root, *BoCYP83B1* was expressed during the seedling stage in the vascular cylinder, and a high expression was observed in the lateral root primordium (Fig. 4*E*). In the adult plants, GUS activity was predominantly detected in the vascular tissue of the petioles, flower stalks, and leaf veins, and hardly any GUS signal was observed in the roots (Fig. 4*F*). In flower stalks, GUS staining was observed in the vascular bundle and the middle of the pitch (Fig. 4*G*). In the mature leaf, the GUS signal was clearly observed in the mid-vein and more or less in the side veins (Fig. 4*H*). As concerns the reproductive organs, GUS activity was detected in the vascular tissue of the sepals and the filaments of the stamen, and an extremely strong GUS signal was present in the style of the pistil (Fig. 4*I*,*J*). In the silique, *BoCYP83B1* was expressed in the young carpels but not in the mature carpels (Fig. 4*K*,*L*).

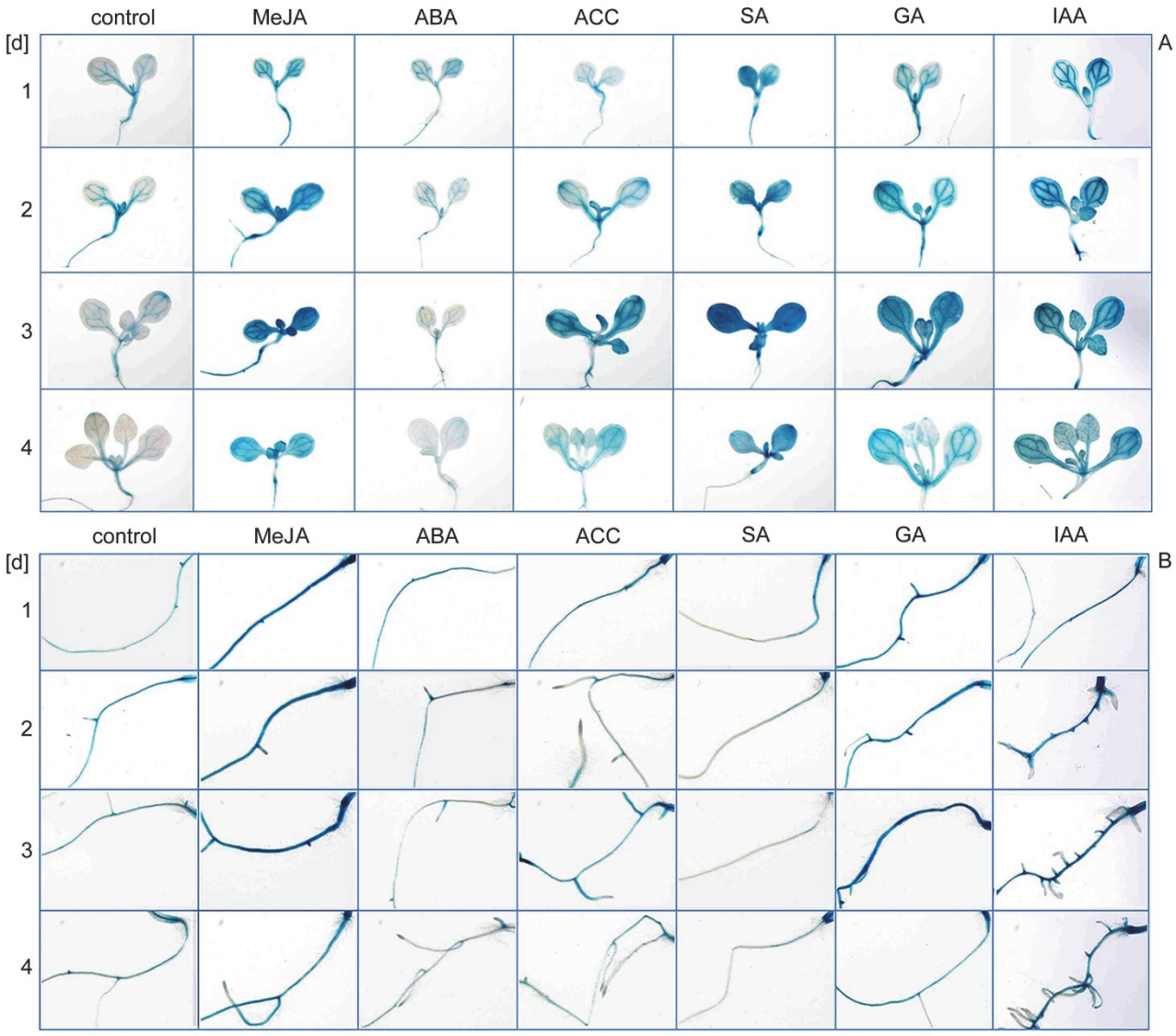

Fig. 5. Expression of *BoCYP83B1* in response to phytohormones. *A* - *BoCYP83B1* expression in leaves. *B* - *BoCYP83B1* expression in roots. Five-d-old plants were transferred to 1/2 MS agar media containing 25 μM methyl jasmonate (MeJA), 200 μM salicylic acid (SA), 10 μM abscisic acid (ABA), 20 μM 1-amino-1-cyclopropanecarboxylic acid (ACC), 10 μM gibberellic acid (GA), or 5 μM indole-3-acetic acid (IAA) and grown for 4 d. Plants transferred to the 1/2 MS agar medium without hormone addition were used as a control. Activity of β-glucuronidase (GUS) was detected at the same time each day. Three independent transgenic lines were used for GUS activity analysis. All the transgenic lines showed a similar staining pattern and responding tendency. The result (repeated three times) of one line is shown.

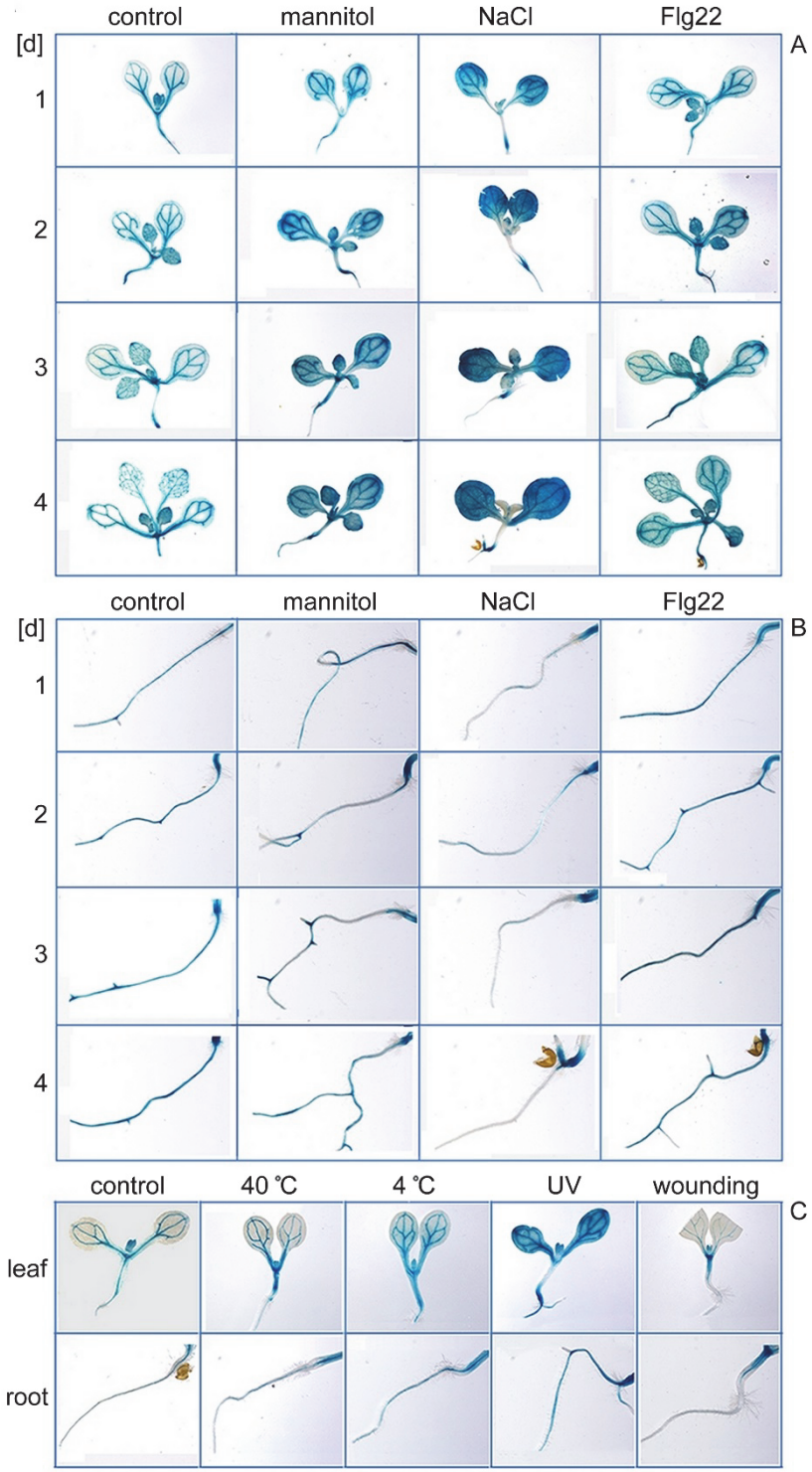

Fig. 6. Expression of *BoCYP83B1* in response to different stresses. *A* - *BoCYP83B1* expression in leaves in response to mannitol, NaCl and Flagelin 22 (Flg22). *B* - *BoCYP83B1* expression in roots in response to mannitol, NaCl and Flg22. *C* - *BoCYP83B1* expression in response to a high temperature, a low temperature, UV, and wounding. 5-d-old plants were transferred to 1/2 MS agar media containing 200 mM mannitol, 150 mM NaCl, or 1 μM Flg22 and grown for 4 d. Activity of β-glucuronidase (GUS) was detected at the same time each day during treatment. For the other stress treatments, 5-d-old plants were exposed to UV radiation for 1.5 h, 40 °C for 2 h, and 4 °C for 2 h. The wounding treatment was performed by cutting the cotyledons in half. Seedlings without any treatment were used as a control. Three independent transgenic lines were used for GUS activity analysis. All the transgenic lines showed a similar staining pattern and responding tendency.

To better understand how environmental factors affect the catalysis of IAOx by BoCYP83B1 and to identify related signaling pathways where BoCYP83B1 is involved, we evaluated the response of *BoCYP83B1* expression to different environmental conditions and treatments with plant hormones. Firstly, the promoter sequence of *BoCYP83B1* was analyzed with *PlantCARE* (bioinformatics.psb.ugent.be/webtools/plantcare/html/) to identify potential *cis*-elements. Several hormone responsive elements and stress-related elements were found (Table 3 Suppl.). To investigate whether *BoCYP83B1* responded to these biotic and abiotic stresses and was involved in predicted hormone signaling pathways, 5-d-old seedlings of the transgenic plant expressing $P_{BoCYP83B1}$*::GUS* were treated with MeJA, SA, ABA, ACC, GA, and IAA, and a series of abiotic and biotic stresses including NaCl, mannitol, 40 °C, 4 °C, UV, wounding, and a plant bacterial defense elicitor Flg22. The expression of *BoCYP83B1* was evaluated according to GUS staining *in vivo* (Figs. 5 and 6) and a quantitative assay of GUS activity in the seedlings (Fig. 6 Suppl.).

After 4-d treatments, *BoCYP83B1* expression was significantly affected by all hormones except ABA (Fig. 5*A* and Fig. 6 Suppl.). The MeJA, ACC, SA, GA, and IAA strongly induced *BoCYP83B1* in the shoots, and the induction steadily increased over the first 3 d. The induction began to weaken on the fourth day. The *BoCYP83B1* did not appear to be very sensitive to ABA because only a slight inhibition was observed after 4 d of ABA treatment. In the roots, the effect of these hormones resembled the effects observed in the shoots, except for SA (Fig. 5*B*). In contrast to a strongly induced expression of *BoCYP83B1* in the shoots, SA significantly inhibited *BoCYP83B1* expression in the roots (Fig. 5*B*). The organ specific responses of *BoCYP83B1* indicate that BoCYP83B1 might play different roles in different organs in response to SA. In addition to affecting *BoCYP83B1* expression, MeJA, ABA, and SA largely inhibited growth and development of the seedlings. After 4 d, the control seedlings and those treated with ACC, GA, and IAA had four leaves, whereas the seedlings treated with MeJA, ABA, and SA had only two small euphyllas. The SA treatment inhibited formation of lateral roots, whereas IAA significantly promoted formation of them (Fig. 5*B*). Under stress treatments, *BoCYP83B1* was continually induced by mannitol, NaCl, and Flg22 in the shoots, but no significant induction was observed in the roots (Fig. 6*A*,*B*). Interestingly, a strong *BoCYP83B1* expression activated by NaCl was observed only in the cotyledons and the junction of the hypocotyl and root (Fig. 6*A*,*B*). However, *BoCYP83B1* expression was significantly inhibited in the emerging euphyllas, hypocotyl, and roots in response to NaCl (Fig. 6*A*,*B* and Fig. 7 Suppl.). This tissue-specific expression pattern was quite similar when the plant was treated with SA.

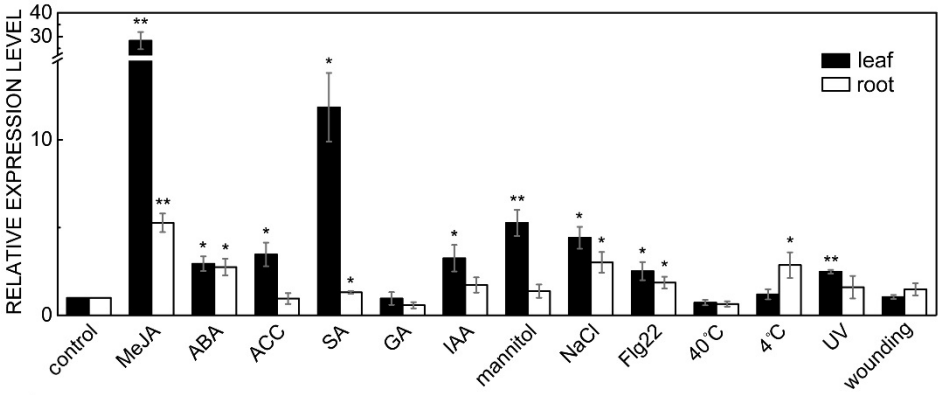

Fig. 7. Expression of *BoCYP83B1* in response to exogenous hormones and stresses in broccoli. Transcription of *BoCYP83B1* was determined by real time reverse transcription PCR. 5-d-old seedlings were treated as described above. The experiment included three biological repeats, and three technical repeats; mean values (± SEs) are shown. Data were analyzed statistically using the *t*-test, * and ** indicate significant differences from the corresponding control at $0.01 < P < 0.05$ and $P < 0.01$, respectively.

Because treatment with high temperature (40 °C), low temperature (4 °C), UV radiation and wounding causes wilting, no time course experiments were performed. As shown in Fig. 6*C*, *BoCYP83B1* was significantly induced by UV radiation and slightly enhanced by a high or low temperature. A position-specific responsive expression pattern to wounding was again observed. After the cotyledons were cut in half, a condensed GUS signal was observed at the cotyledonary node suggesting a strong expression of *BoCYP83B1*, whereas expression was inhibited in the cotyledons and roots.

The expression pattern of *BoCYP83B1* presented by GUS staining was confirmed and quantified by quantitative analysis of GUS activity in the seedlings of the transgenic plant $P_{BoCYP83B1}$*::GUS*. Activity of GUS significantly increased after all the treatments except ABA (Fig. 6 Suppl.), which is perfectly consistent with the results obtained from GUS staining.

To determine whether the expression pattern of *BoCYP83B1* observed in *Arabidopsis* represents that in

broccoli, quantitative real time PCR was performed in broccoli. The 5-d-old broccoli seedlings were treated as described above and transcriptions of *BoCYP83B1* in the leaves and roots were detected (Fig. 7). Consistently MeJA, ACC, SA, IAA, mannitol, NaCl, Flg22, and UV significantly up-regulated *BoCYP83B1* expression in the leaves as observed in *Arabidopsis*. Comparing to the leaves, *BoCYP83B1* expression seemed to be less affected by all the treatments in the roots, and the responsive expression pattern basically coincided with that shown in *Arabidopsis*. Driven by the promoter of *BoCYP83B1*, *GUS* transcription in *Arabidopsis* can largely represent the *BoCYP83B1* expression in broccoli. However, some differences were found between the observations in *Arabidopsis* and broccoli. In *Arabidopsis*, *GUS* expression driven by the *BoCYP83B1* promotor was slightly repressed by ABA and significantly induced by GA, whereas in broccoli, *BoCYP83B1* expression was promoted by ABA and not affected by GA. For both *Arabidopsis* and broccoli, the 5-d-old seedlings were used in gene expression analysis. They were not possibly in the perfectly equivalent development stage, and therefore responded to the same stimuli differently. Another possibility is that the regulatory proteins controlling *BoCYP83B1* promoter activity in *Arabidopsis* are not completely identical with those in broccoli. Comparing to GUS activity observed in transgenic *Arabidopsis* $P_{BoCYP83B1}$*::GUS*, transcription level detected in broccoli can represent the expression of *BoCYP83B1* more authentically. However, the transcription level is not as visualized as GUS signal presented in the image and thus may lose some detailed information.

In this study, we isolated the broccoli *BoCYP83B1* gene, which was an ortholog of the *Arabidopsis CYP83B1*. Phylogenetic analysis proved that the CYP83B1s were highly conserved in *Brassicaceae* plants. In *Arabidopsis*, CYP83B1 has been shown to catalyze conversion of aromatic oximes derived from Phe, Trp, and Tyr to corresponding S-alkyl-thiohydroximates and further to indolic glucosinolates (Bak and Feyereisen 2001, Bak *et al.* 2001, Hansen *et al.* 2001). However, *BoCYP83B1* overexpression in *Arabidopsis* did not result in an increased production of indolic glucosinolates, which was unexpected. However, this result is consistent with a previous study in which transgenic *Arabidopsis* overexpressing *AtCYP83B1* showed an indolic glucosinolate content similar to that in a wild type (Naur *et al.* 2003). Similar results were found in other studies. For instance, overexpression of *CYP83B1* alone in Chinese cabbage does not affect indolic glucosinolate accumulation, whereas overexpression of *CYP83B1* together with genes *CYP79B2* or *CYP79B3* significantly increases indolic glucosinolate biosynthesis (Zang *et al.* 2008). Based on these studies, we speculate that CYP83B1 is not a rate-limiting enzyme in indolic glucosinolate production.

Interestingly, the *35S::BoCYP83B1* transgenic *Arabidopsis* showed an increased content of aliphatic glucosinolates. This can possibly be explained by promoted expressions of genes encoding several enzymes concerning aliphatic glucosinolate biosynthesis in the *35S::BoCYP83B1* transgenic plants. Another explanation is that BoCYP83B1 likely catalyzes an aliphatic oxime and promotes production of aliphatic glucosinolates. In *Arabidopsis*, aliphatic oximes derived from methionine are thought to be metabolized by CYP83A1 rather than by CYP83B1 (Bak and Feyereisen 2001, Hemm *et al.* 2003, Naur *et al.* 2003). However, CYP83B1 metabolizes the aliphatic oximes with a low efficiency (Bak and Feyereisen 2001, Hemm *et al.* 2003). Considering that content of aliphatic glucosinolates is much higher than that of indolic glucosinolates, the excessive BoCYP83B1 in *35S::BoCYP83B1* could possibly choose aliphatic oximes with higher concentrations as substrates and promote aliphatic glucosinolate production.

In *Arabidopsis*, *CYP83B1* is functional as a repressor of IAA biosynthesis due to consumption of IAOx, which is the common intermediate of IAA and indolic glucosinolates. Thus, *CYP83B1* is involved in many aspects of growth and development (Barlier *et al.* 2000, Mikkelsen *et al.* 2000, Bak *et al.* 2001, Smolen and Bender 2002). A knock-out mutant of *CYP83B1* (*sur2-8*) presents a severe high-auxin "supperroot" phenotype (Delarue *et al.* 1998, Barlier *et al.* 2000, Bak and Feyereisen 2001, Bak *et al.* 2001, Naur *et al.* 2003). A missense mutation with a one amino acid change in *CYP83B1* lead to a tall phenotype due to the long hypocotyl (Maharjan *et al.* 2014). The CYP83B1 can also be involved in stem branching through regulation of auxin homeostasis under a high ratio of red to far-red radiation (Hoecker *et al.* 2004, Zhang *et al.* 2014). However, whether the IAOx-dependent IAA biosynthesis pathway is conserved in broccoli and whether BoCYP83B1 is involved in plant growth and development are unknown. Our study shows that *BoCYP83B1* was not only induced by MeJA and SA, which suggests its function in biotic stress resistance, but it was also strongly induced by IAA and GA, indicating that *BoCYP83B1* is possibly involved in growth and development.

The expression pattern analysis shows that *BoCYP83B1* was expressed in the roots, leaves, flower stalks, flowers, sepals, pistils, stigma, and young siliques. This expression pattern was quite similar to that of *AtCYP83B1* (Mizutani *et al.* 1998, Barlier *et al.* 2000, Maharjan *et al.* 2014), suggesting the conservation of promoter elements between the two orthologs. The *BoCYP83B1* was found to be responsive to several hormones, which are closely related to stress resistance and plant development. The *BoCYP83B1* was strongly induced by MeJA, SA, ACC, GA, and IAA, and slightly repressed by ABA. These responses indicate that *BoCYP83B1* is involved in complex signal transduction pathways and support a suggestion that like its *Arabidopsis* ortholog, BoCYP83B1 plays a role not only

in defense against pathogens and herbivores but also in plant growth and development.

In *Arabidopsis*, biosynthesis of indolic glucosinolates is affected by many environmental factors, especially biotic and abiotic stresses (Mikkelsen *et al.* 2003, Mewis *et al.* 2005, 2006, 2012 Alvarez *et al.* 2008, Pfalz *et al.* 2009). Consistently, *cis*-acting elements involved in defense and stress responses were found in the promoter sequence of *BoCYP83B1*. Therefore, we detected expression of *BoCYP83B1* in response to several biotic and abiotic stresses. Under our experimental conditions, *BoCYP83B1* was strongly induced by treatments with mannitol, NaCl, Flg22, and UV radiation.

Although BoCYP83B1 has the same catalytic activity as AtCYP83B1, it displays distinctive expression patterns. In *Arabidopsis*, *CYP83B1* has been reported to be preferentially expressed in roots and induced by wounding (Mizutani *et al.* 1998, Reymond *et al.* 2000). Conversely, *BoCYP83B1* was preferentially expressed in the shoots. When challenged with wounding, *BoCYP83B1* expression in the cotyledons and roots decreased. However, *BoCYP83B1* was significantly induced by SA in the cotyledons and emerging euphyllas, but it was largely repressed in the roots. When treated with NaCl, *BoCYP83B1* expression was strongly induced in the cotyledons but significantly repressed in the emerging euphyllas, hypocotyls, and in roots, expression appeared to be highly concentrated at the junction of the radicle and hypocotyl. Since CYP83B1 affects production of several different metabolites, indolic glucosinolates, camalexin, and IAA, this gene may execute different functions in different cells.

In summary, we identified a *BoCYP83B1* gene from broccoli and analyzed its expression pattern. Since the substrate of CYP83B1 (IAOx) is a precursor of important natural products and the plant hormone auxin, the gene should be studied in economically relevant vegetables as well as in model plants. In this study, we present abundant data concerning the *BoCYP83B1* expression pattern, which will provide useful information and further understanding the function and regulation of this gene in broccoli.

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