Chapter 3

Molecular and Functional Characterization of *BjMYB28* Paralogs

3.1 Introduction

In *A. thaliana* it is well established that MYB28 is involved in the biosynthesis of aliphatic glucosinolates. In our previous chapter, we have isolated four full-length paralogs of *BjMYB28* from the allopolyploid *B. juncea*. The close similarity and phylogeny of *BjMYB28* to *AtMYB28* and other members of subgroup-12 of R2R3-MYB family, which are also involved in the positive regulation of glucosinolates biosynthesis in Arabidopsis, indicates the possible role of *BjMYB28* in the regulation of glucosinolates biosynthesis.

Hence, the current chapter was aimed at the functional characterization of four *BjMYB28* paralogs to study their biological functions. First we checked the subcellular localization of *BjMYB28* using both transient and stable transformation systems. Next, we developed both over-expression and RNAi cassettes of all the four *BjMYB28* paralogs. Since genetic transformation and development of homozygous transgenic stock is a time taking process in a crop system like *B. juncea*, we initially tested these constructs in the closely related model system, *A. thaliana*. The four *BjMYB28* over-expression constructs were transformed into two different genetic backgrounds of Arabidopsis viz., Col-0 (wild-type) and BRC_H161 (*AtMYB28* knock-down mutant). The homozygous *BjMYB28* over-expression lines in both the genetic backgrounds were analyzed for both leaf and seed glucosinolates phenotype to
ascertain the role of each BjMYB28 paralogs towards glucosinolates regulation. Further to confirm their function, BjMYB28 RNAi constructs were also developed and tested in Arabidopsis wild-type, Col-0. The glucosinolates contents and profiles were estimated in the homozygous RNAi lines. We further tested the trans-activation or trans-repression of glucosinolates biosynthetic pathway genes in BjMYB28 over-expression and RNAi lines of A. thaliana. As glucosinolates are an important component of plant defence, these lines were also analyzed for their resistance/susceptibility towards Pseudomonas syringae, in the later part of this chapter.

Finally, we also developed and analyzed over-expression lines of BjMYB28 paralogs in B. juncea, as these results would ideally reflect the near absolute roles of different BjMYB28 paralogs towards controlling specific glucosinolates in the native host plant.

3.2 Materials and Methods

3.2.1 Plant materials and growth conditions

A. thaliana plants (ecotype Col-0) and the AtMYB28 gene (At5g61420) loss of function mutant BRC_H161b (kindly provided by Dr. Piero Morandini) were grown in growth rooms set at 22 °C with 16 hrs light/8 hrs dark cycle. Pots were prepared by filling agropeat: vermiculite mixture (3:1) and were transferred to trays containing water for saturating overnight. Seeds were sown directly onto water saturated pots and covered with cling film to maintain humidity. Trays were initially kept at 4 °C in dark for three days to favour uniform seed germination and later transferred to the growth rooms. The plants were supplied with nutrient media and water on regular basis. For B. juncea transformation, high glucosinolates Indian cultivar Varuna was used.

3.2.2 Experimental methods

3.2.2.1 Generation of BjMYB28:YFP and GFP fusion constructs

To generate BjMYB28-YFP fusion constructs, coding DNA sequence (CDS) of BjMYB28 paralog BjMYB28-1 was cloned into C-terminal YFP fusion vector pEarleygate 101 (Earley et al., 2006) under the control of CaMV35S promoter through gateway cloning. AtMYB28:YFP construct was also generated, which was used as a
positive control and empty vector was used as a negative control for the experiment. Constructs were transformed into onion epidermal cells using particle bombardment. The BjMYB28:GFP fusion constructs were prepared by fusing the CDS of BjMYB28-4 paralog with mGFP protein under the control of CaMV35S promoter in pMDC83 gateway binary vector and transformed into A. tumefaciens and later into A. thaliana through floral dip method.

3.2.2.2 Transient expression and sub-cellular localization of transgene in onion epidermal cells by particle bombardment

For sub-cellular localization studies, C-terminal YFP fusion of the desired gene in a suitable vector were transformed into onion epidermal cells through particle delivery system (PDS 1000, Bio-Rad) according to manufacturer’s instructions. For transformation of the onion epidermal cells, epidermis of onion bulb were peeled off and placed on MS agar plates (3% sucrose and 1% agar) with inner side facing upward. Approximately 3 mg of gold particles were weighed and soaked in 100 µl of 70% ethanol for 15 min. After vortexing vigorously for 5 min, tubes were centrifuged for 5 sec at maximum speed at room temperature. Supernatant was discarded and 100 µl of sterile MQ water was added to the tubes. Tubes were again vortexed for 1 min and the contents were allowed to settle for 1 min, followed by a spin at maximum speed at room temperature for 5 sec. Supernatant was discarded and washing process was repeated for two more times. For coating DNA on gold particles, 50 µl of 50% glycerol was added to the tubes and vortexed for 5 sec in a platform vortex. While vortexing 5-6 µl of 1 mg ml\(^{-1}\) plasmid DNA, 50 µl of 2.5 M CaCl\(_2\), and 20 µl of 0.1 M spermidine were added subsequently and quickly. Vortexing was continued for another 3 min and was then allowed to settle for 1 min followed by a spin for 2 sec. Supernatant was discarded and 140 µl of 70% ethanol was added to the tubes. Contents were again spun for 2 sec. After discarding the supernatant, 48 µl of 100% ethanol was added and a spin was given. The coated particles were then resuspended in 48 µl of 100% ethanol and constantly vortexed at moderate speed till bombardment.

For bombarding the particles on onion epidermal cells, macro carriers were placed on to their holders after cleaning with 100% ethanol. Approximately 8 µl of DNA coated gold particles were pipetted out and placed on to the macro carrier at the centre and
allowed to dry. These were then placed along with stopping screen and rupture disc, and tightened within the gene gun. Pressure of 26.5-27.5 inches of Hg was applied and bombardment was performed with 1100 psi of helium. Plates were incubated in dark at 21 °C for 48 hrs and observed under confocal laser scanning microscope (Leica).

3.2.2.3 Generation of plant transformation constructs of BjMYB28 paralogs

The modified binary vector, pPZP200:lox (Arunugam et al., 2007) containing the ‘lox’ tandem repeats for marker excision selection was used for preparing constructs. A PCR amplified 35Sde-bar-ocspA fragment conferring resistance to herbicide phosphinothricin (commercially available as Basta) was cloned between the lox repeats at EcoRV site to create the basic binary vector pPZP200lox:35Sde-bar-ocspA [pPZP200:lox(bar)] for developing all other constructs.

For development of over-expression and mutant complementation lines, CDS of BjMYB28 paralogs were cloned into pPZP200:lox(bar) binary vector under the control of CaMV35S promoter. The BjMYB28 over-expression constructs were independently transformed into both wild-type (Col-0) and the homozygous loss-of-function mutant of AtMYB28 (BRC_H161b). Transformants were selected on soil by spraying herbicide Basta (active ingredient phosphinothricin) as described above. Designing of RNAi constructs is described in detail in Chapter 5; section 5.2.2.1.

3.2.2.4 Preparation of competent cells and genetic transformation of A. tumefaciens

Competent cells were prepared according to the protocol described by Nishiguchi et al. (1981) with minor modifications. A single colony of A. tumefaciens was inoculated in 5 ml of YEB with proper antibiotics and grown overnight in a rotary shaker at 28 °C and 250 rpm. About 2 ml of overnight culture was transferred in to 50 ml YEB in a 250 ml conical flask and grown until OD$_{600}$ = 0.5. The culture was centrifuged at 5000 rpm for 5 min at 4 °C and the supernatant was discarded. The pellet was resuspended in 10 ml of pre chilled 0.5 M NaCl. The contents were again centrifuged at 5000 rpm for 5 min at 4 °C and the supernatant was discarded. The pellet was resuspended in 1 ml of 20 mM ice cold CaCl$_2$. Competent cells were divided in to aliquots of 50 µl each in microcentrifuge tubes and stored at -80 °C.
The competent cells were thawed on ice for 20 min. Approximately 0.5 – 1.0 µg of plasmid DNA was added to the competent cells and mixed gently. The tubes were incubated on ice for 30 min. The cells were frozen in liquid nitrogen for 1-5 min followed by thawing at 37 °C in a water bath for 1-5 min. One ml of YEB was added to the tubes and incubated with gentle shaking at 28 °C for 2-4 hrs. The contents were then centrifuged at 6000 rpm for 5 min at room temperature. Supernatant was discarded and the pellet was resuspended in 100 µl of the YEB medium and plated on YEB agar plate supplemented with proper antibiotics.

3.2.2.5 Genetic transformation of A. thaliana by floral dip

Genetic transformation of A. thaliana was carried out using floral dip method (Clough and Bent, 1998) A. thaliana plants were grown in culture rooms as described above until flowering. A single colony of A. tumefaciens carrying gene of interest was inoculated in 5 ml liquid medium containing appropriate antibiotics. From this primary culture, a large liquid culture (250-300 ml) was grown at 28 °C for overnight (mid log phase to recent stationary) with antibiotics to select for the binary vector. Bacterial cells were harvested by centrifuging the culture at 6000 rpm at room temperature for 7 min. Agrobacterium cells were then resuspended to a final OD<sub>600</sub> of ~0.8 in 5% sucrose solution. Before dipping the plants, silwett L-77 was added to the suspension at a final concentration of 0.05%. All the above ground part of the plants was dipped in the bacterial suspension for 15-30 sec with gentle agitation. Dipped plants were placed under a polythene cover in a tray (with the plants laid on their side) for 16-24 hrs to maintain high humidity. Plants were transferred to growth rooms next day, watered and grown normally till harvesting the seeds.

3.2.2.6 Seed sterilization protocol for Arabidopsis

Seeds were taken in a microcentrifuge tube and 1 ml of 70% ethanol was added and mixed for 2 min followed by rinsing twice with sterile distilled water. Tubes were refilled with a 1 ml solution of 30% bleach (chlorec and 0.1% Triton X-100). The contents of the tubes were shaken vigorously to develop a layer of foam and the tubes were continued incubating on a slow rocking shaker for 15 min. Seeds were allowed to settle and the bleach solution was decanted off. Bleach was completely removed by washing the seeds in sterile distilled water for 4-5 times. Seeds were then plated on to suitable MS plates supplemented with appropriate selective agent.
3.2.2.7 Selection of Basta resistant Arabidopsis transformants in soil

Basta resistant Arabidopsis transformants were directly selected on soil. Seeds were sprinkled on to the pots saturated with water and covered with cling film to maintain humidity. Pots were kept in dark for 2-5 days at 4 °C, and then transferred to growth rooms. After ten days film was removed and plants were sprayed with Basta (120 mg l⁻¹) on alternate day’s interval. Three to four sprays were given to confirm transformants. Transformants were transferred to fresh pots after selection, and grown normally.

3.2.2.8 Genetic transformation of B. juncea

Adequate amount of seeds (~500 seeds/experiment) were taken in a conical flask and a few drops of Teepol was added. The contents were shaken for 10 min and then rinsed thoroughly in running tap water for approximately 30 min. The seeds were further treated with 70% ethanol for 2 min and washed twice with sterile distilled water. Surface sterilization was done with 0.05% HgCl₂ for 10 min followed by washing with sterile distilled water for at least five times. Seeds were then inoculated on to basal MS media and grown in culture rooms set at 10 h light (day) / 14 h dark (night) cycle, at a temperature of 22 °C / 15 °C and 70% relative humidity, respectively. Hypocotyls of five day old seedlings cut into 0.5 to 1.0 cm pieces were used as explants. Explants were pre-cultured in MS liquid media supplemented with 1 mg ml⁻¹ each of NAA and BAP (N1B1) for 24 hrs. Agrobacterium harbouring desired constructs were grown in YEB medium supplemented with proper antibiotics. Bacterial cells at OD₆₀₀ of 0.5 were harvested and resuspended in N1B1 medium to a final OD₆₀₀ of 0.3. Explants were incubated with the bacterial suspension for 30 min and co-cultivated at 23 °C, 110 rpm for 16-24 hrs. After co-cultivation the explants were washed with liquid N1B1 supplemented with augmentin (200 mg l⁻¹). Total of four washes; first two of which were given for 1 min and remaining two were given for 30 min each with gentle agitation. The explants were then blotted with sterile Whatman no. 1 filter paper to remove residual media and plated on to shoot induction media containing 1 mg l⁻¹ each of NAA, BAP, 200 mg l⁻¹ augmentin, 20 μM AgNO₃ and 10 mg l⁻¹ of herbicide Basta. Regenerated shoots were transferred on to rooting media supplemented with 2 mg l⁻¹ IBA along with augmentin and Basta.
3.2.2.9 Extraction and estimation of glucosinolates

Total and component glucosinolates profiles were determined by High Performance Liquid Chromatography (HPLC) as per the protocols described earlier (Kraling et al., 1990) with minor modifications for both Arabidopsis and Brassica. Samples were analyzed in Shimadzu CLASS-VP V6.14 HPLC machine (Luna C18 RP column of 150 x 4.6 mm with 0.5μ internal diameter). Benzyl glucosinolate, glucotropaeolin (GTL, Applichem, Germany) was used as the internal standard.

**Extraction of glucosinolates:** For glucosinolates extraction, approximately 200 mg freeze dried leaf sample or seed sample was ground to fine powder with mortar and pestle. From this exactly 200 mg tissue was weighed and transferred to a polypropylene tube and 2-3 ml of 70% boiling methanol was added. Then 200 µl GTL (6 mM) was added carefully to each tube from the centre. Tubes were vortexed for 10 sec and kept in water bath maintained at 75 °C for 5 min, vortexed again for 10 sec and incubated in water bath for another 5 min. Samples were centrifuged at 5000 rpm for 5 min at room temperature and the supernatant was transferred to a fresh tube. Extraction was repeated with the settled pellet using 10% methanol for seeds. For leaf samples, both the extractions were carried out in 70% methanol. Finally both the supernatants collected were mixed and again centrifuged to pellet down any contaminating tissue debris.

**On-column desulfonylation of glucosinolates:** Columns were prepared by placing silane treated glass wool in the form of a cup inside a shortened pasteur pipette with the help of a glass rod. About 1 ml of sephadex-A25 solution (20 mg ml⁻¹) was loaded on to the column and allowed to settle. To the column, 500 µl imidazole formiate (40 g imidazole in 100 ml of 30% formic acid and 1% rhodamine B) was added followed by washing twice with 1 ml of HPLC grade water (filtered through 0.22 µm filter) each time. Extracted sample (500 µl) was then loaded on to the column and left for some time. Column was washed twice with 1 ml of HPLC grade water each time and 75 µl of purified sulfatase (25 mg ml⁻¹, Sigma-Aldrich) was added. Columns were then incubated at 20-22 °C for 10-12 hrs. Desulforylated glucosinolates were eluted by washing the columns thrice with 500 µl of HPLC grade water each time. Eluent was filtered through 0.22 µm filter and collected in assay vials for injection in to the HPLC machine.
**Estimation of glucosinolates:** For detection and estimation of glucosinolates, a gradient of water (solvent A) and acetonitrile (solvent B) was used with a flow rate of 1 ml min⁻¹ at 25 °C oven temperature. The 30 min HPLC run was programmed at 1.5% B (1 min), 1.5-5% B (2 min), 5-7% B (2 min), 7-21% B (7 min), 21-29% B (3 min), 29-43% B (5 min), 43-93% B (0.5 min), 93% B (hold for 4 min), 93-1.5% B (0.5 min), and a 5 min hold at 1.5% B. Glucosinolate components were detected at 229 nm, UV and peaks were identified with reference to the retention time of already published chromatograms. Concentrations of individual glucosinolates were calculated in micromole per gram seed/leaf dry weight (μmol g⁻¹ DW) relative to the area of the internal standard peak (GTL) applying their respective response factors (Brown *et al.*, 2003; Clarke, 2010).

**3.2.2.10 Flood inoculation method for *Pseudomonas syringae* infectivity assay**

To check the response of Arabidopsis transgenic lines with altered glucosinolate profiles towards *Pseudomonas* infection, flood inoculation method (Ishiga *et al.*, 2011) was carried out. Both over-expression as well as RNAi lines developed in *A. thaliana* were used for the assay along with wild-type (Col-0) as control. Homozygous seeds were surface sterilized and plated on to deep culture plates and sealed with micropore tapes. Approximately 7 plants were inoculated per plate. Plants were grown under 12 h light / 12 h dark cycle at 21 °C for three weeks and used for the assay. *Pseudomonas syringae* pv. tomato DC3000 was grown on Kings medium B containing appropriate antibiotics for 24-36 hrs. Bacterial culture was grown till OD₆₀₀ reached 0.5. The bacterial cells were pelleted down at 6000 rpm for 15 min at room temperature. The cells were resuspended in sterile distilled water to a final OD₆₀₀ of 0.01 and used for infection.

For flood inoculation, 30 ml each of the culture suspension containing 0.025% silwett L-77 was added to the plates containing seedlings and allowed to stand for 3 min with gentle agitation. Bacterial culture was decanted and plants were transferred to culture room. Observations were taken till 5 days post infection (dpi) and recorded. Infected seedling were harvested on 5 dpi and weighed. Tissues were then surface sterilized with 5% H₂O₂ for 3 min followed by thoroughly rinsing with sterile distilled water. Sterilized tissues were then homogenised in sterile distilled water and different serial dilutions (10⁻⁴-10⁻⁶) were plated on Kings medium B, supplemented with proper
antibiotics and plates were incubated at 28 °C. Colonies were counted on third day and infectivity was expressed as cfu mg⁻¹ (colony forming unit) of tissue for normalization. Three independent experiments with four replicates for each line were carried out.

3.3 Results

3.3.1 Nuclear localization of BjMYB28

MYB28 belongs to the R2R3 transcription factor family and hence is expected to be localized in the nucleus. The AtMYB28 as well as the four BjMYB28 proteins isolated in the current study lack the typical nuclear localization signal, when queried against the PredictProtein (http://www.predictprotein.org/). However, a conserved SV40-type putative nuclear localization motif ‘LKKRL’ (van der Krol and Chua, 1991), adjacent to the R3 repeat; between amino acid position 112-116 of these proteins was observed (Chapter 2, Fig. 2.10).

To investigate the subcellular localization, a representative BjMYB28 protein namely, BjMYB28-1 was fused in frame to the 5’ terminus of a yellow fluorescent reporter protein (YFP) tag under the control of CaMV35S promoter in pEG101 binary vector. The BjMYB28-1:YFP fusion construct was introduced in the onion epidermal cells (Allium cepa) by particle bombardment and analyzed under confocal laser scanning microscope after 48 hrs of transformation. The AtMYB28:YFP fusion construct was used as a positive control which is shown to be localized in the nucleus of cultured A. thaliana cells (Gigolashvili et al., 2007a). The nuclei of cells transformed with BjMYB28-1:YFP fusion construct showed a strong yellow fluorescent signal as also observed for AtMYB28:YFP protein (Fig. 3.1), thus demonstrating the nuclear localization of BjMYB28 protein. The YFP vector control showed cytoplasmic distribution as expected.
Figure 3.1

Sub-cellular localization of BjMYB28:YFP fusion construct in onion epidermal cells. The YFP filter, bright field and merged images of (A) YFP vector control (B) BjMYB28:YFP, and (C) AtMYB28:YFP fusion proteins are shown.

Simultaneously, the subcellular localization of one of the BjMYB28 protein, BjMYB28-4 was also analyzed in transgenic A. thaliana lines stably expressing the BjMYB28:GFP fusion protein. Five day old T2 seedlings were analyzed under confocal microscope. GFP was visible in the nucleus of the cells of root tissue which also showed co-localization with nucleus specific DAPI stain (Fig. 3.2).

Figure 3.2

Sub-cellular localization of BjMYB28:GFP fusion construct in stable Arabidopsis transgenic lines. (A) GFP filter (B) DAPI staining for the nucleus and (C) merged images of BjMYB28:GFP fusion protein are shown.
These results of subcellular localization of BjMYB28 proteins based on both transient and stable transformation systems clearly demonstrate that BjMYB28 proteins are nuclear localized.

### 3.3.2 Generation of BjMYB28 over-expression constructs and development of functional complementation and over-expression lines of BjMYB28 paralogs in A. thaliana

To determine the functional role of the BjMYB28 paralogs, over-expression constructs were developed in pPZP200:lox(bar) binary vector under the control of CaMV35S promoter for all the four BjMYB28 paralogs. Functional complementation lines of all BjMYB28 paralogs were initially generated in AtMYB28 mutant BRC_H161b, an Atmyb28 knock-out T-DNA insertion line of A. thaliana (Beekwilder et al., 2008). The BjMYB28 over-expression constructs were also transformed to A. thaliana wild-type, ecotype Col-0. Around 2-6 homozygous lines for both mutant complementation and over-expression phenotypes were obtained in T3/ T4 generation on Basta selection (Table 3.1). Only two independent homozygous lines for each of the four BjMYB28 paralogs were analyzed for their leaf and seed glucosinolate profiles using HPLC. Schematic representation of the over-expression constructs developed is given in Fig. 3.3. A representative HPLC chromatogram is also depicted showing the identification of the peaks and retention times of individual glucosinolate components (Fig. 3.4).

![Schematic representation of T-DNA cassette of an over-expression construct developed](image-url)

**Figure 3.3**

*Schematic representation of T-DNA cassette of an over-expression construct developed.* The CDS of BjMYB28 paralogs were cloned under the control of the constitutive promoter CaMV35S. bar gene was used as the plant selection marker.
A representative chromatogram showing detection of individual fractions of Arabidopsis leaf glucosinolates as identified by HPLC. Glucosinolates were detected with UV at 229 nm. HPLC was performed in Shimadzu CLASS-VP V6.14 SP2 HPLC machine with Luna C18 column (150 x 4.6 mm, 5µm). (A) Glucosinolates profile of A. thaliana wild-type Col-0; (B) Glucosinolates profile of one representative BjMYB28 over-expression line.

Table 3.1. Summary of BjMYB28 mutant complementation (MC) and over-expression (OE) lines developed in A. thaliana.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Construct</th>
<th>No. of homozygous lines obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OE (Col-0)</td>
</tr>
<tr>
<td>1.</td>
<td>pt35S:BjMYB28-1</td>
<td>2</td>
</tr>
<tr>
<td>2.</td>
<td>pt35S:BjMYB28-2</td>
<td>4</td>
</tr>
<tr>
<td>3.</td>
<td>pt35S:BjMYB28-3</td>
<td>4</td>
</tr>
<tr>
<td>4.</td>
<td>pt35S:BjMYB28-4</td>
<td>6</td>
</tr>
</tbody>
</table>

3.3.3 Analysis of functional complementation lines of BjMYB28 paralogs in AtMYB28 mutant BRC_H161b

In order to check the function of BjMYB28 paralogs, functional complementation lines were developed in AtMYB28 mutant BRC_H161b background. The BRC_H161b lines have been shown to accumulate about 30-40% less of total aliphatic glucosinolate pools in the leaves than wild-type Col-0. The BjMYB28 complemented lines in Arabidopsis mutant background were analyzed for their leaf glucosinolate contents in 25 d old rosette leaves. The total aliphatic glucosinolates were found to be elevated up to 2.5 fold compared to the mutant H161 background, similar to that of wild-type,
Col-0 (Fig. 3.5A). The mutant complementation lines of BjMYB28 paralogs showed 1.4 to 2.5 fold increase in the levels of short-chain glucosinolates viz., 3-methylsulfinylpropyl-glucosinolate (3MSOP) and 4-methylsulfinylbutyl-glucosinolate (4MSOB) (Fig. 3.5B and 3.5C). The two long chain glucosinolates; 6-methylsulfinylhexyl-glucosinolate (6MSOH) and 7-methylsulfinylheptyl-glucosinolate (7MSOH) were undetected in the mutant complemented lines and the reference control H161. However, 8-methylsulfinyloctyl-glucosinolate (8MSOO) was increased by 1.6-2.6 folds in the mutant complementation lines (Fig. 3.5C). The mutant complementation of BjMYB28 also resulted in the production of 5-methylsulfinylpentyl-glucosinolate (5MSOP) fraction which was undetected in the H161 mutant (Fig. 3.5C).

---

**Figure 3.5**

Glucosinolates accumulation in rosette leaves of Arabidopsis myb28 mutant (BRC_H161b) over-expressing BjMYB28 paralogs. The glucosinolates content and profile (in µmol g⁻¹ dry weight) was determined in 25 days old rosette leaves. The individual graph shows accumulation of (A) total aliphatic glucosinolates (GSL); (B) 4MSOB; (C) 3MSOP, 5MSOP and 8MSOO; (D) total indolic glucosinolates. Abbreviation used: 4MSOB- 4-methylsulfinylbutyl-GSL; 3MSOP- 3-methylsulfinylpropyl-GSL; 5MSOP- 5-methylsulfinylpentyl-GSL; 8MSOO- 8-methylsulfinyloctyl-GSL. At least two independent mutant-complemented lines for each BjMYB28 paralog were analyzed and the average foliar glucosinolates are represented along-with their standard error.
The indolic fractions namely, indol-3-ylmethyl-glucosinolate (I3M), 1-methoxy-3-ylmethyl-glucosinolate (1MOI3M) and 4-methoxyindol-3-ylmethyl-glucosinolate (4MOI3M) were found to be unaltered or slightly elevated in the mutant complemented lines (Fig. 3.5D).

3.3.4 Analysis of over-expression lines of BjMYB28 paralogs in A. thaliana wild-type Col-0

The BjMYB28 over-expression constructs developed were later transformed to the wild-type Arabidopsis background, Col-0. The wild-type Arabidopsis was selected because the wild-type Col-0 plants have optimal accumulation of all the aliphatic glucosinolate components which were otherwise not present in the AtMYB28 mutant genetic background (BRC_H161b) used earlier for the functional complementation, described in the above section. Total as well as individual glucosinolate fractions of BjMYB28 over-expression lines in A. thaliana wild-type (Col-0) were estimated at 25 d old rosette leaves using HPLC. BjMYB28 over-expression lines showed approximately 1.5-1.9 fold increase in total leaf aliphatic glucosinolate accumulation compared to the wild-type plants (Fig. 3.6A). Levels of 4MSOB, the major glucosinolate fraction present in Arabidopsis was found to be increased by approximately 1.4-1.8 fold (Fig 3.6B). Contents of short chain aliphatic glucosinolates like 3MSOP and 5MSOP were also found to be increased up to 2.4 and 1.7 folds, respectively (Fig. 3.6C). Concomitantly, the level of precursor 4-methylthiobutyl-glucosinolate (4MTB) showed significant reduction in over-expression lines of at least two BjMYB28 paralogs, thereby reflecting an increased flux of 4C-glucosinolates towards 4MSOB in the biosynthesis pathway. Accumulation of long-chain aliphatic glucosinolates namely 7MSOH and 8MSOO were also increased by 1.7-2.9 fold, whereas 6MSOH showed an increase up to 1.6 fold, compared to the wild-type plants (Fig. 3.6D). Contents of the indolic glucosinolates such as I3M, 1MOI3M and 4MOI3M were found to be unaltered or slightly elevated (Fig. 3.6E and 3.6F).
Glucosinolates accumulation in rosette leaves of *BjMYB28* over-expressing Arabidopsis lines (Col-0). The glucosinolates content and profile (in µmol g⁻¹ dry weight) was determined in 25 days old rosette leaves. The individual graph shows accumulation of (A) total aliphatic glucosinolates; (B) major glucosinolate 4MSOB; (C) short chain aliphatic glucosinolates including 3MSOP, 4MTB and 5MSOP; and (D) long-chain aliphatic glucosinolates including 6MSOH, 7MSOH, 8MSOO. Abbreviation used: 4MSOB- 4-methylsulfinylbutyl-GSL; 3MSOP- 3-methylsulfinylpropyl-GSL; 5MSOP- 5-methylsulfinylpentyl-GSL; 6MSOH- 6-methylsulfinylhexyl-GSL; 7MSOH- 7-methylsulfinylheptyl-GSL; 8MSOO- 8-methylsulfinyloctyl-GSL; (E) Total indolic (F) Component fractions of indolic glucosinolates such as I3M- Indol-3-yl methyl, 4MOI3M-4-Methoxyindol-3-ylmethyl, and 1MOI3M-1- Methoxyindol-3-ylmethyl glucosinolate. At least two independent over-expression lines for each *BjMYB28* paralogs were analyzed and the averages of foliar glucosinolates are represented along-with their standard error.
Further, the homozygous T4 seeds of BjMYB28 over-expression lines of Arabidopsis were also analyzed for the seed glucosinolates content using HPLC. Total glucosinolates were estimated in mature seeds. HPLC analysis showed that there was an increase in the total glucosinolates content from 1.2-1.6% in the BjMYB28 over-expression lines in Arabidopsis compared to the wild-type control, Col-0 (Fig. 3.7). Almost all the individual aliphatic glucosinolate fractions were elevated in the seeds as also observed for the leaf glucosinolates.

![Figure 3.7](image)

**Figure 3.7**

Total glucosinolates accumulation in the seeds of BjMYB28 over-expressing Arabidopsis lines (Col-0). The glucosinolate content and profile (in µmol g\(^{-1}\) dry weight) was determined in mature seeds. Two independent over-expression lines for each BjMYB28 paralogs were analyzed and the average values are represented along-with their standard error.

Based on both functional complementation and over-expression studies in *A. thaliana*, it is evident that the over-expression of BjMYB28 paralogs resulted in the over-accumulation of methionine-derived aliphatic glucosinolates, albeit with different over-expression phenotype among themselves. The four BjMYB28 paralogs control accumulation of both short and long-chain aliphatic glucosinolates with almost similar over-expression phenotype, thereby representing functional redundancy when expressed in a heterologous system. Interestingly, A-genome (*B. rapa*) specific paralogs showed relatively higher levels of leaf and seed glucosinolates accumulation in the over-expression lines which reflects their higher participation towards glucosinolates accumulation. We presume that detailed characterization of BjMYB28
paralogs in native (*B. juncea*) system will provide a definitive clue of their functional variance towards controlling glucosinolates content and profiles in Brassica species.

### 3.3.5 Growth phenotypes of *BjMYB28* over-expression lines

The over-expression lines were tested for their seed germination and survival under normal and in the presence of various hormonal and stress conditions. The over-expression lines of *BjMYB28* paralogs in both wild-type (Col-0) and *AtMYB28* mutant line (BRC_H161b) backgrounds showed no visible effects on seed germination, plant growth or development (Fig. 3.8). These lines grew normally and did not provide any significant advantage in response to different abiotic stress conditions (such as salt, heat, dehydration, cold treatments) compared to their corresponding reference background. Root length, lateral roots, hypocotyl length etc. were also analyzed. There was no difference between the wild-type and transgenic lines for the parameters tested. Thus *BjMYB28* paralogs showed specific role towards regulating the aliphatic glucosinolate pools without affecting any other plant processes or phenotypes.

![Figure 3.8](image)

**Figure 3.8**

**Growth phenotype of over-expression lines of *BjMYB28* paralogs in Arabidopsis.** Transgenic plants were grown on 3:1 agaropeat: vermiculite mixture and selection was done with herbicide Basta. Homozygous lines were generated and analyzed for growth phenotype. There was no difference in the growth and development of transgenic lines compared to the wild-type. *AtMYB28* mutant BRC_H161b is also compared.
3.3.6 Trans-activation of aliphatic glucosinolate biosynthesis pathway genes in *BjMYB28* over-expression lines of *A. thaliana*

*AtMYB28* is known to be the master regulator of glucosinolates biosynthesis in Arabidopsis regulating almost all the biosynthetic genes of the pathway. In order to test the effect of *BjMYB28* over-expression on the steady-state mRNA levels of the Arabidopsis native genes involved in glucosinolates biosynthesis, real-time quantitative RT-PCR analysis was performed. A representative over-expression (in Col-0) and mutant complementation (in BRC_H161b) line of both A- and B-genome specific *BjMYB28* paralogs, showing highest level of aliphatic glucosinolates accumulation were selected for the transcript profiling of glucosinolates biosynthesis genes in the rosette leaves of three weeks old Arabidopsis transgenic lines.

![Graph showing fold expression values](image)

**Figure 3.9**

Transcript levels of glucosinolates pathway genes in rosette leaves of representative *BjMYB28* mutant complementation and over-expression lines of *A. thaliana*. (A) Real-time RT-PCR analysis of aliphatic glucosinolates pathway genes in *BjMYB28* mutant complemented lines. (B) Real-time RT-PCR analysis of aliphatic glucosinolates pathway genes in over-expression lines of *BjMYB28*. The relative transcript accumulation was measured with reference to the BRC-H161b in (A) and wild-type Col-0 in (B), set at 1. Values are means of three technical replicates and showing standard error bars.
As shown in Fig. 3.9, expression of genes involved in both side-chain elongation (\textit{MAM1} and \textit{MAM3}) and core biosynthesis (\textit{CYP79F1}, \textit{CYP79F2}, \textit{CYP83A1}, \textit{AtST5b}, and \textit{AtST5c}) steps of aliphatic glucosinolates biosynthesis pathway were considerably increased in the over-expression and mutant complemented lines compared to their corresponding reference controls. The increased glucosinolates accumulation in the over-expression lines corresponds well with the increased mRNA transcript levels of putative target genes. The BjMYB28-4(OE) and BjMYB28-4(MC) lines with highest aliphatic glucosinolates content in over-expression (Col-0) and mutant-complementation (H161b) backgrounds respectively showed highest transcript accumulation of most of the glucosinolates biosynthesis genes.

### 3.3.7 Knock-down of MYB28 in \textit{A. thaliana}

Alternatively, to confirm the function of BjMYB28 paralogs towards controlling aliphatic glucosinolates accumulation, RNAi lines of \textit{MYB28} were generated in the heterologous system, \textit{A. thaliana}. Two different RNAi constructs were developed to target the \textit{AtMYB28} gene. A 320 bp fragment from the 3' region of the BjMYB28 paralogs showing maximum sequence conservation with the \textit{AtMYB28} gene was selected for RNAi construct design. The RNAi construct were driven by two different BjMYB28 promoters namely, BjMYB28-3 and BjMYB28-4. These promoters of two A-genome specific paralogs were selected because in the previous section of the current chapter, we found that A-genome specific paralogs contribute more towards aliphatic glucosinolates accumulation (These constructs corresponds to C3 and C15 constructs described in chapter 5, Table 5.1. Details of construct design are given in Annexure-III).

Homozygous seeds of the RNAi lines in Arabidopsis were generated and their leaf glucosinolates content were analyzed using HPLC in 25 d old rosette leaves. The results of analysis showed that there was a significant reduction of total aliphatic glucosinolates in the RNAi lines by 73% - 85% of the wild-type (Col-0) level. Data of two best performing lines of each RNAi constructs are given (Fig. 3.10). The levels of indolic glucosinolates were found to be unaltered in these RNAi lines.
**Figure 3.10**

**Leaf glucosinolates content of BjMYB28 (RNAi) lines of Arabidopsis.** The glucosinolates content and profile (in µmol g⁻¹ dry weight) was determined in 25 days old rosette leaves. The individual graph shows accumulation of (A) total aliphatic glucosinolates; (B) total indolic glucosinolates in the best performing RNAi lines. Two RNAi lines each of BjMYB28-3 and BjMYB28-4 are given. Values represent mean of two biological replicates with their standard error.

Molecular basis of the low glucosinolates accumulation was investigated by measuring steady state mRNA levels of AtMYB28 as well as the candidate genes involved in the biosynthesis of aliphatic glucosinolates by real-time quantitative RT-PCR. The *A. thaliana* RNAi lines showed reduced expression of AtMYB28. Concomitantly, the expression levels of aliphatic glucosinolates biosynthesis pathway genes were also found to be significantly reduced. Expression of AtST5c, the last enzyme of the pathway was found to be least affected followed by AtST5b (Fig. 3.11). Thus based on knock-down analysis in *A. thaliana*, it is evident that BjMYB28 paralogs are positive regulators of aliphatic glucosinolates biosynthesis in *B. juncea* and not that of indolic glucosinolates.
Figure 3.11

Transcript levels of glucosinolates pathway genes in rosette leaves of representative MYB28 RNAi lines in *A. thaliana*. Real-time RT-PCR analysis of aliphatic glucosinolates pathway genes in RNAi lines of BjMYB28-3(RNAi 6) and BjMYB28-4(RNAi1) are given. The relative transcript accumulation was measured with reference to the wild-type (Col-0) as calibrator (set at 1). Values are means of three technical replicates with their standard error bars.

3.3.8 Response of *BjMYB28* over-expression and knock-down lines to infection by *P. syringae*

Glucosinolates are well known for their role in plant resistance to insects and pathogens. In order to investigate whether the altered accumulation of aliphatic glucosinolates in Arabidopsis over-expression/knock-down lines impart resistance/susceptibility to pathogenic invaders, *Pseudomonas syringae* pv. tomato strain DC3000 was tested on these lines. One of the best model pathosystems for studying plant-pathogen interactions is *A. thaliana*- *P. syringae* interaction system because of its pathogenicity in Arabidopsis (Katagiri et al., 2002). This model system has been widely used to understand a number of dynamic and complex molecular events in both resistance and susceptible interactions. In addition, *P. syringae* pv. tomato DC3000 can infect and induce disease symptoms on both Arabidopsis and Brassica spp. (Whalen et al., 1991; Abramovitch et al., 2004). Two best performing lines each of both over-expression as well as knock-down lines of *BjMYB28* generated in Arabidopsis were subjected to *Pseudomonas* infection. Infection was carried out in seedlings after three weeks of growth in MS agar media in petriplates. Flood
inoculation method was performed for infectivity assay. Mock control, flooded with water, was also set aside for each line including the wild-type control. Symptoms started developing on the third day post infection (dpi) and observations were taken on every day and recorded. There was no visible difference in symptom development and severity of infection, in the transgenic Arabidopsis lines compared to the wild-type, Col-0. Symptom development in representative over-expression and knock-down lines are shown (Fig. 3.12).

**Figure 3.12**

Symptom development in Arabidopsis seedlings infected with *Pseudomonas syringae*. Flood inoculation with the cultured pathogen was carried out in three week old seedlings and symptoms were recorded. Seedlings of 5 day post infection are shown. (A) *BjMYB28* over-expression lines; (B) *BjMYB28* knock-down lines. Representative plants from an infected population are given along with mock control. Experiment was repeated three times to confirm the results.
From these results it becomes evident that indolic glucosinolates may be the key determinants of plant-*Pseudomonas* interaction, the level of which were unaltered in both over-expression and knock-down lines of Arabidopsis. The detailed explanation of these observations is discussed later in Chapter 5, along with some in-depth analysis in *B. juncea*.

### 3.3.9 Development and characterization of BjMYB28 over-expression lines in *B. juncea*

The *BjMYB28* over-expression constructs were parallely transformed into *B. juncea* cv. Varuna using standard protocols. A representative *B. rapa* (A-genome) and *B. nigra* (B-genome) specific *BjMYB28* paralogs were used and tested for their specific role towards controlling the profile of glucosinolates in *B. juncea*. Eight transgenic lines each were developed for the *BjMYB28*-2 (B-genome specific) and *BjMYB28*-4 (A-genome specific) (Table 3.2). Plants were grown in containment growth facility during the growing season from November-April.

**Table 3.2.** Over-expression lines developed in *B. juncea* cv. Varuna.

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Construct</th>
<th>Target gene</th>
<th>No. of lines developed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pt35S:BjMYB28-2 (OE)</td>
<td><em>BjMYB28</em>-2</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Pt35S:BjMYB28-4 (OE)</td>
<td><em>BjMYB28</em>-4</td>
<td>8</td>
</tr>
</tbody>
</table>

To check the levels of glucosinolates in the over-expression lines of *B. juncea*, HPLC was performed. Two months old leaf tissue of T0 transgenic lines were harvested and lyophilized. About 100 mg lyophilized powder was used for the HPLC analysis. The *BjMYB28* transgenic lines showed a variable range of leaf aliphatic glucosinolates content ranging from 22.29-79.86 µmol g⁻¹ DW (Table 3.3). Out of the eight independent transgenic lines developed using each of the BjMYB28(OE) constructs, we found that only 2-3 lines showed significant increase (1.5- 2.0 fold) in the total leaf glucosinolate content and profiles compared to the wild-type (Varuna) and vector control line. The individual fractions of aliphatic glucosinolates were also found to be increased in these over-expression lines (Table 3.3). Thus our preliminary data clearly indicates that over-expression of *BjMYB28* paralogs in *B. juncea* can increase accumulation of leaf aliphatic glucosinolates.
Table 3.3. Leaf glucosinolates content and profiles of *BjMYB28* over-expression lines of *B. juncea*. Two months old leaves of T0 transgenic lines were analyzed for glucosinolates estimation. SIN- sinigrin, GNA- Glucanapin. Lines marked with * denotes plants with elevated glucosinolates accumulation.

<table>
<thead>
<tr>
<th>Plant/Transgenic line</th>
<th>SIN</th>
<th>GNA</th>
<th>Total GSL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Varuna</td>
<td>6.22</td>
<td>32.56</td>
<td>40.82</td>
</tr>
<tr>
<td>Vector control</td>
<td>5.83</td>
<td>34.42</td>
<td>40.25</td>
</tr>
<tr>
<td><em>BjMYB28-2 (OE)-1</em></td>
<td>11.84</td>
<td>43.08</td>
<td>57.34</td>
</tr>
<tr>
<td><em>BjMYB28-2 (OE)-2</em></td>
<td>6.16</td>
<td>30.74</td>
<td>40.15</td>
</tr>
<tr>
<td><em>BjMYB28-2 (OE)-3</em></td>
<td>3.20</td>
<td>19.25</td>
<td>22.29</td>
</tr>
<tr>
<td><em>BjMYB28-2 (OE)-4</em></td>
<td>6.23</td>
<td>30.84</td>
<td>38.58</td>
</tr>
<tr>
<td><em>BjMYB28-2 (OE)-5</em></td>
<td>5.06</td>
<td>23.54</td>
<td>29.54</td>
</tr>
<tr>
<td><em>BjMYB28-2 (OE)-6</em></td>
<td>6.03</td>
<td>27.26</td>
<td>34.09</td>
</tr>
<tr>
<td><em>BjMYB28-2 (OE)-7</em></td>
<td>5.18</td>
<td>30.39</td>
<td>36.57</td>
</tr>
<tr>
<td><em>BjMYB28-2 (OE)-8</em></td>
<td>9.54</td>
<td>49.72</td>
<td>59.26</td>
</tr>
<tr>
<td><em>BjMYB28-4 (OE)-1</em></td>
<td>13.02</td>
<td>63.93</td>
<td>77.96</td>
</tr>
<tr>
<td><em>BjMYB28-4 (OE)-2</em></td>
<td>5.43</td>
<td>29.00</td>
<td>34.43</td>
</tr>
<tr>
<td><em>BjMYB28-4 (OE)-3</em></td>
<td>5.40</td>
<td>20.68</td>
<td>26.08</td>
</tr>
<tr>
<td><em>BjMYB28-4 (OE)-4</em></td>
<td>4.56</td>
<td>21.6</td>
<td>26.16</td>
</tr>
<tr>
<td><em>BjMYB28-4 (OE)-5</em></td>
<td>11.91</td>
<td>49.635</td>
<td>53.14</td>
</tr>
<tr>
<td><em>BjMYB28-4 (OE)-6</em></td>
<td>6.74</td>
<td>29.48</td>
<td>36.22</td>
</tr>
<tr>
<td><em>BjMYB28-4 (OE)-7</em></td>
<td>4.90</td>
<td>17.40</td>
<td>22.30</td>
</tr>
<tr>
<td><em>BjMYB28-4 (OE)-8</em></td>
<td>9.68</td>
<td>43.78</td>
<td>53.14</td>
</tr>
</tbody>
</table>

Since the leaves of primary T0 transformants were analyzed for the glucosinolates content, we presume that it may not reflect the true over-expression phenotype as the primary transformants are bound to harbour somaclonal variations and growth abnormalities in T0 generation. Glucosinolates profile was then analyzed in the T1 seeds for getting a better insight. Upon analyzing the seed glucosinolates content, all the transgenic lines developed using *BjMYB28*(OE) constructs showed increased accumulation of glucosinolates. Total aliphatic glucosinolate contents of the over-expression lines increased by 1.2-2.0 folds, thereby reflecting the specific role of *BjMYB28* paralogs towards aliphatic glucosinolates accumulation (Fig. 3.13A). However, few transgenic lines showed somewhat reduced levels of indolic glucosinolates accumulation probably suggesting putative cross-talk between the
aliphatic and indolic glucosinolates biosynthesis pathways in *B. juncea* (Fig. 3.13B). In general, the lines with higher accumulation of seed glucosinolates also showed higher accumulation of leaf glucosinolates. It was also clear from the data that, there was no paralog specific difference with respect to glucosinolate profiles. Both paralogs increased all fractions in the same manner, but at different levels. In general, *B. rapa* specific paralogs, (*BjMYB28-4*) was found to be contributing at a higher level than the *B. nigra* specific paralog (*BjMYB28-2*) toward total glucosinolate pools.

![Figure 3.13](image)

**Seed glucosinolates content of *BjMYB28* over-expression lines of *B. juncea.* (A) Total aliphatic glucosinolates (B) total indolic glucosinolates. T1 seeds of *BjMYB28* over-expression lines were analyzed using HPLC with three technical replicates for each line. The average values are represented along-with their standard deviation.
Our data advocates a major concern regarding analyzing the transgenic events in the T0 generation, which generally have lots of growth related abnormalities. The transgenics must be analyzed for the desired trait only once the transgenic event has completed one cycle of vegetative and reproductive growth. Detailed investigation of these lines in advance homozygous T2 generation will be done in future.

3.4 Discussion

Earlier studies have shown that AtMYB28 is the principal positive regulator of the genes involved in aliphatic glucosinolates biosynthesis in A. thaliana (Gigolashvili et al., 2007a; Hirai et al., 2007; Sonderby et al., 2008; 2010). In the current study, over-expression of BjMYB28 paralogs in two different genetic backgrounds of closest model system A. thaliana, demonstrated that BjMYB28 is involved in aliphatic glucosinolates biosynthesis in B. juncea also. Further, all the four BjMYB28 paralogs showed positive regulatory function towards controlling aliphatic glucosinolate contents and profiles (Fig. 3.5, 3.6). Both functional overlap and redundancy was observed for the BjMYB28 paralogs. Analysis of gain-of-function in AtMYB28 mutant demonstrates that ectopic over-expression of BjMYB28 paralogs caused accumulation and hence restoration of aliphatic (Met-derived) glucosinolates without directly affecting the indolic glucosinolates pathway. The A. thaliana over-expression line in wild-type (Col-0) clearly suggests that the BjMYB28 paralogs have sub-functionalized roles toward regulating aliphatic glucosinolate pools in B. juncea. Further, over-expression of A-genome specific BjMYB28 paralogs showed relatively higher accumulation of leaf and seed glucosinolates content, compared to the B-genome specific paralogs.

Both over-expression and functional complementation analysis revealed that BjMYB28 paralogs are able to activate the promoters of target genes starting from the first enzymes of chain-elongation reactions (MAM1 and MAM3) up to the last enzymes of aliphatic glucosinolates biosynthesis (AtSt5b and AtST5c) (Fig. 3.9). However, differences in the regulation level of expression of the genes were observed. Further analysis revealed that CYP79F1 and CYPF2 genes, responsible for the oxidation of short and long chain glucosinolates were strongly up-regulated by
BjMYB28, MAM1 and MAM3 genes, contributing to the side-chain elongation of aliphatic glucosinolates were also up regulated significantly by BjMYB28. The last enzymes of the pathway namely AtST5b and AtST5c were the least affected transcripts. Results show that BjMYB28 paralogs strongly regulate the early enzymes of the pathway than the enzymes involved in side chain modifications. Similar results were also obtained in the knock-down lines of Arabidopsis wherein MAM1, MAM3, CYP79F1, CYP79F2 and CYP83A1 showed strongest reduction in their transcript levels compared to the wild-type plants (Fig. 3.11).

In contrast to mutants defective in CYP79F1 or CYP79F2 functions (Tantikanjana et al., 2004), BjMYB28 RNAi plants did not completely showed the absence of aliphatic glucosinolates (Fig. 3.10). This may be either due to the partial knock-down of AtMYB28 in these RNAi plants or complementation of AtMYB28 function by other transcription factors with similar roles, e.g. AtMYB76 or AtMYB29. However, both AtMYB76 and AtMYB29 were unable to fully compensate for the reduced AtMYB28 activity in RNAi lines. In A. thaliana, AtMYB28 RNAi knock-down mutants reduced accumulation of aliphatic glucosinolates significantly (Gigolashvili et al., 2007a) but couldn’t completely abolish the synthesis of aliphatic glucosinolates. In the same study, it is also reported that constitutive over-expression of AtMYB28 led to either an almost unaltered growth phenotype or a moderate to strong effect on Arabidopsis plant growth depending on the content of indolic glucosinolate fraction I3M and auxin. In the lines with decreased I3M, growth was severely affected. In our current study, no visible phenotype was observed in the case of over-expression or knock-down lines. This may be due to the unaltered levels of indolic fraction in the transgenic lines expressing BjMYB28.

Recent association of glucosinolates pathway genes with QTL studies on seed-glucosinolates in B. juncea and B. napus have also suggested that the ‘A-genome’ of these two complex allopolyploid genomes have prominent role towards controlling the glucosinolates variability (Ramchiary et al., 2007b; Bisht et al., 2009; Feng et al., 2012). For example, in B. juncea, four out of six QTLs for seed-glucosinolates trait are from A-genome, with LG-J3 consisting of two QTLs, occupying a total of four glucosinolates pathway genes including BjuA.Myb28.a (BjMYB28-4 of this study). Feng et al. (2012) identified 105 metabolic QTL (mQTL) in B. napus for glucosinolates concentration. Most of the mQTL and epistatic loci (> 60%) for leaf
and seed glucosinolates could be assigned to the A- genome linkage groups, but the corresponding loci within the C- genome were not identified. It was also evident from the studies that there is more genetic variation in the A- genome than in the B- or C- genomes of these Brassica species.

Duplicated genes, besides providing redundancy to a system, act as potential resources for enhancing quantitative variation of a particular trait. The occurrence of four *BjMYB28* paralogs in *B. juncea* with sub-division of gene functions; in all possibility suggest a complex glucosinolate regulation in *B. juncea*. It will be interesting to see how the four *BjMYB28* paralogs are differentially regulated to control the glucosinolate contents and profiles in *B. juncea*. 