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Gene expression analysis during turnip tuber formation in *Brassica rapa*

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Abstract

Brassica rapa comprises economically important vegetates grown worldwide. There are three different morphotypes in *Brassica rapa* such as leafy, oil, and turnip. Turnip (*Brassica rapa* L. subsp. Rapifera) forms a storage organ which is a modified stem (tuber). The molecular background of turnip tuber formation is not explicitly known. Two sets of gene expression experiments were conducts to identify the causal genes for turnip formation by using quantitative real time polymerase chain reaction (qRT-PCR). In Experiment 1, expression of 29 genes in DH-VT-117 was studied. Eight genes were among candidate genes that were differentially expressed during swede (*B. napus*) formation from literature. The remaining genes were randomly selected genes. In experiment 2, seven (five swede formation genes and two random) genes that were differentially expressed in DH-VT-117 were studied in six different turnip accessions (DH-VT-117, VT-053, VT-044, VT-007 and VT-008) of different places of origin. The qRT-PCR gene expression assays were conducted on hypocotyl tissue at different growth and developmental stages such as 7, 14, 21, 28, 35 and 42 days after sowing in Experiment 1 and at 4, 7, 11, 14, 18, 21, 25 and 28 days after sowing in Experiment 2. The results showed that out of the 29 tested genes in Experiment 1, 24 genes were differentially ($p<0.05$) expressed. Seven genes which were studied in Experiment 2 were also differentially ($p<0.05$) expressed. Genes, Bra035787 and Bra040904 of MATH protein domain, were highly expressed (compared to the reference gene) in six turnip accessions. An increased by 5 fold for Bra 035787 and 10 fold for Bra040904 until 21 days after sowing was observed. High gene expression values were observed at 18 days after sowing comparing to other time points for all the genes. This suggests that a major change in cellular components in turnip tissue happened at this stage. Bra008132 (sulfotransferase gene), known to be involved in glucosinolate (GLS) biosynthesis pathway, was differentially expressed indicating that it could be involved in GLS biosynthesis during turnip development. A gene, Bra004117 (flowering time gene) was expressed at the later growth stage (42 days after sowing) in DH-VT-117 explaining the mechanistic basis of storage organ formation and flowering in turnip. In order to explicitly understand genes that are specific to turnip formation, it would be helpful to undertake a comparative gene expression analysis in turnip and other non-turnip forming species of *B. rapa*.

Contents

1. Introduction

The genus *Brassica* belongs to the family Brassicaceae (also called Cruciferae) and consists of various species that are grown worldwide. Species of *Brassica* differ in genome composition, chromosome number and morphological appearance. Most Brassicas are diploids and some are amphiploids (an inter-specific hybrid having a complete diploid chromosome set from each parent). The diploid species are *Brassica rapa* (2n=20; genome composition AA), *Brassica nigra* (2n=16; genome composition BB) and *Brassica oleracea* (2n=18; genome composition CC). The amphidiploid species are *Brassica juncea* (2n=36; genome composition AABB), *Brassica napus* (2n=38; genome composition AACC) and *B. carinata* (2n=34; genome composition BBCC). *B. juncea* comprises the A and B genomes, *B. napus* comprises the A and C genome, and *B. carinata* has B and C genomes (U, 1935).

Brassica comprises economically important vegetable species such as broccoli, cabbage, cauliflower, Chinese cabbage (heading and non-heading), mustards, rape seed and turnip. They are consumed as vegetables and condiments; used to extract oil; and used as animal feed. They are adapted to a range of cultivation and agro-climatic conditions.

B. rapa is the first domesticated *Brassica* specie. It consists of various morphotypes which differ in morphological appearances. These morphotypes form three groups: oil types, leafy types and turnip type. Turnip (*Brassica rapa* L. subsp. Rapifera) is one of the most economically important vegetable especially in Europe and East Asia. It is grown for food and fodder in Europe, New Zealand, North America and Australia (Neilsen et al., 2008). Turnip accessions have been collected from different centre of origins. They are mainly from Asia (e.g. Japan, India), Europe (e.g. Netherlands, Germany and Italy) and Soviet Union (Zhao et al., 2005).

Turnip gives rise to underground storage organ which is developed from a secondary cambial growth of taproot and hypocotyl (Lu et al., 2008; Zhao et al., 2005; Kubo, 2010). Formation and development of the storage organ is a result of complex interaction between environmental, genetic and physiological factors after its initiation by sucrose supply and growth regulation (Gupta et al., 2001; Rouhier and Usuda, 2001).

Studies on nutritional and metabolite content of turnips indicate that turnip storage organ contains high concentration of health benefiting gluconasturtiin and glucosinolates compounds which can reduce the risk of cancer (Li et al., 2007). Turnip contains different level of sugar composition (Table 1).

Despite the great variability in morphology of *Brassica* species, the molecular basis behind this variability is mostly unknown. The genetic background of turnip tuber formation is also not well known (Kubo, 2010; Lu et al., 2008).

1.1. Research goal

In *B. rapa* turnips form a thickened hypocotyl (tuber) as storage organ. However, the specific gene(s) that regulate formation of this storage organ is not explicitly known. Aim of this study was to identify genes underlying turnip tuber formation.

Specific objectives

- To validate microarray gene expression data with qRT-PCR and select candidate genes.
- To examine expression of the selected candidate genes during growth and development of turnip tuber in six turnip accessions.

Research questions

- Which genes are differentially expressed during turnip tuber formation in DH-VT-117?
- Do the expression patterns of the selected genes differ in different turnip accessions?

2. Literature review

2.1. Gene expression

Almost every cell contains a set of chromosomes and identical genes. Not all of these genes are turned on at a time; rather a few subsets are expressed and determine cell types. Such differential gene expression regulates the development of cells and tissues. Thus, analysis of gene expression profile helps to detect molecular background of a specific trait (Birnbaum et al, 2003).

Genes are expressed when DNA is transcribed in to messenger RNA (mRNA). mRNA is then translated in to proteins that carryout functions of cells. Gene expression is a tightly regulated complex process that enables cells to drastically respond to change in the environment and internal cues as 'on/off' switch. This mechanism guides the type of genes to be expressed and also controls level of particular gene expression.

Several technologies have been developed to deduce and quantify gene expression. These technologies are generally summarized as hybridization and sequence based. Hybridization based approaches are suited for high throughput and most of them are inexpensive (Wang et al., 2009). Microarray is one of the hybridization based approach.

Microarray can generate enormous data at a time but has limitations concerning reliability of the data and it needs further validation, new computational and statistical techniques to analyse the data (Wu, 2001).

Sequence based methods directly determine a cDNA sequence. Tag-based methods such as, Serial Analysis of Gene Expression (SAGE), Cap Analysis of Gene Expression (CAGE) and Massively Parallel Signature Sequencing (MPSS) are better than aforementioned method in providing precise digital gene expression levels. However, they rely on an expensive Sanger sequencing techniques and a portion of the short tags cannot be uniquely mapped to the reference genome. RNA sequencing (RNA-Seq) method of transcriptome profiling is developed to determine and quantify RNA expression levels using deep-sequencing. It is expensive and requires prior knowledge on the genome sequence. RNA-Seq gene expression results are more precise than microarrays results and have been shown to be highly accurate as determined using quantitative real time PCR (Wang et al., 2009).

Quantitative reverse transcriptase Polymerase chain reaction (qRT-PCR) is distinguished from aforementioned methods with regard to sensitivity, accuracy, reliability, and a time it takes to

give results (Derveaux, 2010). RT-PCR is a standard method for accurate, highly sensitive, specific and broader quantification range of moderate number of gene expression profiling (Exposito-Rodriguez et al., 2008). It amplifies and simultaneously quantifies DNA molecule in a sample. It is named qRT-PCR because detection of the amplified DNA is made in real time while the reaction is running.

qRT-PCR data can be analysed in two different methods: absolute and relative (Livak and Schimittgen, 2001). Absolute method directly quantifies the input copy number by relating the obtained PCR signal to the standard curve. Relative quantification determines the change in gene expression by relating the PCR signal in a test sample with that of a reference sample (control). However it can also be calculated in absence of control treatment by directly normalising the cycles to threshold (Ct) of target gene with Ct of reference gene and following an appropriate equation ($2^{-\Delta Ct}$) (Livak and Schmittgen, 2011). Ct is a unit of measurement representing the number of cycles that an amplified DNA reaches a threshold to be detected. Ct value is in logarithmic scale and indicates inversely proportional to the amount of DNA in the sample. Higher Ct represents lower expression than lower Ct. The same holds true for ΔCt (Goni et al., 2009). The relative gene expression values ($2^{-\Delta Ct}$) can be transformed to fold change. The fold change results in negative or positive log values. Positive values indicate that the gene is up-regulated, and negative values indicate the gene is down-regulated (Livak and Schimittgen, 2001).

2.2. Genetic findings

Genetic evaluation of turnip root/tuber traits in different turnip populations under different environment was conducted by researchers to make comparisons and assess consistency of the detected QTLs for the same trait in various genetic backgrounds (Lou et al., 2007; Lu et al., 2008; Rémi, 2010, unpublished data). A major QTL, explaining 24-40% of phenotypic variation, for turnip traits such as turnip formation, shoot, length, width and weight was detected and mapped at the top of linkage group R02 (Lou et al., 2007). A mapping population used in their study was BC/ DH F2/3 obtained from a cross between Rapid Cycling line RC-144 and a vegetable type Chinese cabbage line CC-156.

A genetic map was constructed on F2/3 mapping populations from a cross between two morphologically different species for root characters, Chinese cabbage (*B. rapa* ssp. chinensis) and turnip (*B. rapa* ssp. rapifera), using AFLP and RAPD markers (Lu et al.,

2008). QTL analysis for root traits was also done by Lu et al. (2008) resulting in total of 18 QTLs with 8.4-27.4% of the phenotypic variation for root thickness, length and weight.

A study carried out on F3 population derived from a cross between vegetable turnip (VT-115) and Rapid Cycling (RC-144), identified six QTL for turnip diameter, turnip weight and turnip length (Rémi, Unpublished data). In a population of *B. napus* L. F3 obtained from a cross between oilseed rape and swede, 13 QTLs on six LGs were detected for three root characters (root fresh weight, root dry matter and root diameter) with 7.0-14.5% phenotypic variation (Lange et al., unpublished data).

A member of Brassicaceae family, *B. napus*, forms an underground storage organ known as Swede. Swede is composed of root, hypocotyl and parts of above ground shoot. Differentially expressed genes during Swede formation were identified (Lange et al. Unpublished). These genes are similar to 11 *Arabidopsis thaliana* genes based on sequence identity. Forty *B. rapa* genes are homologous to the 11 *A. thaliana* genes.

Microarray gene expression data set from previous experiment on DH-VT-117 during six different growth stages of turnip tuber is available from Ningwen Zhang (unpublished data). Microarray experiments showed that largest change in up-regulated genes occurs between 14 and 21 days after sowing, while between 35 and 42 days gene expression was stable. Such information helped us to deduce essential time points to design a gene expression experiment.

Flowering locus T (FT) is a component of mobile signals which is shown to regulate floral transition and tuberization in potato under seasonal fluctuations in day length (Navarro et al., 2011). Flowering time QTLs were detected in co-location with turnip formation QTLs (Lu et al., 2008; Rémi, Unpublished data) suggesting a tight linkage in regulation of these traits.

3. Materials and methods

Two experiments (Experiments 1 and 2) were carried out in this study. Experiment 1 was conducted to select best stable reference gene in developing turnip tissue, to validate an existing microarray dataset, and to study expression of candidate genes in DH-VT-117 and select differentially expressed genes to study in Experiment 2. During candidate gene selection, priority was given for the differentially expressed genes with different expression patterns. The aim of Experiment 2 was to examine the expression of seven target genes in six different turnip accessions.

3.1. Plant materials and growing conditions

Experiment 1 was conducted on previously isolated RNA samples of DH-VT-117 at six different growth and development stages of turnip tuberization by Ningwen Zhang using RNeasy mini kit (Qiagen, Milden, Germany). DH-VT-117 is a doubled haploid (DH) line obtained from a donor plant of a Japanese turnip accession VT-117 (CGN15201). DH-VT-117 genome has been re-sequenced to a depth of 27x and sequence information is available in the Laboratory of Plant Breeding, WUR. The plants used for RNA extraction were grown in standard pot soil in a climate chamber, with 20/18 ^{0}C day/night temperature and 16h/8h day/night length. For each time point, two biological replicates were included and each contains a mixed sample from three individual turnip tubers.

Experiment 2 was conducted on the RNA samples isolated from hypocotyl/thickened turnip tuber of six different accessions of different places of origin. All the accessions were obtained from the Dutch Crop Genetics Resources Centre (CGN) in Wageningen. Plants were grown in climate chamber with the same condition as DH-VT-117 in Experiment 1.

Table 1. Turnip accessions used in this study

Accession†	Gene bank ID	Cultivar name	Origin	Cluster ‡	Sugar composition (mg/100g)¥ Glucose	Fructose	Sucrose
DH-VT-117	CGN15201	Toya	Japan	T1	22.14	10.99	2.69
VT-053	CGN07167	Teltower Kleine	Germany	T2	3.08	0.59	23.41
VT-044	CGN06859	Soloveckaja	Soviet Union	T2	18.25	11.51	10.62
VT-007	CGN06710	Maiskaja	Soviet union	T1	10.32	4.32	15.62
VT-008	CGN06711	Pusa Chandrina	India	T2	15.40	26.45	35.66
FT-047	CGN06866	Moskovskij	Soviet Union	T2	13.67	17.42	13.73

†Only VT-117 is a double haploid (DH) fixed line. Five accessions are vegetable Turnip (VT) and one is Fodder Turnip (FT).
‡ Clustering was made based on AFLP fingerprinting (Zhao et al., 2005). The same labels in the 5th column indicate the accessions are genetically closely related.
¥ Sugar composition data obtained from Menamo T (Personal communication)

In addition to the difference in place of origin and sugar composition, the six turnip accessions studied vary in morphological appearance (colour, shape and texture). Some are reddish (DH-VT-117 and FT-047), greenish (VT-053, VT-044 and VT-007) and whitish (VT-008). They also differ in growth habit (underground, aboveground and half buried in soil) (Figure 1).

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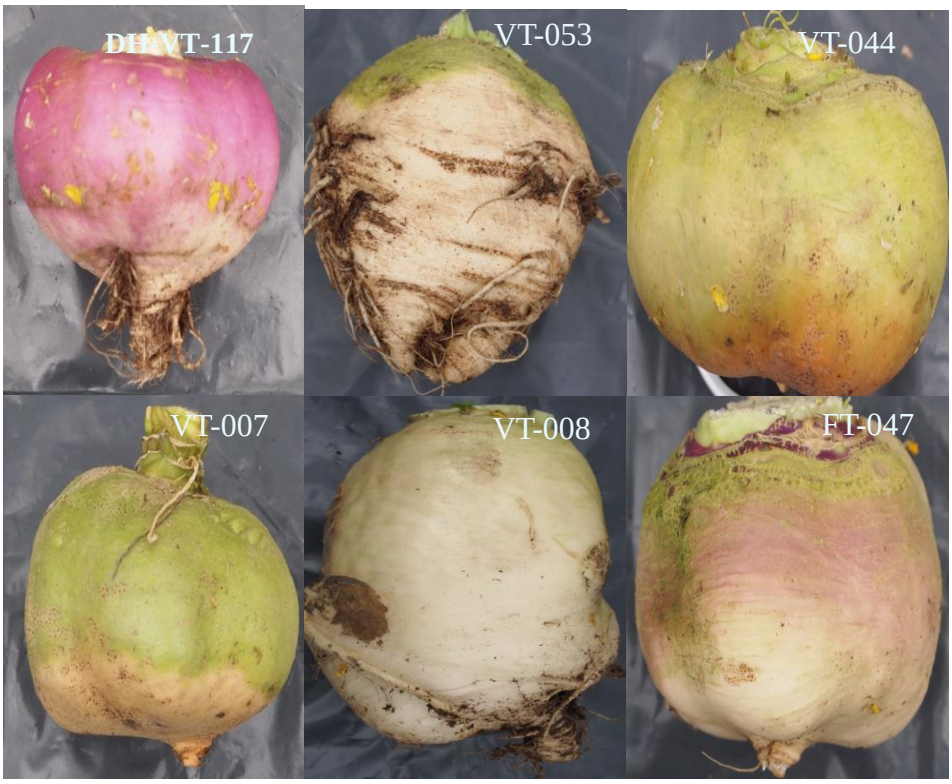


Figure 1.Six turnip accessions used in this study. Courtesy: Images by Ningwen Zhang.

3.2. Sample collection of experiment 2

Plant sample collection for RNA isolation was performed at different growth and development stages (4, 7, 11, 14, 18, 21, 25, and 28 days after sowing) from each turnip accession. The plant part harvested was hypocotyl tissue (below cotyledon and above lateral root) during early growth stages and at later growth stages, entire enlarged turnip tissue was collected (Figure 2). For each time point (days after sowing) two samples were taken and used as biological replicates. Two to ten plants were pooled to make one sample. The number of pooled plants per sample depending on the growth stage of the plants. Hypocotyl tissues detached from plants were immediately immersed in to liquid nitrogen and kept in freezer (-80 ^{0}C) till RNA isolation to prevent synthesis of new mRNA due to tissues wounded during detaching.

Figure 2. Plant part (hypocotyl tissue) harvested for RNA isolation. The labels indicate the days (after sowing) at which samples were taken.

Phenotypic data was collected to evaluate the morphological variation among the accessions grown. Hypocotyl diameter was measured from images which were taken at all studied development stages from each accession. The measurement was done on two randomly selected plants and the average was plotted on the graph. Because not all plants harvested for

gene expression study were measured, as a result the data was not further analysed but only serve as an indication of the growth curve for each accession.

3.3. RNA extraction

Total RNA was extracted using RNeasy mini kit (Qiagen, Milden, Germany). Simultaneous to RNA extraction, elimination of genomic DNA (gDNA) contamination was done by applying on-column DNase treatment. An additional off-column DNase I treatment (Invitrogen, Carlsbad, CA, USA) was also performed after the on-column DNase treatment to completely eliminate the residues of gDNA. RNA concentration and quality were measured on Nanodrop spectrophotometer and Agarose gel (1%). Additional purification step was also performed on the samples with low quality (a 260/280 and 260/230 ration less than 2.0 and 1.8, respectively). Detailed protocol for RNA extraction, purification and DNase I treatment can be found in Appendix A1-3.

3.4. cDNA synthesis

Reverse transcription reaction was done from 50ng/µl of total RNA to synthesize cDNA using to iScript^{TM} cDNA Synthesis kit (Bio-Rad). The master mix for iScript cDNA synthesis reaction per each sample is presented in Table 2.

Table 2. cDNA synthesis reaction master mix

Component	Volume per reaction (µl)
5x iScript reaction mix	4
iScript reverse transcriptase	1
Nuclease free water	4
RNA template	11
Total volume	20

The complete reaction mix of iScript was incubated in the PCR machine for 5 minutes at 25 ^{0}C, 30 minutes at 42 ^{0}C, and 5 minutes at 85 ^{0}C. The obtained cDNA template was diluted 20x for Experiment 1 and 40x for Experiment 2 to adjust the concentration to 50 ng/µl and 20 ng/µl, respectively.

3.5. Gene expression assay

3.5.1. Selection of reference gene

A gene used as reference gene for both experiments was selected from 13 candidate reference genes (ACTIN, ACTIN 1, ACTIN 2, GAPDH, CYP, EF1-α, TUB-α, 18SrRNA, UBC30, UBQ, PPR, PP2A and MDH) that were validated in non-heading Chinese cabbage tissue derived from different developmental stages, organs and stress treatments (Dong et al., 2012). The selection was made by carrying out an amplification experiment on standard PCR program and qRT-PCR program (Table 3) followed by loading on 2% agarose gel to check if the gene gives an amplified product for all cDNA samples.

Table 3. Different PCR program set on PCR machine during reference gene screening

Standard PCR program			qPCR program		
Cycle(s)	TM (^{0}C)	Time (min.)	Cycle(s)	TM (^{0}C)	Time (min.)
1	94	5	1	95	3
33	94	½	40	95	¼
	60	½		60	1
	72	½			
1	72	½			
Hold	10	∞	Hold	10	∞

The PCR reactions with standard PCR and qRT-PCR programs) were performed using same component of reaction mix. The reaction mix was prepared using DreamTaq^{TM} DNA polymerase kit (Table 4).

Table 4. DNA polymerase reaction mix for standard SSR and qPCR program

Components	Volume per reaction (µl)
Primer Forward	1.0
Primer Reverse	1.0
dNTPs	0.4
Taq reaction buffer	1.0
Taq DNA polymerase	0.1
MQ water	5.5
cDNA template	1.0
Total volume	10

Three reference genes which gave amplification on all of the cDNA samples were further tested on qRT-PCR program and the most stable gene selection was performed based on gNORM algorithms (Derveaux, 2010). The expression value of the selected reference gene was used to normalize the expression of target genes.

3.5.2. Selection of candidate genes

Available microarray gene expression data obtained from experiment conducted on RNA extracted from DH-VT-117 was validated using qRT-PCR. The microarray experiment was performed on RNA samples isolated from hypocotyl and root (combined) tissues at six different growth and development stages (7, 14, 21, 28, 35 and 42 days after sowing). Twenty one genes were randomly selected (Table 5) from the microarray data set to a) validate the reliability of the microarray data, b) select candidate genes to be studied in detail in Experiment 2. A flowering time gene was included the randomly selected genes list.

During the time of this MSc thesis, however, we realized that the designs of the used microarray were wrongly printed by Agilent, so the features that were presumed differentially expressed in the array, did not correspond to the presumable design. As a consequence, we did not validate these features. Therefore, out of twenty one tested genes only two genes with unknown expression patterns were randomly selected from the microarray data set, to be studied in Experiment 2. Even though the corresponding genes are unknown, expression pattern observed in the microarray data set was useful to understand gene expression changes observed among the studied time points. An example of microarray gene expression pattern of 20 randomly selected probes at six growth and development stages is indicated in Figure 3. Based on this information, Experiment 2 was designed to comprehend changes of gene expression in more intense time points (4, 7, 11, 14, 18, 21, 25 and 28 days after sowing).

In total, we found 40 *B. rapa* orthologs for those 11 Arabidopsis genes that were described as differentially expressed during swede tap root formation process (Lange et al., Unpublished data). The 40 orthologoues genes of the 11 *A. thaliana* genes were retrieved from Brassica database (http://brassicadb.org/brad/index.php). Eight out of forty genes were randomly selected (Table 5) and tested in DH-VT-117 turnip tuber formation. Five differentially expressed genes out of eight genes were selected for Experiment 2.

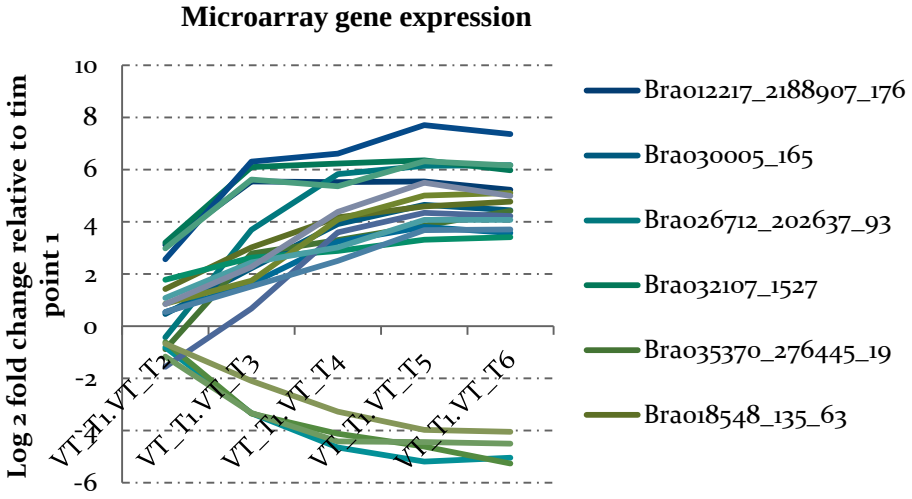


Figure 3. An example of gene expression patterns for a few set of genes in the available microarray data set. The graphs are plotted based on the Log 2 transformed fold change values relative to the first time point (7 days after sowing). On the X-axis, VT represents vegetable turnip, T1-T6 represent time points 1- 6 (7, 14, 21, 28, 35 and 42 days after sowing). Different coloured lines represent different genes. Accession number of few of plotted genes with probe sequence position is included in the legend.

3.6. Primer design

Forward and reverse primer pairs were designed on Primer3Plus software with qPCR active setting. The parameters set to the program include; PCR amplicon length range of 100-150 base pair (bp), primer size of 18-23 bp primer annealing temperature of 59-61^{0}C, and primer Guanine Cytosine (GC) content of 45-55%. Primer pairs were designed for 29 candidate genes (Table 5). Out of 29 genes, the primer pairs of 21 genes were designed either at the start or end of the 60 mer probe sequences to make sure that the same region in the genome was amplified in the previous (microarray) and validation experiments. DNASTAR, Lasergene 9.1 (Lasergene, Madison, WI, USA) was used to locate the position of the probe sequence.

For the 8 genes tested from *A. thaliana* orthologues in *B. rapa*, each *A. thaliana* gene corresponds to two to nine paralogoues genes. Sequences of these genes were downloaded from Brassica database. For candidate genes from these paralogues, primer pairs were designed at the paralogue specific regions by aligning all the paralogue genes sequence on MegAlign software. For the reference gene screening experiment, the primer pairs of 13 reference genes were designed by Dong et al. (2012).

For each gene, three alternative primer pairs were designed and the best primer combination (Appendix B) was selected from pre-screening on qRT-PCR machine using few samples and including negative control (RNA) and Non template control (MQ water). Primer pairs that gave double melt curve peaks and signal on the water and RNA control were discarded. Primers were dissolved as per described on the fact sheet from the manufacture and 20x dilution was made to reach a concentration of 3 µM.

Table 5. List of all *Brassica rapa* genes studied, chromosomal position and *A. thaliana* orthologous genes with corresponding functional annotation

Gene (Bra ID)	Chromosomal position/Linkage group number	*A. thaliana*	
		Orthologues genes	Locus description
Bra002177	A10	At5g18660	Carotenoid biosynthetic
Bra003397	A07	At3g59970	Mthfr1,methylenetetrahydrofolate reductase 1
Bra004053	A07	At1g68060	microtubule-associated proteins
Bra004117	A07	At4g20370	Encodes a floral inducer that is a homolog of FT
Bra004827	A05	At2g17750	Nep-interacting protein 1, nip1
Bra006354	A03	At5g16440	pyrophosphate isomerase 2, IPP2
Bra008132	**A02**	**At1g74100**	**sot16(sulfotransferase)**
Bra011488	**A01**	**At4g34110**	**Polyadenylate binding protein 2, pabp2**
Bra011568	A01	At4g34870	Protein peptidyl-prolyl isomerization
Bra013079	A03	At2g16370	Thymidylate synthase 1
Bra015220	A10	At4g34110	RNA binding translational initiation
Bra017852	A03	At4g37750	Transcription factor
Bra018150	A06	At3g47420	Carbohydrate transmembrane transport
Bra018669	A06	At5g60390	Gtp binding elongation factor tu family protein
Bra020425	**A02**	**At5g57660**	**Sequence-specific DNA binding transcription factor**
Bra021925	A04	At2g34650	Protein kinase, catalytic domain, abr protein
Bra026945	A09	At1g12950	Drug transporter activity
Bra027035	**A09**	**At1g62540**	**flavin-containing monooxygenase (FMO)**
Bra029257	A02	At4g28360	Ribosomal protein l22p/l17e family protein
Bra032107	**A04**	**At2g24300**	**Calmodulin binding protein-like**
Bra034603	A08	At4g34110	Polyadenylate-binding protein 2/ pabp2
Bra034715	A05	At3g12940	Dioxygenase-like protein
Bra035787	**A05**	**At3g20370**	**plasmodesma, math, traf-like family protein**
Bra036428	A07	At3g03910	Glutamate dehydrogenase 3
Bra038088	A08	At4g15210	Starch catabolic process
Bra038412	A08	At1g31170	DNA binding
Bra038542	A09	At4g36800	Protein binding,
Bra038726	A01	At3g12500	Athchib (basic chitinase)
Bra040904	**Scaffold000303**	**At3g20370**	**plasmodesma, math, traf-like family protein**

Seven genes studied in Experiment 2 are written in bold font

3.7. qRT-PCR runs

qRT-PCR experiments were performed in duplicates for each samples as a technical replicate and including reference gene in parallel as an internal control to normalize expression of the target genes. A Bio-Rad CFX thermocycler using iQ SYBR green reaction mix (Table 6) was used for the qRT-PCR experiments. Thermocycling reaction conditions were; 95^0C for 3 minutes, 40 cycles of 95^0C for 30 seconds and 60^0C for 30 seconds.

Table 6. qRT-PCR reaction mixture

Component	Volume per reaction (µl)
IQSyber green reaction mix	5.0
MQ water	1.8
Primer Forward	0.6
Primer Reverse	0.6
cDNA template(20 ng/µl)	2.0
Total volume	10.0

Experimet 1 was performed on the 96-wells qPCR plate (Bio-Rad Laboratories) while in Experiment 2, Invitrogen 384-wells plate was used for convenience to handle larger number of samples per run. The master mix, total reaction volume and run protocol was similar for both kinds of setups but the cDNA template concentration varies.

3.8. Data analysis

Cycle threshold (Ct) values obtained from the RT-PCR run for the target genes were normalized and converted to quantities using Ct values of the reference gene. Values of ΔCt were obtained using Equation 1.

$$\Delta Ct = Ct_{target\ gene} - Ct_{reference gene} \quad (1)$$

For each time point, two biological and two technical replicates were included in the RT-PCR experiments. ΔCt was calculated per each well in the target gene with the Ct of corresponding well in reference gene. Relative gene expression (RGE) was calculated using Equation 2 (Livak and Schmittgen, 2011).

$$RGE = 2^{-\Delta C_t} \quad (2)$$

The relative expression values were Log10 transformed to get a fold change (Goni et al., 2009). Positive values of the log transformed fold change indicate the miRNA transcripts are up-regulated whereas negative values represent down-regulation of the miRNA. Statistical significance at 5% P-value was analysed using IBM SPSS 19 to determine differentially

($p<0.05$) expressed genes. Two biological and two technical replicates were included in all analyses. Technical replicates with standard deviation of above 0.5 (indicating a half cycle difference) were repeated to minimize technical errors and get trustable data.

Selection of stable reference gene was done based on ranking using gNORM program. gNORM is a program used to identify the most stable reference gene among the tested candidate reference genes (Ahn et al., 2008). The program assumes identical expression ratio of two internal control genes in all tested samples to perform average pair-wise variation comparison per a single gene with all other tested genes and calculates gene stability measure (M). M value of <1.5 is acceptable indicating that the gene is stably expressed and can be a good reference gene (Dong et al., 2012).

Multiple experiments viewer (MeV v4.8) software was used to present relative gene expression data with range of colours. Orthologous genes search, functional annotations and sequence search and sequence blast was carried out on online databases such as Brassica databas (http://brassicadb.org/brad/index.php), *A. thaliana* (http://www.arabidopsis.org/) and National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

4. Result

Experiment 1 comprises results of validation of reference genes in turnip samples, validation of microarray dataset, and gene expression study for selected genes in turnip samples (DH-VT-117). Experiment 2 presents gene expression study in six turnip accessions at eight growth and developmental stages of turnip tuber.

4.1. Experiment 1: Validation of reference genes in turnip

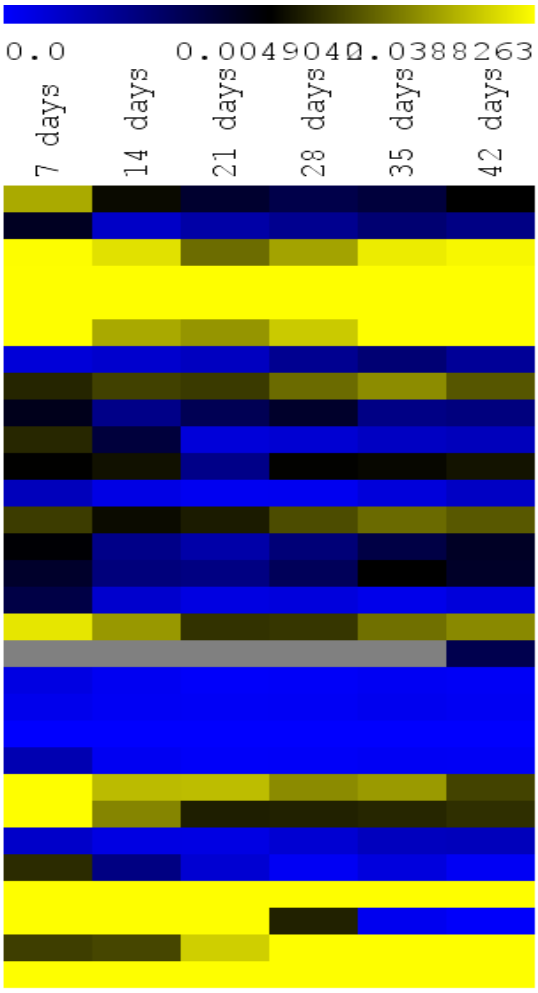
Thirteen reference genes were studied using standard PCR and gel-electrophoresis. Only three genes which gave amplification product (~150bp) on 2% agarose gel were selected for further study on qRT-PCR to select the stable gene to be used for normalisation of expression of target genes. Table 7 shows the Ct values obtained from reference genes when tested on turnip tissue samples of different growth and developmental stages. All tested genes had Ct values ranging more than 2 cycles, suggesting none of them is an ideal reference gene for target samples but the expression stability value (M) less than 1.5 indicates these genes can be acceptable as a reference gene. The M value observed for CYP was the lowest, suggesting that CYP is the most stable reference gene among the tested three reference genes. Therefore, CYP was used in this study to normalize the qRT-PCR experiments and to calculate relative gene expression values.

Table 7. Ct values of the three reference and expression stability values (M) calculated by gNORM software

Samples	CYP	EFlα	GAPDH
VT-T1R1	20.1	22.75	22.76
VT-T1R2	21.01	22.98	22.89
VT-T2R1	19.74	21.91	25.11
VT-T2R2	19.96	22.27	23.01
VT-T3R1	18.94	21.16	22.98
VT-T3R2	18.89	21.37	23.17
VT-T4R1	19.47	21.10	24.20
VT-T4R2	19.19	20.94	24.93
VT-T5R1	20.01	21.36	25.72
M<1.5	0.058448	0.065962	0.095622

4.2. Experiment 1: Gene expression in DH-VT-117 samples that were also used for microarray experiment

Initially we aimed to validate the available microarray dataset to confirm the reliability of the dataset for further study. As described in the material and methods, the microarray was not printed correctly, and thus the features that were differentially regulated and selected for validation, did not represent the genes for which we designed primers. As a result, the 21 tested genes should be considered as a set of 21 randomly picked genes. The pattern and level of expression of the 21 genes were tested in the DH-VT-117 samples, which were used for the microarray experiments, and 16 out of the 21 genes were differentially ($p<0.05$) expressed (Figure 4). Only two differentially expressed genes (Bra032107 and Bra020425) were selected to be studied in Experiment 2.



Gene	P-value	LG
Bra002177	0.202	10
Bra003397	0.679	7
Bra004053	0.000	7
Bra011568	0.000	1
Bra018669	0.000	6
Bra038542	0.137	9
Bra013079	0.000	3
Bra017852	0.000	3
Bra018150	0.007	6
Bra020425	0.000	2
Bra021925	0.840	4
Bra026945	0.000	9
Bra029257	0.001	2
Bra032107	0.000	4
Bra034715	0.001	5
Bra036428	0.000	7
Bra038412	0.000	8
Bra004117	-	7
Bra004827	0.019	5
Bra006354	0.010	3
Bra021925	0.840	2
Bra038726	0.001	1
Bra008132	0.000	2
Bra011488	0.000	1
Bra015220	0.094	10
Bra027035	0.000	9
Bra034603	0.001	8
Bra035787	0.000	5
Bra038088	0.000	8
Bra040904	0.000	-

Figure 4. A hitmap constructed using Multiple experiment viewer software. Relative gene expression ($2^{-\Delta Ct}$) of 29 genes in DH-VT-117 is represented in colours (yellow, blue and black). Yellow represents high expression while blue represents low expression. The rows represent individual genes tested and the columns describe different turnip tuber growth stages at which gene expression study was done. The expression levels are continuously mapped on the colour scale provided at the top of the figure. The $p<0.05$ indicates the significantly

different expression of the gene in different time points. Genes selected for experiment two are underlined. LG: linkage group/ position of the gene.

Different expression pattern were observed from the 29 genes (21 randomly selected and 8 from Lange et al.) tested in DH-VT-117 turnip tuber. Eight genes from bottom to top in Figure 4 are from Lange et al., genes. Figure 4 shows constitutively higher (represented in yellow) or lower (in blue) expressed, transient up-regulation and down-regulation of genes. Four genes (Bra011568, Bra018669, Bra034603 and Bra040904) were constantly highly expressed while three genes (Bra004827, Bra006354 and Bra038726) were constantly lowly but significantly ($p<0.05$) expressed over growth stages of turnip tuber. Five out of 29 genes showed less fluctuation in expression and resulted in non significant, at $p<0.05$, expression at different turnip tuber growth stages. A flowering time gene (Bra004117) was not expressed until tuber growth stage 35 days after sowing but gave low signal of expression at 42 days after sowing. Transient down-regulation in expression of genes Bra035787, Bra027035, Bra020425, Bra008132 and Bra011488 was observed and these genes were among the selected to be studied in detail in Experiment 2. A constitutively up regulated gene, Bra040904, with significant difference at different turnip tuber growth stages was also selected for Experiment 2.

Eight genes, Bra008132, Bra011488, Bra027035, Bra034603, Bra035787, Bra038088 and Bra040904, were from Lange et al (Unpublished). Except Bra015220, all are differentially ($p<0.05$) expressed in DH-VT-117 at different turnip tuber growth stages (Figure 4). Five genes (Bra027035, Bra008132, Bra040904 Bra011488 and Bra032107) out of seven differentially expressed genes were studied in detail in Experiment 2 in six different turnip accessions.

In total seven genes were selected to be tested in Experiment 2 in six different turnip accessions. Only these seven genes were selected considering the time limit during the period of the master thesis. The relative gene expression values of the seven genes that were selected to be studied in Experiment 2 were log 10 transformed and presented in Figure 5.

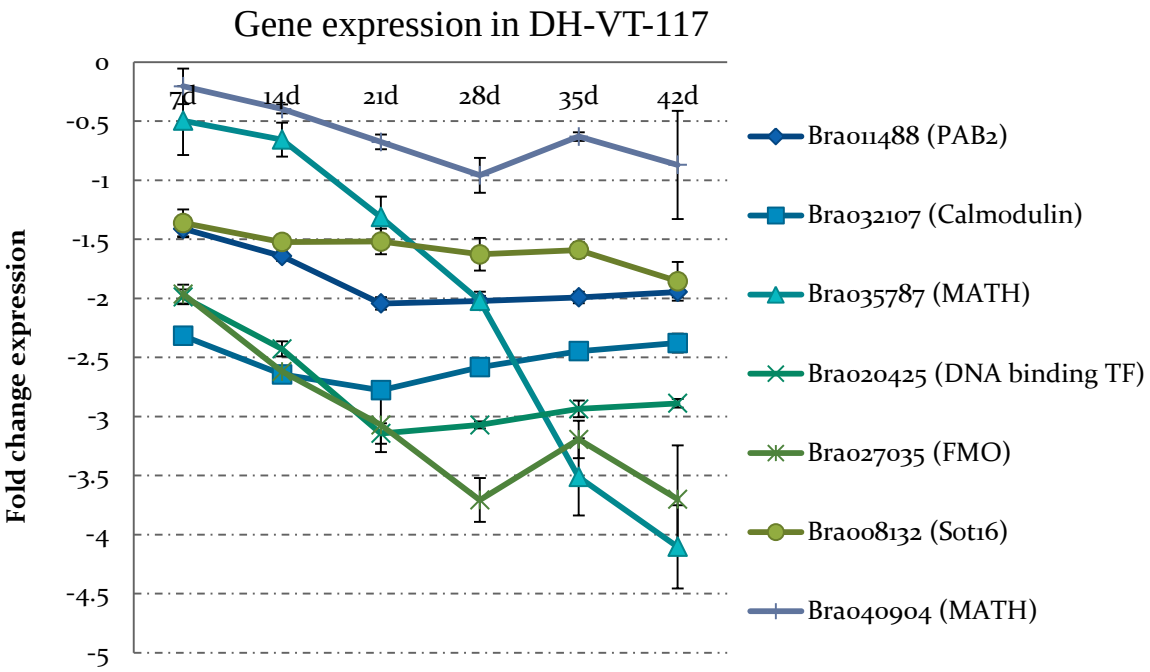


Figure 5. Log 10 transformed relative gene expression values of the candidate genes tested in Experiment 1 on DH-VT-117 turnip samples. The X-axis represents days at which samples harvested for gene expression. Error bars represent standard deviation (n=4).

Figure 5 shows that all the seven genes showed differential ($p<0.05$) expression levels during turnip tuber development. Higher fluctuation in expression pattern of the genes was observed until 21 days after sowing followed by a decrease or constant expression pattern except for a gene Bra035787. Expression level of the seven genes was relatively higher at the earliest growth stage (7 days after sowing) than the latest time points. Higher change in expression (more than 30 fold change) was observed for a gene Bra035787 during growth and development of turnip tuber. Bra027035 exhibited more than 20 fold change of expression in developing turnip tuber.

4.3. Experiment 2: Phenotype and gene expression analysis of six turnip accessions

4.3.1. Phenotypic data

Hypocotyl tissue thickening was measured at each time points to assess the phenotypic variation among the studied accessions (Figure 6). Increase in turnip tuber enlargement was observed after 18 days stage and stayed constant after 25 days after sowing. DH-VT-117 accession was the most vigorous in turnip tuber width followed by VT-007 accession. Fodder turnip FT-047 and VT-053 showed less vigorous turnip tuber growth.

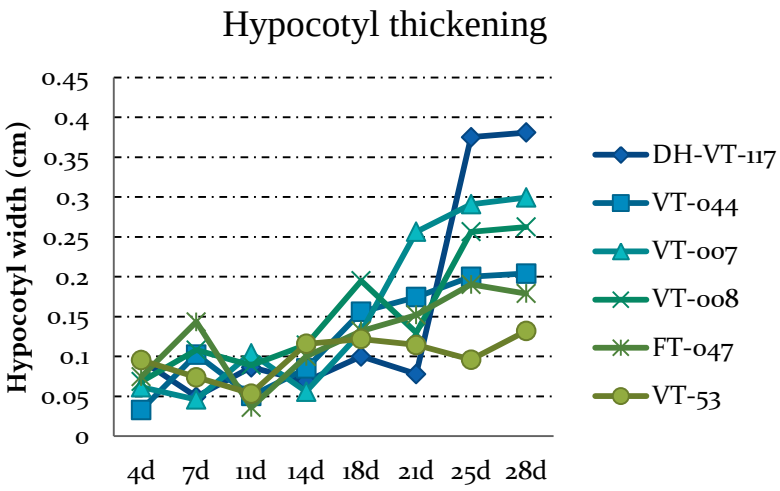


Figure 6. Phenotypic variation among turnip accessions in hypocotyl width. The average was calculated from two measurements taken from images for each time point and accessions.

4.3.2. Gene expression in six turnip accessions

Expression of seven genes was examined in six turnip accessions at eight growth and developmental stages of turnip tuber. Similar to Experiment 1, all the seven genes were differentially expressed ($p<0.05$) at different growth stages in all turnip accessions (Appendix D). Some of the genes resulted in comparable expression pattern only in some of the accessions and expressed differently in the other accessions. Expression pattern of the seven genes in DH-VT-117 in both experiments are dissimilar. Possible reason could be that in Experiment 1, expression was studied in root and hypocotyl (combined) tissue whereas in Experiment 2, only hypocotyl tissue was studied. The other reason could be any difference during sampling process. Among the data, relative expression of VT-044 at 21 days growth stage is doubtful as a wide variation was observed in raw Ct values for biological replicates in both target and reference genes.

The flavin-containing monooxygenase (FMO) gene, Bra027035, was differentially ($p<0.05$) expressed in all studied turnip accessions during tuberization (Figure 7). Transient up-regulated expression pattern was observed in most of the accessions. Expression level of this gene at 18 and 21 days after sowing was highest (10-100 folds) for all accessions.

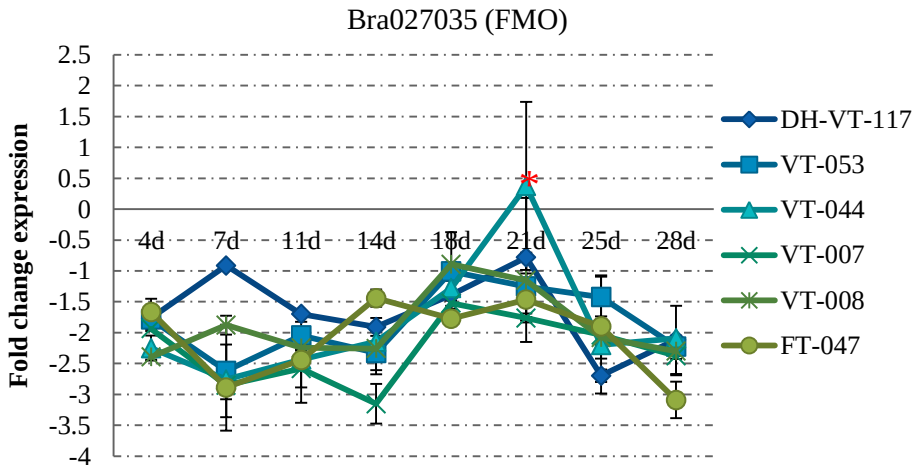


Figure 7. Log 10 transformed (fold change) relative gene expression of Bra027035 in Six turnip accessions at eight turnip development stages. The error bars represent standard deviation (n=4). *: the data point is not trustable due to wider variation between biological replicates.

Expression of a sulfotransferase Sot16 (Bra008132) gene was transient up-regulation and reached higher at 18 days stage, and then decreased by 10 fold after 18 days of growth stage in all accessions (Figure 8). In accessions, VT-053, VT-044 and VT-007, its expression reached higher earlier at 11 days after sowing and decreased (>10 fold change decrease) at 14 days stage. This gene showed more fluctuating expression pattern in VT-053 accession.

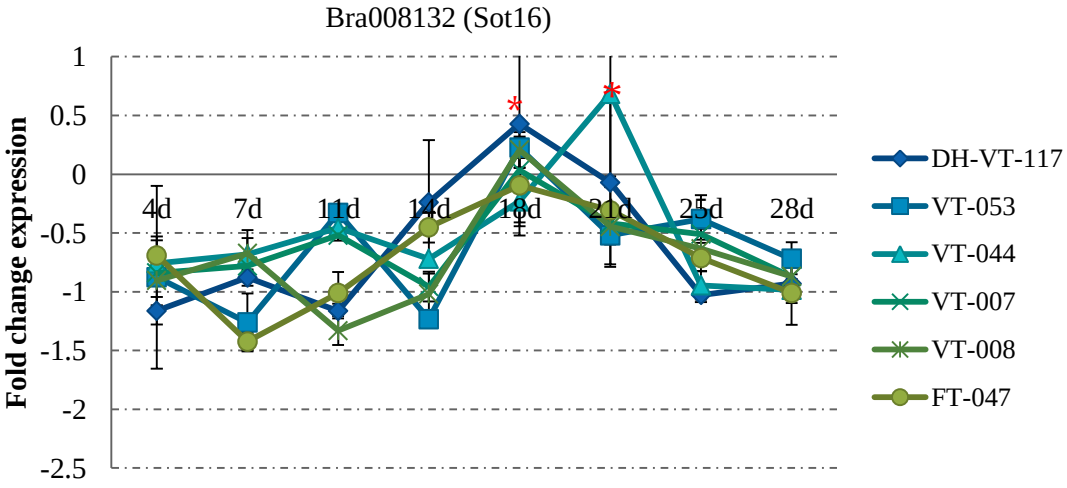


Figure 8. Log 10 transformed (fold change) relative gene expression of Bra008132 in Six turnip accessions. The error bars represent standard deviation. *: at DH-VT-117 at 18d and VT-044 at 21d, the data points are less trustable due to wider variation between biological replicates.

Bra040904 gene was highly expressed comparing to reference gene (Figure 9). This gene showed very uniform expression pattern among all tested accessions. The lowest expression level observed at the latest time points, indicating a down-regulated expression pattern. Higher expression (up to 10 fold increase) was observed in all the accessions at 18 days stage. This gene was down-regulated (up to 10 fold decrease) in all the accessions after 25 days after sowing.

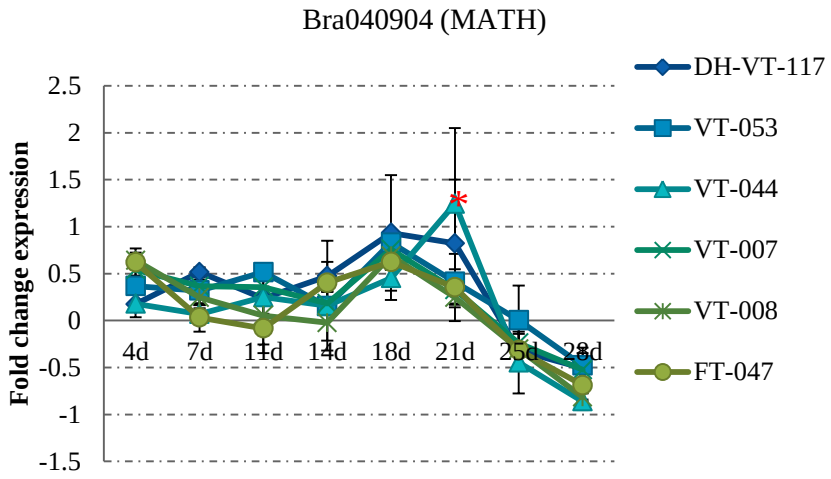


Figure 9. Log 10 transformed (fold change) relative expression of Bra040904 in six turnip accessions. The error bars represent standard deviation (n=4). High standard deviation for DH-VT-117 (at 18d and 21d) and *: at VT-044 (21d) indicates the data point is less trustable due to wider variation between biological replicates.

Figure 10 shows that Bra011488 (polyadenylate bining protein 2) was expressed uniformly in all accessions except the higher expression at 4d stage in VT-008, and 7d and 11d in VT-053. The expression was higher (<10 fold change decrease) at 18 days stage and decreased expression (>150 fold change decrease) was observed after 25 days stage in all turnip accessions.

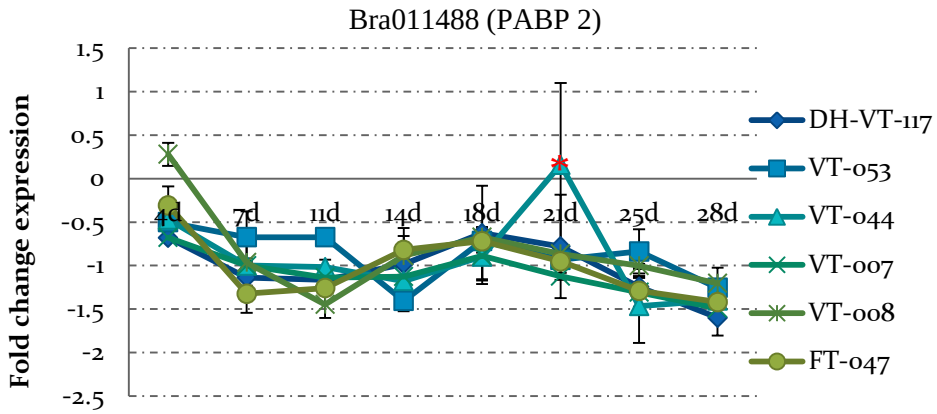


Figure 10. Log 10 transformed (fold change) relative gene expression of Bra011488 in six turnip accessions. The error bars represent standard deviation (n=4). High standard deviation for VT-008 at 4 days, and for VT-044 at 21 days stage (*) indicate the data at these time points are less trustable due to wider variation between biological replicates.

Figure 11 indicates that Bra032107 (Calmodulin binding protein) gene was lowly expressed compared to the refernce gene in all the turnip accessions. A relatively higher expression was observed in early growth stage (4 days) and up to 350 fold change decrease in expression of this gene was observed in the later turnip development stages. The expression of this gene in DH-VT-117 at 28 days stage was undetectible.

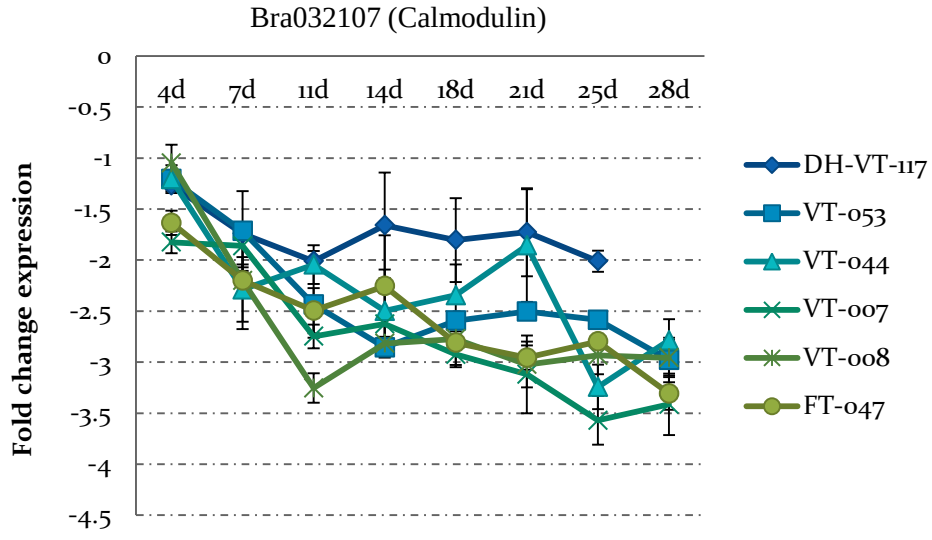


Figure 11. Log 10 transformed (fold change) relative gene expression of Bra032107 in Six turnip accessions. The error bars represent standard deviation (n=4).

Figure 12 shows that a MATH protein gene (Bra035787) was highly expressed than reference gene in growth stages from 4d to 21d (except in DH-VT-117 and VT-044). Little change in expression (<10 fold increase or decrease) was observed from day 4 to 21, but from 21 to 28 days expression decreased 10 to 100 fold in DH-VT-117, while between 25 and 28 days after sowing, expression is similar again in all accessions.

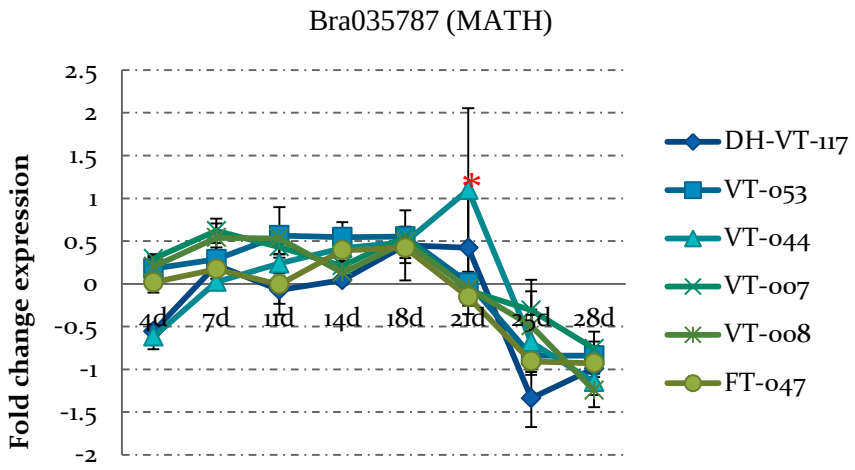


Figure 12. Log 10 transformed (fold change) relative gene expression of Bra035787 in six turnip accessions. The error bars represent standard deviation (n=4). *: data point is less trustable due to wider variation between biological replicates.

Bra020425 (sequence specific DNA binding transcription factor) gene showed lower expression than the reference gene. Transient down-regulation was observed in all turnip accessions (Figure 13). Its expression levels fluctuated over the growth stages and in different accessions. It was differently expressed in FT-047 (higher expression at 11d and 14 d) and in VT-008 (decreased expression early at 11 days after sowing). High reduction in expression (up to 300 fold decrease) was observed at 14, 21 and 25 days after sowing. The expression values changed from 350 to 50 fold change at the latest stage (28 days) of growth and development in all accessions (Figure 13).

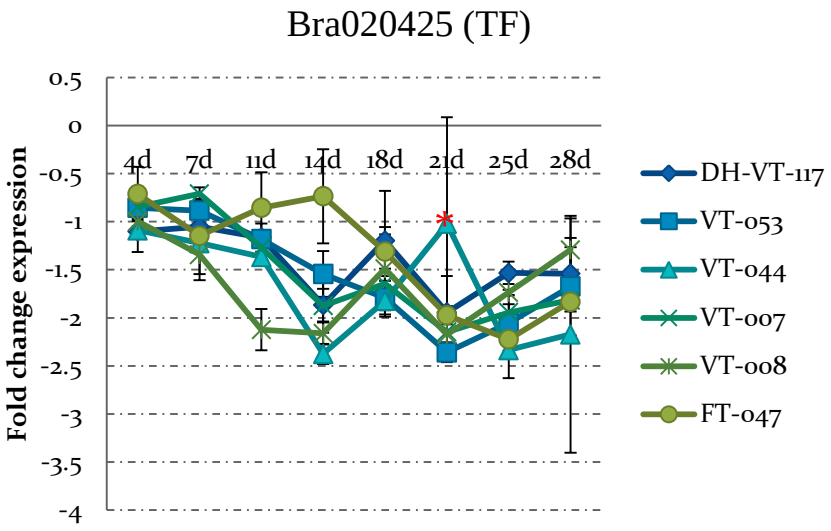


Figure 13. Log 10 transformed (fold change) relative gene expression of a DNA binding transcription factor (TF) gene, Bra020425, in six turnip accessions. The error bars represent standard deviation (n=4). *: the data point is less trustable due to wider variation between biological replicates.

5. Discussion and Conclusions

The aim of this study was to identify genes that play role in turnip formation in *B. rapa*. Expression of seven *B. rapa* genes (Bra027035, Bra008132, Bra040904, Bra011488, Bra032107, Bra035787 and Bra020425) in different growth and developmental stages of turnip tuber in six different accessions. All genes were differentially expressed ($p<0.05$) at the studied growth and developmental stages in all accessions. Out of seven genes, two genes (Bra035787 and Bra040904) of MATH protein domain were highly expressed than the reference gene by, 5 fold increase for Bra035787 (Figure 12) and 10 fold increase for Bra040904 (Figure 9) until 21 days stage.

High expression levels of genes Bra040904 and Bra035787 from 4 to 21 days after sowing and followed by a significant ($p<0.05$) decrease of the expression levels in the later stages in all accessions (Figure 9 and Figure 12), suggests that these genes may play vital role in the growth and development of turnip. The expression of these genes was substantially decreased after 21days. This could be because of the molecular initiation of turnip growth is happened earlier than 21 days. These genes are orthologs to *A. thaliana* gene At3g20370, which belongs to MATH (Merpin and TRAF homology) domain protein family. In total 59 genes are identified to code for MATH domain protein in Arabidopsis genome. Cellular localisation of several MATH proteins is predicted to be in ER-Golgi-plasma membrane (Weber et al., 2005). Function of MATH protein in plants is not explicitly known.

For all studied genes, distinctive (high or low) gene expression levels that were observed at 18 days after sowing in all accessions, suggests that a major change in cellular process happened at this stage. The phenotypic data on hypocotyl width that was measured from the six turnip accessions during different growth stages (Figure 6) also showed that turnip tuber enlargement starts at 18 days after sowing. This information together with the gene expression results suggests that 18 and 21 days after sowing growth stages are important to further study on and the genes tested in our study are essential during turnip tuberization.

An *A. thaliana* gene, Sot 16 is a Desulfoglucosinolate sulfotransferase gene. Expression of its orthologoues gene in *Brassica rapa* (Bra008132) was investigated in this study. This gene was highly expressed (comparing to reference gene) in turnip tissue at 18 days of growth and development stage in all the accessions. This indicates that this gene could take part in glucosinolate synthesis in turnip tuber. However metabolites synthesis is a result of

involvement of several genes from different pathways, therefore, these other genes co-regulate the glucosinolate synthesis should be investigated. Low expression of the remaining genes may indicate that these genes do not play a major role during turnip formation.

The expression of flowering time (FT) gene Bra004117 in DH-VT-117 in Experiment 1 was undetectable in early time points and gave low signal of expression at latest time point (42 days) (Figure 4 and Appendix C). This gene could probably be highly expressed in turnip hypocotyl tissue at the later growth and development stages that were not included in this study. This result could support the finding on interaction between Flowering Locus T (FT) and tuberization in potato under seasonal fluctuations in day length. In potato, the FT genes were highly expressed in leaf tissue, not only in tubers (Navarro et al., 2011). Since leaf tissue was not included in this study, as a result we are unable to elaborate the expression of FT gene in turnip tuberization process.

Twenty one turnip formation related QTLs were detected in F2/3 population derived from a cross between Vegetable Turnip (VT-115) and a Rapid Cycling (RC 144) explaining 8.6 to 19.9% of the total variation (Rémi, Unpublished). These QTLs covered 6 LGs (1, 2, 5, 6, 8 and 10). Two main regions (middle of LG 2 and 10) covered most of the QTLs. These regions also showed co-location with flowering time QTLs in the same study suggesting the tight regulation of flowering time and storage organ formation processes.

In F2/3 population of Chinese cabbage (*B. rapa* ssp. chinensis) and turnip (*B. rapa* ssp. rapifera), five QTLs influencing turnip tap root length and five QTLs for turnip diameter were detected explaining 11.6-27.4% and 9.2-19.3% of the phenotypic variation respectively (Lu et al., 2008). These QTLs are co-located at LG 1, 2, 3, 4, 6, 7 and 9 suggesting that these loci control turnip root formation process. In our study, we found 14 differentially ($p<0.05$) expressed genes during tuberization in six turnip accessions. These genes are physically positioned on the aforementioned LG. This could suggest the role of these loci in control of turnip tuberization process.

A comparable expression patterns was observed for most of the tested genes in all accessions (Figure 9, 10 and 12). A unique expression in time and pattern of some genes in accession FT-047 was observed (Figure 7, 8, 11 and 13). This could suggest that those genes have a different role in fodder turnip than in vegetable turnip. VT-053 was the less vigorous vegetable turnip comparing to other accessions (Figure 6). A different expression pattern of

some genes was observed in VT-053 (Figure 8, 10 and 13) suggesting that late maturation of the accession had influence on the expression pattern of the studied genes.

6. Recommendation

Gene expression study on flowering time (FT) genes at later growth and development stages of turnip will provide a better idea on source-sink translocation of turnip plants and the role of FT genes in the process. Hypocotyl tissue samples harvested and kept from later time points (4-6 weeks) can be used to design this experiment. In addition, the expression study of FT genes in turnip leaf tissue could help to understand their putative function.

To identify genes that play explicit roles in turnip formation, comparative gene expression on turnip forming and non-turnip forming morphotypes of *B. rapa* will be a helpful approach. The RNA samples isolated from Chiifu (Chinese cabbage) and YS-143 (oil type) root and hypocotyl tissues in previous study (by Dr. Ningwen) are ready to be used for the recommended study.

RNA isolation from VT-044 and VT-053 accessions at earliest time points was challenging resulting in low quality due to unknown reason. Harvesting of extra samples as a backup could be helpful in the future.

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Appendices

Appendix A: RNA isolation

Appendix A: RNA isolation procedure

RNA isolation and on-column DNAse treatment was done using RNeasy Mini Kit (Qiagen, Germany).

Before starting:

- All materials to be used and the working area were cleaned with 0.5% SDS buffer to minimize any risk of contamination with RNase (an enzyme able to degrade RNA).
- Frozen hypocotyl tissues were grinded (homogenized) in liquid nitrogen frozen mortar and pistil. Hypocotyl tissues harvested at early time points (before 2 weeks growth stages) were very little in size. Therefore homogenization and RNA isolation was performed in 1.5ml eppendorf tube.
- Extraction buffer was prepared by add 10 µl of β-Mercaptoethanol (β-ME) per 1ml Buffer RLT
- Buffer RPE was diluted with 4 volumes of ethanol (99%) to obtain a working solution
- DNase I stock solution was prepared for on-column DNase treatment: Lyophilized DNase I was dissolved in 550 µl of RNase-free water followed by gently inverting the vial
- DNase I incubation mix was prepared from 10 µl DNase I stock solution and 70 µl Buffer RDD

Procedure:

The homogenized tissue lysate was transformed in to RNase free, pre-cooled tube. 450 µl of extraction buffer (RLT) was added on to the powder in the tube. Following vigorous vortexing, the lysate was transformed to QIAshredder spin column (lilac). The spin column was centrifuged for 2 minutes at full speed. The spin column takes away the cell debris and homogenise the lysate that passes through the membrane. A flow through contains pellet (left over cell debris) and the supernatant. Only the supernatant was used in the subsequent procedures. Half volume of ethanol was added to the supernatant and immediately transferred to the RNeasy spin column that was placed in 2ml collection tube. At this stage the RNA is binded to the membrane and the flow through was discarded.

In the meantime on-column DNase digestion was carried out (Appendix A3) followed by the remaining RNA isolation steps. 700 µl Buffer RW1 was added to the RNeasy spin column then centrifuged for 15 seconds at 10,000 revolutions per minute (rpm) to wash the spin column. The flow through was discarded and two successive washing steps was performed using 500 µl of Buffer RPE each time. The flow through was discarded and an optional spin column drying was performed by placing the spin column in new 2 ml collection tube and centrifuging for 1 minute. The RNeasy spin column was placed in a new 1.5 ml collection tube and the RNA was eluted by adding 30µl RNase free water to the spin column, and centrifuging for 5 minutes at 10,000 rpm. The elusion step was repeated by adding the elute RNA to get high yield of RNA.

Appendix A2: RNA quality and concentration

Concentration and quality of RNA samples isolated from six turnip accessions at eight time points (Experiment 2)

Sample§	Concentration (ng/µl)	A260	A280	260/280	260/230
DH-VT-117-T1B1	64.67	1.642	0.804	2.04	2.56
DH-VT-117-T1B2	63.15	1.579	0.776	2.03	2.25
VT-053-T1B1	64.73	1.118	0.552	2.03	2.01
VT-053-T1B2	64	1.775	0.844	2.1	2.02
VT-044-T1B1	60.85	1.521	0.729	2.42	2.29
VT-044-T1B2	62.39	1.685	0.906	1.86	1.28
VT-007-T1B1	63.98	1.6	0.802	2	2.36
VT-007-T1B2	64.23	1.606	0.78	2.06	2.37
VT-008-T1B1	64.56	1.614	0.774	2.08	2.46
VT-008-T1B2	62.94	1.573	0.708	2.22	2.07
FT-047-T1B1	62.88	1.572	0.754	2.09	1.86
FT-047-T1B2	64.78	1.645	0.797	2.06	1.84
DH-VT-117-T2B1	60.23	1.456	0.655	2.22	2.01
DH-VT-117-T2B2	64.01	1.678	0.788	2.13	2.2
VT-053-T2B1	66.86	1.672	0.972	1.72	0.43
VT-053-T2B2	59.01	0.669	0.303	2.21	1.51
VT-044-T2B1	59.12	1.453	0.694	2.09	0.36
VT-044-T2B2	30.11	0.753	0.357	2.11	0.13
VT-007-T2B1	59.84	1.471	0.706	2.08	0.54
VT-007-T2B2	63.56	1.589	0.736	2.16	2.16
VT-008-T2B1	59.29	1.205	0.577	2.09	1.99
VT-008-T2B2	62.56	1.564	0.747	2.09	1.69
FT-047-T2B1	61.8	1.745	1.057	1.65	0.65
FT-047-T2B2	60.19	1.655	1.112	2.05	0.52
DH-VT-117-T3B1	64.74	1.619	0.761	2.13	2.24
DH-VT-117-T3B2	64.62	1.691	0.79	2.14	1.81
VT-053-T3B1	59.51	1.413	0.668	2.11	0.34
VT-053-T3B2	59.82	1.253	0.569	2.2	2.01
VT-044-T3B1	61.42	1.535	0.698	2.2	2.69
VT-044-T3B2	59.22	1.43	0.646	2.22	0.73
VT-007-T3B1	64.4	1.668	0.79	2.11	2.71
VT-007-T3B2	59.5	1.108	0.522	2.12	1.51
VT-008-T3B1	64.38	1.61	0.762	2.11	1.77
VT-008-T3B2	59.76	1.494	0.809	1.85	1.81
FT-047-T3B1	64.34	1.733	0.826	2.1	2.33
FT-047-T3B2	62.3	1.558	0.728	2.14	2.55
DH-VT-117-T4B1	63.84	1.596	0.759	2.1	1.92

DH-VT-117-T4B2	59.96	1.449	2.578	0.56	1.5
VT-053-T4B1	59.02	1.176	0.575	2.05	1.14
VT-053-T4B2	61.48	1.537	0.735	2.09	1.94
VT-044-T4B1	64.35	1.634	0.783	2.09	2.34
VT-044-T4B2	61.89	1.547	0.728	2.13	2.17
VT-007-T4B1	64.92	1.773	0.903	1.96	2.53
VT-007-T4B2	59.9	1.347	0.631	2.13	0.79
VT-008-T4B1	59.73	1.418	0.666	2.13	1.89
VT-008-T4B2	64	1.6	0.743	2.15	1.96
FT-047-T4B1	64.84	1.721	0.787	2.19	0.21
FT-047-T4B2	59.65	1.319	0.654	2.02	2.13
DH-VT-117-T5B1	64.1	1.603	0.771	2.08	2.25
DH-VT-117-T5B2	65.18	1.654	0.836	1.98	1.8
VT-053-T5B1	60.63	1.516	0.736	2.06	1.25
VT-053-T5B2	63.47	1.587	0.743	2.13	2.05
VT-044-T5B1	63.9	1.697	0.807	2.1	1.8
VT-044-T5B2	59.87	1.419	0.678	2.09	1.97
VT-007-T5B1	65.1	1.627	0.788	2.06	1.66
VT-007-T5B2	58.63	1.466	0.69	2.12	2.25
VT-008-T5B1	61.27	1.532	0.737	2.08	1.98
VT-008-T5B2	61.18	1.529	0.728	2.1	1.68
FT-047-T5B1	59.6	1.367	0.645	2.12	0.39
FT-047-T5B2	59.62	1.44	0.684	2.11	0.31
DH-VT-117-T6B1	63.95	1.599	0.76	2.1	2.27
DH-VT-117-T6B2	65.54	1.663	0.778	2.14	1.62
VT-053-T6B1	59.38	1.484	0.704	2.11	2.24
VT-053-T6B2	63.32	1.583	0.748	2.11	2.47
VT-044-T6B1	65.28	1.657	0.768	2.16	1.82
VT-044-T6B2	63.33	1.583	0.759	2.09	2.4
VT-007-T6B1	62.87	1.572	0.734	2.14	2.65
VT-007-T6B2	63.15	1.804	0.837	2.16	2.17
VT-008-T6B1	64.53	1.688	0.783	2.16	2.13
VT-008-T6B2	62.56	1.564	0.748	2.09	1.77
FT-047-T6B1	64.43	2.261	2.134	1.06	0.63
FT-047-T6B2	64.69	1.667	0.779	2.14	2.16
DH-VT-117-T7B1	61.8	1.545	0.758	2.04	2.84
DH-VT-117-T7B2	63.19	1.58	0.727	2.17	2.03
VT-053-T7B1	63.02	1.575	0.757	2.08	2.47
VT-053-T7B2	63.33	1.583	0.758	2.09	0.57
VT-044-T7B1	63.43	1.586	0.773	2.05	2.78
VT-044-T7B2	62.82	1.57	0.726	2.16	0.39
VT-007-T7B1	64.17	1.604	0.762	2.11	2.07
VT-007-T7B2	64.73	1.618	0.745	2.17	1.01
VT-008-T7B1	62.67	1.567	0.729	2.15	2.31
VT-008-T7B2	60.76	1.519	0.704	2.16	2.03
FT-047-T7B1	64.88	1.647	0.774	2.13	0.95
FT-047-T7B2	62.49	1.562	0.727	2.15	2.51
DH-VT-117-T8B1	60.43	1.511	0.74	2.04	1.75
DH-VT-117-T8B2	63.75	1.594	0.777	2.05	1.96
VT-053-T8B1	62.68	1.567	0.739	2.12	1.94
VT-053-T8B2	61.4	1.535	0.731	2.1	2.24
VT-044-T8B1	62.85	1.571	0.758	2.07	1.64
VT-044-T8B2	64.82	1.621	0.772	2.1	2.07
VT-007-T8B1	60.14	1.504	0.727	2.07	2.1
VT-007-T8B2	64.58	1.614	0.766	2.11	1.64
VT-008-T8B1	64.32	1.633	0.779	2.1	2.02
VT-008-T8B2	63.92	1.598	0.775	2.06	2.36
FT-047-T8B1	63.35	1.584	0.762	2.08	2.15
FT-047-T8B2	62.3	1.558	0.754	2.07	1.86

§ In sample nomenclature, T stands for time point, B stands for biological replicate

Appendix A3: DNase treatment

All the RNA samples recieved DNase treatment to eliminate genomic DNA (gDNA) contamination. DNase treatment was performed twice; first during RNA isolation (on-column) and later separately (off-column). The second DNase treatment was done because all the volunteer gDNA was not eliminated during on-column DNase treatment.

On-column using RNeasy Mini kit

On-column DNase treatment was performed during RNA isolation with the following procedure: DNase I incubation mix (preparation, see Appendix A1) was added on to the spin column membrane and incubated for 15 minutes at room temperature.

Of-column using Deoxyribonuclease I, Amplification Grade (Invitrogen)

Before cDNA synthesis, efficiency of on-column DNase treatment was checked by loading the RNA samples on qRT-PCR. But the majority of RNA samples gave a Ct value of around 33-36 indicating that there could a remaining gDNA contamination. Therefore, additional of-colum DNase treatment was performed treating the RNA samples with Deoxyribonuclease I (Invitrogen).

Reaction mix for Deoxyribonuclease I :

Component	Volume per reaction (µl)
RNA template	8
DNase I	1
10x DNase I Reaction Buffer	1
Total volume	11

Procedure:

DNase I enzyme and DNase I reaction buffer were added to a diluted RNA (62.5ng/µl) samples and incubated for 15 minutes at room temperature. After 15 minutes of incubation, EDTA was added to stop the reaction. Then the mixture was incubated for 10 minutes at 65^{o}C in the PCR machine.

Appendix B: Primer design

Primer pairs designed for all the genes tested

Gene	sequence (in 5'----> 3' order)	Length (base pairs)	TM (^{0}C)	GC (%)	Product size (base pairs)
Bra002177_F	AAGAGGCTTCAACGTGATCG	20	60.4	50	
Bra002177_R	CGTCGGAGAAACAGACGTTAG	21	59.9	52.4	113
Bra003397_F	AAAAGAGCCACACCACTTGC	20	60.3	50	
Bra003397_R	TGTCGACAACCTTCATCGTC	20	59.7	50	125
Bra004053_F	GGCTTGTGATGAAGCGAAAG	20	60.9	50	
Bra004053_R	CGGGACATTGTCTTCAGTTG	20	59.1	50	112
Bra004117_F	GGTTACATACGGCCAAAGAGAG	22	60	50	
Bra004117_R	CCTTAGGTCTTCTCCACCAATC	22	59.1	50	100
Bra004827_F	CATCAAGAGTCCGGATGGAG	20	60.6	55	
Bra004827_R	TGGTCTCATCTGAATGGTGTG	21	59.5	47.6	103
Bra006354_F	ACCTGTCTTTCGCGCTTTC	19	60.5	52.6	
Bra006354_R	GGATGCATATGAGTCGTCTCTG	22	59.7	50	92
Bra008132_F	TCGTGCCTTACGTGGAGATAG	20	58.5	50	
Bra008132_R	ATCTTGCAACCGGAGTTCAC	20	60.3	50	106
Bra011488_F	GCAGCTCTTCGAAGCCTTTAG	21	60.8	52.4	
Bra011488_R	AGTTCCTGGATTGCTCTTGC	20	59.4	50	141
Bra011568_F	ACTCTGGTCCCAACACCAAC	20	61	55	
Bra011568_R	CAACCTTCTCAATCGCCTTC	20	60	50	105
Bra013079_F	TTCCGGTGGGTTTGACATAG	20	60.7	50	
Bra013079_R	TTCCCTCAATATATCACCACCTC	23	59.2	43.5	145
Bra015220_F	GACGTTCTCAGAAGTGTTGCTG	22	60.1	50	
Bra015220_R	AGAGACAAGGTCGTCGTTGAG	21	59.5	52.4	78
Bra017852_F	CCATGCTCAAGTTTCATCTCC	21	59	50	
Bra017852_R	TGTTTGGCTCATGAGTGGTG	20	59.9	50	112
Bra018150_F	AGCGCTCTTGATCCACAATC	20	59.5	50	
Bra018150_R	GAGCCCAACCACCATTTTC	19	59	50	102
Bra018669_F	GCTGCTGAGATGAACAAGAGG	21	60.1	52.4	
Bra018669_R	AGCGTCAATAACCGTGCAG	19	59.9	52.6	141
Bra020425_F	GCCGTGTACTGCCGATTC	18	59	48	
Bra020425_R	CTTGTTCGCAGACTTCACAGAG	22	60	50	97
Bra021925_F	CAACAAGGATCCGAGTAAACG	21	59.6	47.6	
Bra021925_R	ACTTCTTCGGTCTCCTGACG	20	59.5	55	146
Bra026945_F	GCTTCTGCTGTCATGCTTTG	20	59	50	
Bra026945_R	GCTAGCTCCAAGCTCATTTGAC	22	59	51	112
Bra027035_F	ATGTGGTTGTCAGCCTGTTG	20	59	50	
Bra027035_R	TCATCGCGGTAATTTTCAGG	20	61	52	102
Bra029257_F	AGAAGCTGAGATTGCGAAGC	20	59.9	50	
Bra029257_R	ACGGTTCCAGATTGGAGTTG	20	60	50	109

Bra032107_F	TTCTGAGATGCTCGAGACTGAC	22	59.8	50	
Bra032107_R	TTGGTACGGCATATTGTAGCC	21	59.9	47.6	136
Bra034603_F	ACTGCTAACGGCTCCAGTG	19	59	57.9	
Bra034603_R	CTAAACGCCTCGAAAAGCTG	20	60.1	50	116
Bra034715_F	TCAGGGAGTTCCGTTTGTTC	20	60.1	50	
Bra034715_R	TCAGACCATCCTGGCTCATAC	21	60.1	52.4	87
Bra035787_F	CAAAGGGGTTTCTTGTGGATG	21	61.6	47.6	
Bra035787_R	GTGGCCGATAACAAAGCAAG	20	60.6	50	141
Bra036428_F	TGGATCTTTGGGGAGAGATG	20	60	50	
Bra036428_R	AACTTAGCCGCCCAAGAAC	19	59.3	52.6	144
Bra038088_F	ACTTAATGCGAGGCCTAACG	20	59.4	50	
Bra038088_R	CAGCGTGCATTTTCCTCAC	19	60.4	52.6	146
Bra038412_F	TTGCCGCTGGAGAAGATAAG	20	60.5	50	
Bra038412_R	ATCACATCGATCGGGACTTG	20	60.9	50	119
Bra038542_F	GGCTAATGTCAAGAGGGCTATG	22	60.1	50	
Bra038542_R	GAGCAAACACCACACAAGTCTC	22	59.8	50	133
Bra038726_F	TGTCGCAAGGAAGAAAGAGC	20	60.7	50	
Bra038726_R	ATGCATATGGTCCGTCTGG	19	59.3	52.6	95
Bra040904_F	AAACGATGTGGGGATTCTCTC	21	60.3	47.6	
Bra040904_R	TCAACACCAAACTCGCAGTG	20	60.3	50	100

Appendix C: Relative gene expression values

Relative gene expression ($2^{-\Delta Ct}$) of all candidate genes tested in Experiment 1 on DH-VT-117

Gene§	7d	14d	21d	28d	35d	42d	Mean exp.	Std. Dev	P-value
Bra002177	0.0277	0.0065	0.004	0.0035	0.0037	0.0049	0.0084	0.0095	0.202
Bra003397	0.0042	0.0011	0.0017	0.0022	0.0027	0.0024	0.0024	0.0011	0.679
Bra004053	0.0388	0.035	0.0194	0.0267	0.0366	0.0379	0.0324	0.0077	0.000
Bra004117	N/A	N/A	N/A	N/A	N/A	0.0034	-	-	-
Bra004827	0.0005	0.0002	0.0001	0.0001	0.0002	0.0002	0.0002	0.0002	0.019
Bra006354	0.0004	0.0002	0.0002	0.0002	0.0003	0.0002	0.0002	0.0001	0.010
Bra008132	**0.0434**	**0.0299**	**0.0303**	**0.0236**	**0.0256**	**0.014**	**0.0278**	**0.0097**	**0.000**
Bra011488	**0.0388**	**0.0228**	**0.009**	**0.0095**	**0.0102**	**0.0113**	**0.0169**	**0.0119**	**0.000**
Bra011568	*0.3817*	*0.2292*	*0.3135*	*0.3222*	*0.4297*	*0.5198*	*0.366*	*0.1013*	*0.000*
Bra013079	0.0007	0.001	0.0012	0.0021	0.0027	0.002	0.0016	0.0008	0.000
Bra015220	0.001	0.0005	0.0005	0.0009	0.0012	0.0013	0.0009	0.0003	0.094
Bra017852	0.01	0.0137	0.0128	0.0193	0.0238	0.0165	0.016	0.005	0.000
Bra018150	0.0044	0.0023	0.0033	0.0041	0.0023	0.0025	0.0031	0.0009	0.007
Bra018669	*0.4789*	*0.4562*	*0.5707*	*0.6759*	*0.8302*	*0.6663*	*0.613*	*0.1402*	*0.000*
Bra020425	**0.0103**	**0.0037**	**0.0007**	**0.0008**	**0.0012**	**0.0013**	**0.003**	**0.0037**	**0.000**
Bra021925	1.20e-05	5.94e-06	3.65e-06	4.61e-06	2.79e-06	5.17e-06	5.69e-06	3.28e-06	0.84
Bra026945	0.0013	0.0005	0.0003	0.0003	0.0007	0.0011	0.0007	0.0004	0.000
Bra027035	**0.0109**	**0.0024**	**0.0008**	**0.0002**	**0.0006**	**0.0002**	**0.0025**	**0.0042**	**0.000**
Bra029257	0.0131	0.0066	0.0087	0.0152	0.0192	0.0168	0.0133	0.0048	0.001
Bra032107	**0.0048**	**0.0023**	**0.0017**	**0.0026**	**0.0036**	**0.0042**	**0.0032**	**0.0012**	**0.000**
Bra034603	*0.1025*	*0.0687*	*0.0593*	*0.0833*	*0.092*	*0.093*	*0.0831*	*0.0163*	*0.001*
Bra034715	0.0041	0.0025	0.0024	0.0032	0.0049	0.0041	0.0035	0.001	0.001
Bra035787	**0.3184**	**0.2204**	**0.0488**	**0.0095**	**0.0003**	**0.0001**	**0.0996**	**0.1363**	**0.000**
Bra036428	0.0035	0.001	0.0006	0.0007	0.0004	0.0007	0.0011	0.0012	0.000
Bra038088	0.0134	0.0144	0.0326	0.1528	0.4974	0.5923	0.2171	0.2608	0.000
Bra038412	0.0357	0.0253	0.0118	0.0123	0.02	0.0233	0.0214	0.0089	0.000
Bra038542	0.0624	0.0276	0.0249	0.0319	0.057	0.0501	0.0423	0.0162	0.137
Bra038726	0.0015	0.0002	0.0001	0.0001	0.0002	0.0002	0.0004	0.0005	0.001
Bra040904	**0.623**	**0.4003**	**0.2108**	**0.1101**	**0.2339**	**0.1346**	**0.2855**	**0.1943**	**0.000**

§ Genes written in bold font are the once selected for Experiment 2.

Appendix D. Mean gene expression in six turnip accessions

Mean gene expression in different genotypes for samples harvested in different days

Genotype	Days after sowing (N=4)								Total mean expression (N=32)	Std. dev.	P-value
	4	7	11	14	18	21	25	28			
Bra027035											
DH-VT-117	0.0172	0.1222	0.0200	0.0124	0.0410	0.1651	0.0020	0.0076	0.0484	0.0822	0.012
VT-053	0.0164	0.0024	0.0090	0.0047	0.0981	0.0560	0.0376	0.0060	0.0288	0.0387	0.001
VT-044	0.0056	0.0017	0.0037	0.0071	0.0526	2.3795	0.0063	0.0081	0.3081	1.1704	0.022
VT-007	0.0116	0.0014	0.0026	0.0007	0.0300	0.0172	0.0092	0.0042	0.0096	0.0102	0.001
VT-008	0.0040	0.0132	0.0057	0.0055	0.1277	0.0700	0.0083	0.0050	0.0299	0.0562	0.002
FT-047	0.0216	0.0013	0.0036	0.0362	0.0168	0.0343	0.0125	0.0008	0.0159	0.0180	0.004
Bra008132											
DH-VT-117	0.0689	0.1321	0.0686	0.5768	2.6833	0.8485	0.0940	0.1174	0.5737	1.3307	0.051
VT-053	0.1328	0.0548	0.4684	0.0584	1.6787	0.3024	0.4157	0.1909	0.4128	0.5271	0.001
VT-044	0.1749	0.2076	0.3488	0.1906	0.5883	4.8332	0.1132	0.1037	0.8200	2.2404	0.017
VT-007	0.1446	0.1658	0.3028	0.1105	1.0789	0.3815	0.3091	0.1343	0.3284	0.3669	0.000
VT-008	0.1247	0.2119	0.0467	0.0957	1.6073	0.3578	0.2324	0.1350	0.3514	0.5331	0.000
FT-047	0.2038	0.0377	0.0975	0.3541	0.8019	0.4932	0.1936	0.0978	0.2849	0.3110	0.001
Bra040904											
DH-VT-117	1.4918	3.2711	1.7243	2.9608	8.5722	6.6385	0.4799	0.3102	3.1811	4.4785	0.066
VT-053	2.3264	2.0874	3.2715	1.4179	6.7465	2.5743	1.0082	0.3316	2.4705	1.9892	0.001
VT-044	1.5128	1.1731	1.7770	1.4377	2.8299	17.543	0.3575	0.1376	3.3461	8.0253	0.019
VT-007	3.4757	2.3136	2.2688	1.5191	5.7373	2.1232	0.5754	0.2989	2.2890	1.7789	0.001
VT-008	4.3762	1.7597	1.1304	0.9452	4.8777	1.7611	0.4974	0.1528	1.9376	1.7334	0.001
FT-047	4.1551	1.0777	1.3491	2.5300	4.2113	2.2523	0.4837	0.2049	2.0330	1.6727	0.001
Bra011488											
DH-VT-117	0.2096	0.0728	0.0682	0.1042	0.2359	0.1655	0.0569	0.0251	0.1173	0.1150	0.057
VT-053	0.3163	0.2119	0.2119	0.0391	0.1923	0.1164	0.1454	0.0556	0.1611	0.1045	0.001
VT-044	0.3393	0.1001	0.0959	0.0662	0.1268	1.4431	0.0342	0.0397	0.2807	0.6841	0.031
VT-007	0.2100	0.0999	0.0729	0.0748	0.1298	0.0752	0.0493	0.0322	0.0930	0.0611	0.001
VT-008	1.9036	0.1083	0.0440	0.1299	0.2123	0.1364	0.0997	0.0624	0.3371	0.6308	0.001
FT-047	0.4933	0.0475	0.0550	0.1515	0.1904	0.1119	0.0513	0.0381	0.1424	0.1620	0.001
Bra032107											
DH-VT-117	0.0539	0.0182	0.0098	0.0219	0.0157	0.0188	0.0098	0.0041	0.0200	0.0172	0.001
VT-053	0.0620	0.0193	0.0037	0.0014	0.0026	0.0031	0.0026	0.0011	0.0120	0.0211	0.001
VT-044	0.0621	0.0052	0.0090	0.0032	0.0045	0.0139	0.0006	0.0016	0.0125	0.0210	0.001
VT-007	0.0149	0.0137	0.0018	0.0024	0.0012	0.0008	0.0003	0.0004	0.0044	0.0063	0.001
VT-008	0.0893	0.0063	0.0006	0.0015	0.0017	0.0009	0.0012	0.0011	0.0128	0.0314	0.001
FT-047	0.0231	0.0063	0.0032	0.0056	0.0015	0.0011	0.0016	0.0005	0.0054	0.0077	0.001
Bra035787											
DH-VT-117	0.2781	1.6526	0.8442	1.1048	2.8264	2.6384	0.0459	0.1024	1.1866	1.4852	0.010
VT-053	1.5077	1.9351	3.6754	3.5092	3.5678	1.0325	0.1449	0.1447	1.9397	1.5165	0.001
VT-044	0.2408	1.0526	1.7264	2.6261	3.0660	12.526	0.2082	0.0710	2.6897	5.9094	0.032
VT-007	1.9692	4.1709	2.6995	1.6180	3.5697	0.8377	0.4911	0.1744	1.9413	1.4943	0.001

VT-008	1.5946	3.4547	3.3941	1.3539	3.0602	0.8864	0.3217	0.0568	1.7653	1.7287	0.002
FT-047	1.0405	1.4861	0.9815	2.4724	2.6390	0.7011	0.1242	0.1177	1.1953	1.0439	0.001
Bra020425											
DH-VT-117	0.0790	0.0888	0.0685	0.0136	0.0637	0.0114	0.0294	0.0288	0.0479	0.0359	0.001
VT-053	0.1395	0.1304	0.0659	0.0286	0.0162	0.0044	0.0088	0.0212	0.0519	0.0556	0.001
VT-044	0.0812	0.0599	0.0430	0.0042	0.0150	0.0949	0.0046	0.0067	0.0387	0.0519	0.028
VT-007	0.1422	0.1946	0.0537	0.0134	0.0226	0.0071	0.0114	0.0153	0.0575	0.0685	0.001
VT-008	0.1009	0.0454	0.0075	0.0069	0.0324	0.0067	0.0184	0.0510	0.0337	0.0344	0.001
FT-047	0.1944	0.0715	0.1394	0.1836	0.0490	0.0106	0.0060	0.0147	0.0837	0.1045	0.010