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Change in sugar levels and related gene expression during bilberry fruit development and ripening

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Abstract

Vaccinium myrtillus L., commonly known as European blueberry or bilberry, has been used as food and medicine since ancient times. Bilberries are highly valued in European and Asian markets. Sugar accumulation is responsible for the sweet taste of fruit, which is one of the main determinants of fruit quality. However, sugar metabolism in bilberry has not been studied before. This study was aimed to measure sugar content and to examine the expression of genes encoding the invertase enzymes; which are one of the key enzymes involved in the metabolism of sugars. All the analyses were done from four ripening stages of bilberry fruit (small unripe, large unripe, ripening, and fully ripe). Fructose was found to be predominant sugar in bilberry, followed by glucose. Sucrose was present in lower amount than fructose and glucose. The total sugar content was highest in fully ripe berries. Altogether 32 bilberry specific sugar metabolism genes were identified by bioinformatics tools, and expression of invertases, cell wall invertases (CwINVs), vacuolar invertases (VINVs), and neutral invertases (NINVs) were studied in detail. The different isoforms of invertases were differentially expressed, suggesting that they have specialized functions in the sugar metabolism pathway. *VmCwINV2* and *VmNINV5* were highly expressed in unripe berries while the levels of *VmVINV1* and *VmVINV2* transcripts were high in ripe berries. The findings of this study provide a basis for the further sugar metabolism research in bilberry fruit and deepens understanding of the role of sugar metabolism pathway to the fruit quality.

Keywords: *Vaccinium myrtillus*, Sugar accumulation, Sucrose, Fructose, Glucose, Cell wall invertase, Vacuolar invertase, Neutral invertase

Abbreviations

BLAST	Basic Local Alignment Search Tool
cDNA	Complementary Deoxyribonucleic acid
CDS	Coding Sequence
CwINV	Cell Wall Invertase
dNTP	Deoxynucleoside Triphosphate
dT	Deoxythymine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ExPASy	Expert Protein Analysis System
FK	Fructokinase
HK	Hexokinase
NINV	Neutral Invertase
PVPP	Polyvinylpolypyrrolidone
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
SPP	Sucrose Phosphate Phosphatase
SPS	Sucrose Phosphate Sythase
SRA	Sequence Read Archive
SS	Sucrose Synthase
TAE	Tris-acetate-EDTA
TSA	Transcriptome Shortgun Assembly

UDP	Uridine di-Phosphate
UDPG	Uridine di-Phosphate Glucose
VINV	Vacuolar Invertase

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1 Introduction

1.1 Bilberry (Vaccinium myrtillus L.)

Vaccinium genus; a large and taxonomically diverse genus, consist approximately 450 species. *Vaccinium* species are widely distributed from Northern Hemisphere, mountains of tropical Asia, Central and South America to Africa and Madagascar (Song & Hancock, 2011). Bilberry is a wild low bush shrub of Ericaceae family and is one of the *Vaccinium* species. It is abundant to northern Europe and also found in eastern Europe, northern America and some part of Asia. The commonly known *Vaccinium* species are Northern American highbush (*V. corymbosum*) and lowbush (*V. angustifolium*) blueberries, bilberry (*V. myrtillus*), lingonberry (*V. vitis-idaea*), bog bilberry (*V. uliginosum*), cranberries (*V. macrocarpon*, *V. oxycoccos*) and rabbiteye blueberry (*V. virgatum*). These berries are closely related, and bilberry is often mixed with blueberries due to their similar appearance.

Figure 1 Vaccinium myrtillus L. (Foster, 2006)

Bilberry fruit develops from an inferior ovary. Flowering starts in early spring and it takes around 8-10 weeks to develop and mature to ripe fruit from flower. Ripening phase of 2-3 weeks is characterized by the accumulation of sugar along with accumulation of anthocyanin

pigments (Sjoers, 1989; Zoratti et al., 2016). Bilberry fruit is blue-black due to the accumulation of anthocyanin in both flesh and skin of the fruit (Riihinen et al., 2008).

Bilberry have been part of the human diet as well as used for the medicinal purpose from prehistoric ages to present and are one of the excellent source of anthocyanin (Zoratti et al., 2016). Most of the *Vaccinium* berries along with bilberry are considered beneficial to health. Although there have been only few clinical trials on human, bilberry and it's extracts are being used in treatment and prevention of various diseases and has shown potential antioxidant, geno-protective, anti-cancer, cardioprotective, anti-inflammatory, hypoglycaemic, ocular, neuroprotective and anti-microbial effects (Chu et al., 2011).

1.2 Fruit development and ripening

Based on the high ethylene synthesis and respiration rate in the process of ripening, fruits are classified into two types; climacteric and non-climacteric (Giovannoni, 2001). Climacteric fruit shows increased ethylene production during the process of ripening whereas non-climacteric fruits do not need ethylene for ripening. The plant hormone abscisic acid (ABA) is a regulator of non-climacteric fruit ripening (Jia et al., 2016; Karppinen et al., 2018). Mango, peach, melon, tomato are climacteric fruits and grapes, strawberry, pear, blueberry, and litchi are non-climacteric fruits. Bilberry fruit also is a non-climacteric fruit (Zoratti et al., 2016).

Fruit development and ripening are complex processes which involve highly co-ordinated molecular and biochemical changes. During ripening, fruit undergoes change in firmness (due to cell wall loosening), change in colour (due to chlorophyll degradation and pigment accumulation), development of aroma (due to production of volatile components) and change in taste due to increase in sugar content and decrease in organic acids (Osorio et al., 2013). These changes during fruit development determines quality of fruit which makes them desireable to their potential consumers, such as humans, seed-dispersing birds and animals (Handa et al., 2012; Liu et al., 2015). Fruit development and ripening is a major step in plant biology and horticulture. The physiological and biochemical chages during fruit development and ripening takes place through developmentally regulated expression of genes (Bapat et al., 2010). Thus, various studies in fruits have been done focusing in the context of fruit development and ripening (Zhang et al., 2006; Bapat et al., 2010; Dai et al., 2011; Jia et al., 2013; Karppinen et al., 2018; Aslam et al, 2019).

1.3 Sugar metabolism and accumulation in fruits

1.3.1 Sugar Metabolism

Sugars are known universally as the source of energy in plants. There have been many studies which have highlighted that sugars are not only the source of energy, but they have a regulatory role in different steps of plant growth and development such as seed development and fruit development and ripening (Weber et al., 1997; Li et al., 2012; Jia et al., 2013). Sucrose and glucose has been indicated as signalling molecules. Sucrose possess signalling functions mainly in coordination with ABA during fruit ripening (Jia et al., 2013, 2016; Huang et. al, 2016). Sucrose has been proved to have minor regulatory role in bilberry fruit ripening (Karppinen et al., 2018).

The accumulation of sugar in the fruit is responsible for the sweet taste of fruit, which is one of the main determinants of fruit quality. The fruit shows two types of sugar accumulation during ripening; fruits like oranges, apples and tomatoes show a gradual increase in sugar content until the achieving final maturation, while fruits like grapes, peach and melon show a rapid accumulation of sugars in later stages of ripening (Handa et al., 2012). Almost all the ripe fruit has higher sugar content, but the composition of sugar compounds differs among the fruits. Predominant sugars that accumulate in fruits are sucrose, glucose and fructose. In bilberry fruits, fructose is found to be predominant sugar followed by glucose and sucrose is found in relatively low amount than fructose and glucose (Milivojević et al., 2012).

Sugar metabolism and accumulation during fruit ripening has been studied and well established in various fruits like apple, strawberry, grapes, melon, and peach. Sucrose, the photosynthate is transported from source tissues to sink tissues where it is either hydrolyzed by invertases (cell-wall invertases in the cell wall, neutral invertases in the cytosol and vacuolar invertases in vacuole) to glucose and fructose or hydrolyzed by sucrose synthase (SS) to fructose and uridine diphosphate glucose (UDPG) (Figure 2). The resulting glucose and fructose are stored in vacuole. Fructose and glucose synthesized in cell wall space are transported to the cytosol by hexose transporters where it gets phosphorylated (glucose to glucose 6-phosphate) by hexokinases and (fructose to fructose 6-phosphate) fructokinases (Hu et al., 2016). Fructose 6-phosphate and glucose 6-phosphate can be inter-converted to each other. These inter-conversions are catalyzed by uridine diphosphate glucose pyrophosphorylase (UGPase), phosphoglucoisomerase and phosphoglucomutase (Desnoues et al., 2014). Sucrose 6-phosphate is synthesized from fructose 6-phosphate and UDPG by the action of sucrose

phosphate synthase (SPS) and thus formed S6P is converted to sucrose by sucrose phosphate phosphatase (SPP) (Figure 2).

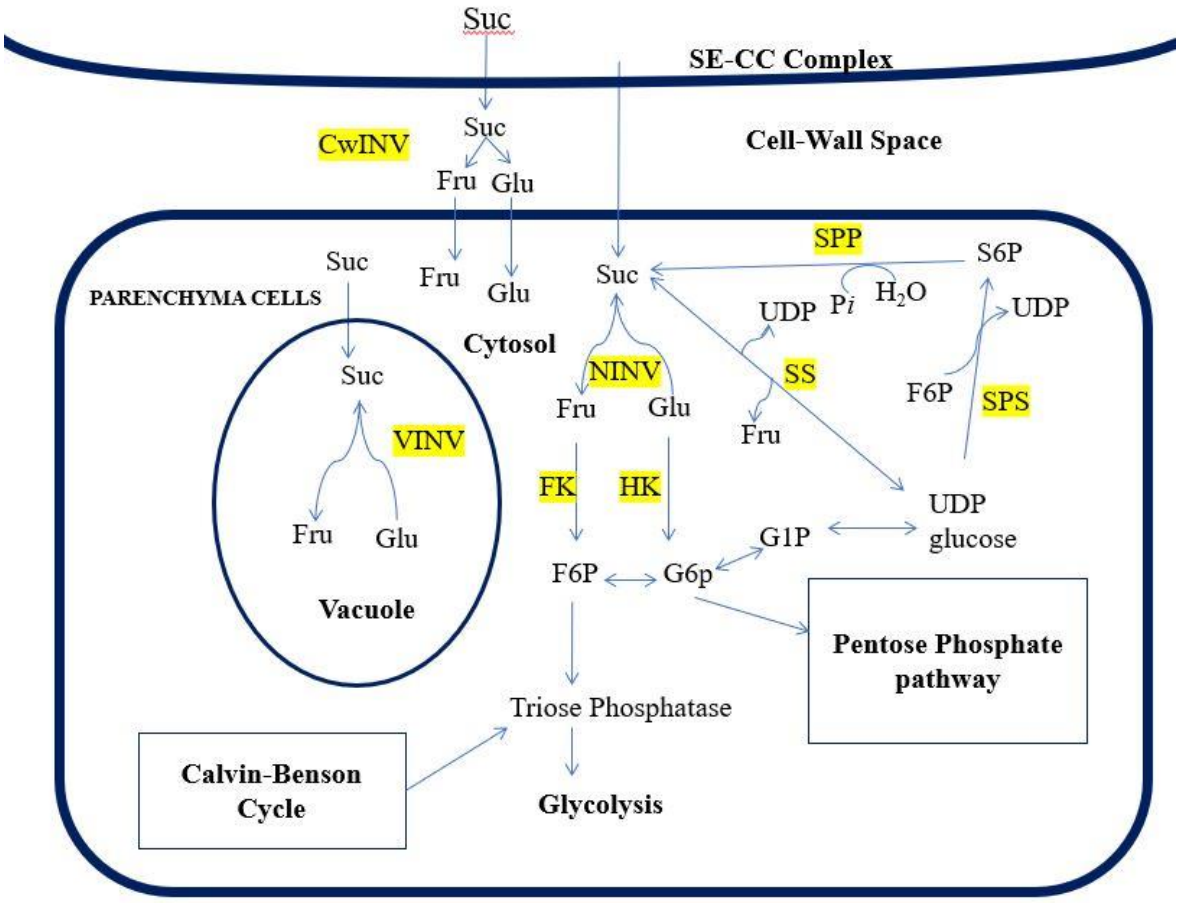

Figure 2 Sugar metabolism in ripening fruitdapted and slightly modified based on (Beshir et al., 2017; Buchanan et al., 2015). Suc: Sucrose, Fru: Fructose, Glu: Glucose, SE-CC Complex: Sieve and companion cell complex, CwINV: Cell wall invertase, VINV: Vacuolar Invertase, NINV: Neutral Invertase, FK: Fructokinase, HK: Hexokinase, F6P: Fructose 6 Phosphate, G6P: Glucose 6 Phosphate, G1P: Glucose 1 Phosphate, UDP: Uridine diphosphate, SS: Sucrose Synthase, SPS: Sucrose Phosphate Synthase, SPP: Sucrose Phosphate Phosphatase, S6P: Sucrose 6 Phosphate.

Studies focusing on sugars and sugar metabolism in fruit development and ripening has been mainly focused on enzymes and related gene expression involved in metabolism. (Dai et al., 2011) identified 42 genes encoding for the enzymatic reaction of sugar metabolism pathway in developing melon fruit and determined their expression pattern, (Li et al., 2012) identified 41 candidate genes encoding key enzymes or transporters involved in sugar metabolism and accumulation in apple fruit and studied their expression patterns in different tissues. Some of the previous studies studied genes related to enzymatic reaction involved in sugar metabolism focusing only on the gene encoding few enzymes (Yang et al., 2013; Zhang et al., 2012; Zhu et al., 2013). Studies have reported enzymes involved in sugar metabolism pathway are coded by multiple gene isoforms (Dai et al., 2011; Hu et al., 2016; Li et al., 2012).

1.3.2 Invertases

During the initial phase of fruit development sucrose is transported into the fruit parenchyma cells either symplastically or apoplastically. In sugar metabolism in fruits, sucrose cleavage is one of the major reactions, the enzyme sucrose synthase and invertases are responsible for the breakdown of sucrose. Sucrose synthase breaks sucrose into UDP glucose and fructose whereas invertases hydrolyses sucrose into fructose and glucose. Previous studies reported invertases as the main enzyme that hydrolyses sucrose and regulates the sugar metabolism fruits. The hydrolysis of sucrose is catalysed by the three isoenzyme of invertases; cell wall invertase (CwINV), vacuolar invertase (VINV) and neutral invertase (NINV). The invertases CwINVs, VINVs, and NINVs, are supposed to be located in the cell wall, vacuole, and cytosol, respectively. The different localization is one of the differentiating features of these isoenzymes.

Cell wall invertase and vacuolar invertase are also called as β-fructofuranosidases as they can hydrolyse sucrose along with other β-Fru-containing oligosaccharides whereas Neutral invertase belongs to glucosidases family (Wan et al, 2018). Cell wall invertase plays role in phloem unloading and apoplastic cleavage of sucrose whereas vacuolar invertase is known to have major role in cell expansion and sugar accumulation (Zhang et al., 2006; Wan et al., 2018). Often, NINVs are attributed to general cellular functions like cell maintenance and growth (Roitsch & González, 2004). The cytosolic hydrolysis of sucrose by neutral invertase is thought to be associated to the response to environmental stress as well as some studies reported NINVs to be important in regulation of root development and reproduction (Vargas & Salerno, 2010).

1.4 Objectives of the Study

Fruit development and ripening is a major step in plant biology and horticulture. Sugar metabolism and accumulation is one of the important biochemical process that occur during fruit development and ripening. The accumulated sugar determines fruit taste and quality. Bilberry has been used as food and medicine since ancient times. Bilberries are highly valued in European and Asian markets. Understanding sugar metabolism during bilberry fruit development and ripening would help to shed light on the important horticultural phenomenon of sugar accumulation. However, sugar metabolism in bilberry has not been studied before.

Thus, the main aim of this study is to study soluble sugar content in different ripening stages of bilberry and to analyse the expression of invertase genes involved in sugar metabolism during fruit development process. The specific objectives of this study was to identify and check genes

involved in sugar metabolism as well as to find total sugar content as well as fructose, glucose and sucrose content during bilberry fruit development.

In sugar metabolism pathway invertases are the major enzymes that synthesize fructose and glucose. Studying the genes encoding enzymes involved in invertases would be important for understanding the phenomena of sugar accumulation in bilberry thus this study will be more focused on studying the genes encoding enzyme invertases during bilberry fruit development.

2 Materials and Methods

2.1 Plant material and sample preparation

Fruits from four different developmental stages of bilberry (Figure 3), that has been described and collected during previous studies on bilberry (Karppinen et al., 2013) were plant material for this study. These wild bilberry fruits were collected in 2011 from a forest in Oulu (65°01' N, 25°28' E), Finland and were kept in -80°C.

S2 S3 S4 S5

Figure 3 Different developmental stages of bilberry (Karppinen et al., 2013) S2, small unripe green fruit; S3, large unripe green fruit; S4, ripening purple fruit; S5, fully ripe blue fruit.

Fruits were grinded in liquid nitrogen to get a fine powder. Approximately 0.12 g of powder of each sample was weighed in five tubes for RNA extraction and remaining powder were kept in separate tubes for sugar content analysis and kept on -80 °C until use .

Samples for sugar content analysis were processed further. The samples stored at -80 °C were placed into the freeze dryer for drying. In a falcon tube, 0.12 g of Polyvinylpolypyrrolidone (PVPP) and 0.1 g of dried sample was placed. Sterile water was added up to 12 ml to the falcon tubes. All the tubes containing samples were vortexed, and mixing was done using a orbital shakerat 200 for 1.5 h. After mechanical mixing centrifugation was done at 4500 g for 10 minutes and 10 ml of the supernatant was transferred to a falcon tube. Supernatant was filtered to a new falcon tube using 0.2 µm filter. The filtered samples were stored in -20 °C until used for sugar concentration analysis.

2.2 Isolation of RNA

RNA was isolated using the Spectrum^{TM} Plant Total RNA Kit (Sigma-Aldrich Co. St. Louis, Missouri, USA) with slight modifications. All the centrifugation during the isolation were done in the refrigerated centrifuge (5424 R; Eppendorf, Hamburg, Germany). RNA extraction was done for four replicates and 500 µl of lysis solution was added to the tubes containing powder,

followed immediately by vortexing. The samples were then incubated at 56 °C for 5 minutes and then centrifuged at maximum speed for 3 minutes. The lysate obtained from the centrifugation was transferred to the filtration column seated in a 2 ml collection tube and centrifuged at maximum speed for 1 minute. An amount of 700 µl of the binding solution was pipetted into the clarified lysate and mixed immediately and thoroughly. Into the binding column seated in a 2 ml collection tube, 700 µl of the mixture was pipetted and centrifuged at maximum speed for 1 minute for binding of RNA, the flow-through in the collection tube was decanted and the residual liquid was drained by tapping on an absorbent paper. This step was done two times. On column DNase digestion was done for complete removal of traces of DNA. Into the column seated on the collection tube, 300 µl of wash solution 1 was pipetted and centrifuged at maximum speed for 1 minute. The flow-through in the collection tube was decanted and the residual liquid was drained by tapping on an absorbent paper. DNase digestion mix was prepared by combining DNase I, DNase I digestion buffer, and water. 50 µl of this mix was pipetted directly into the center of the column and incubated at room tempertature for 15 minutes. Into the column seated on the collection tube, 500 µl of wash solution 1 was pipetted and centrifuged at maximum speed for 1 minute. The flow-through in the collection tube was decanted and the residual liquid was drained by tapping on an absorbent paper. Into the column seated in the collection tube, 500 µl of diluted wash solution 2 was pipetted and centrifuged at maximum speed for 30 seconds, the flow-through was decanted and the column was dried by centrifuging it at maximum speed for 1 minute. this step was done two times.The column was transferred to the new collection tube and 40 µl of nuclease free water was added directly to the centre of the binding matrix inside the column. The tube was incubated in room temperature for 1 minute and then centrifuged at maximum speed for 1 minute and the flow-through eluate containing purified RNA was stored at -80 °C until use.

The concentration and purity of RNA were determined by spectrophotometric analysis by using a nanodrop spectrophotometer (NANODROP 2000c; Thermo Fisher Scientific, Waltham, Massachusetts, USA). To check the quality of the isolated RNA samples, RNA samples were loaded in the gel. The diluted RNA samples were mixed with 1µl of gel loading dye (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for visual tracking of RNA migration during electrophoresis. The RNA samples were run in 1% agarose (Sigma-Aldrich Co. St. Louis, Missouri, USA) gel made on 1X TAE (Tris-acetate-EDTA) buffer (Millipore, Merck KGaA, Darmstadt, Germany) stained with ethidium bromide.The electrophoresis was carried out for around 50 minutes at 50 V in an horizontal electrophoresis system (Bio-Rad, Hercules,

California, USA). The gel was visualized on High-performance UV transilluminator by UVP and documented using DigiDoc-It® Imaging System by UVP (analytikjena, Jena, Germany).

2.3 cDNA preparation

cDNA was prepared by the reverse transcription reaction of isolated RNA. All the heating steps of the reaction was carried out in MJ Mini personal thermal cycler (Bio-Rad, Hercules, California, USA). In the very first step, the oligo dT primer was annealed to the template RNA by combining 1 µl of 50 µM oligo dT_{12}, 1 µl of 10 mM dNTP mix and 4 µg of RNA in nuclease free water (Ambion®; Thermo Fisher Scientific, Waltham, Massachusetts, USA) making a final volume of 14 µl in a reaction tube and then by heating the mixture at 65 ºC for 5 minutes and centrifuged and incubated on ice for at least 1 minute.

To this RNA-primer mixture, a mixture of 4 µl of 5X SSIV Buffer, 1 µl of 10 mM DTT and 1µl of Superscript® IV Reverse Transcriptase (200 U/µl) was added. The combined reaction mixture was incubated at 55 ºC for 30 minutes. The reaction was inactivated by incubating it at 80 ºC for 10 minutes. The cDNA samples were stored at -20 ºC until use.

2.4 Identification of sugar metabolism genes

The key enzymes involved in sugar metabolism pathway were identified from the similar researches on other fruits. The genes encoding those enzymes were searched in NCBI database (.https://www.ncbi.nlm.nih.gov/) The amino acid sequences corresponding to the enzymes involved in sugar metabolism from some related species (*Malus domestica*, *Fragaria vesca* sub species *vesca*, *Prunus persica* and *Vitis vinifera*) were downloaded from the database and used to identify the sugar metabolism pathway genes.

A homologous alignment-based search using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was applied. The downloaded amino acid sequences were used as queries against Transcriptome Shotgun Assembly (TSA) sequence databases of *V. virgatum*. The obtained nucleotide sequences were translated using translate tool of ExPASy (https://web.expasy.org/translate/) and CDS for all genes were identified. The identified *V. virgatum* sequences were used for bioinformatic analysis of sugar metabolism genes.

Thus, obtained *V. Virgatum* genes participating in sugar metabolism were used to identify highly similar short mRNA sequence genes of bilberry by doing SRA-Blast (NCBI) against

SRA experiment set SRX3387852 and SRX3387853. The highly similar sequences obtained from the SRA experiment set were used to design primers.

2.5 Localization Prediction

The deduced amino acid sequences of V. virgatum were used for localization, phylogeny and multiple sequence alignment analysis. Subcellular localization of all the identified isoforms of invertases were predicted using three different prediction tools;

- DeepLoc version 1.0 (http://www.cbs.dtu.dk/services/DeepLoc/),
- Plant-mPLoc version 2.0 (http://www.csbio.sjtu.edu.cn/cgi-bin/PlantmPLoc.cgi), and
- PSI (http://bis.zju.edu.cn/psi/).

2.6 Phylogeny and multiple sequence alignment analysis

The unrooted phylogenetic trees were constructed with MEGA X using the Maximum likelihood method, poisson model, partial deletion method, and a bootstrap test with 1,000 replicates. Multiple alignments of deduced amino acid sequences were performed using EMBL-EBI multiple sequence alignment tools (https://www.ebi.ac.uk/Tools/msa/clustalo/) and viewed and processed in Genedoc 2.7.

2.7 Relative quantification by real-time PCR

2.7.1 Primer Design

The primer designing was done using web-based Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/). The designing parameters were; primer length of 22bp, maximum Tm difference of 2, product length of 90-180 bp, GC content 50-60% and max poly-X 3.

2.7.2 Standard curve analysis

All the PCR reactions of relative quantification of genes encoding enzymes of sugar metabolism pathway were run on C1000^{TM} Thermal cycle (CFX96^{TM} Real-Time System; Bio-Rad, Hercules, California, USA) and the data from qRT-PCR run were visualized and analysed using CFX Manager ^{TM} version 2.1. The samples were analysed in triplicates.

To determine the efficiency of the primers of each gene, a standard curve analysis was done. One cDNA sample from each four fruit developmental stages was mixed (3µl each). Serial

dilution of the mixed cDNA was made by doing three 10-fold dilutions, one 5-fold dilution and three10-fold dilution of 5-fold dilution and one 2-fold dilution of 10-fold dilution. 40µl of 5µM primer was prepared from the 100µM of stock primer. The master mix was prepared by mixing Bio-rad SSO Reaction mix, 5µM Forward Primer, 5µM Reverse Primer in nuclease free water.For each gene, 14 µl of the prepared master mix was pipetted in the 96 well plate PCR and 1 µl of serially diluted cDNA was added to the PCR plate. The plate was briefly centrifuged at speed of 2500 rpm and the plate was run under the following PCR conditions; an initial incubation at 95 °C for 30 seconds followed by 40 cycles at 95 °C(denaturation) for 5 seconds and 60 °C(annealing) for 10 seconds.

2.7.3 qPCR

All cDNA samples were diluted 20-fold and master mix was prepared by mixing Bio-rad SSO Reaction mix, 5µM Forward Primer, 5µM Reverse Primer in nuclease free water. For each gene. For each gene, 14 µl of the prepared master mix was pipetted in the 96 well plate PCR and 1 µl of diluted cDNA was added to the PCR plate. The plate was briefly centrifuged at speed of 2500 rpm and the plate was run under the PCR conditions mentioned above.

2.8 Determination of Sugar Content

2.8.1 Determination of Total sugar content

Total sugars content of the sample was determined by using the phenol-sulfuric acid method (Nielsen, 2010). All the samples were analysed in four replicates. Glucose standards of 10, 20, 40, 60 , 80, and 100 µg/ml were made. Samples stored in -20 °C were used. For the analysis, 100µl of the sample was added to the test-tubes containing 1.9 ml of water. 0.05 ml of 80 % of phenol was added and the mixing was done by vortexing. After vortexing, 5 ml of sulfuric acid was added rapidly and directly to the liquid in the tube. Mixing was done by vortexing and the test tubes were incubated in room temperature for 10 minutes and the tubes were kept in the 25 °C water bath for 10 minutes. The test tubes were vortexed. Samples were poured to the cuvettes from the tubes and absorbance was read at 490 nm with Smartspec^{TM} Spectrophotometer (Bio-Rad, Hercules, California, USA).

2.8.2 Determination of fructose,glucose and sucrose content

The sucrose, fructose and glucose were analysed using Sucrose/D-Glucose/ D-Fructose assay kit (R-biopharm, Darmstadt, Germany). All the samples were analysed in triplicates. All the

absorbances were measured at 340 nm with Smartspec™ Spectrophotometer (Bio-Rad, Hercules, California, USA). Addition of samples and reagents as well as the absorbance readings were done according to the pipetting scheme provided by the producer (Appendix 4). The absorbance difference for each sugar was also calculated using the instructions provided by the producer (Appendix 4). Concentration of each sugar was calculated using the following formula;

$$Concentration\ (C) = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A\ \left[\frac{g}{l}\right]$$

Where,

V = final volume [ml]

v = sample volume [ml]

MW = molecular weight of the substance to be assayed [g/mol]

d = light path [cm]

ε = extinction coefficient of NADPH at 340 nm

The sugar content of each sugar was measured using the following formula;

$$Content = \frac{Concentration\ (C)}{Sample\ weight\ in\ sample\ solution}\ [mg/g]$$

3 Results

3.1 Identification of genes of sugar metabolism pathway

The bilberry genome has not been sequenced yet. The only available nucleotide dataset on bilberry was a GenBank Sequence Read Archive (SRA) (SRX3387852 and SRX3387853) transcriptome with very short length transcripts(Nguyen et al., 2018).

Due to the absence of a sequenced genome, the identification of genes of sugar metabolism in bilberry fruit was done in two steps. At first, the sugar metabolism genes in one of the closely related *Vaccinium* species (*V. virgatum*) were identified. *V. virgatum* genes were used as the the available *V. virgatum* genes were full length. For this, amino acid sequences of the key sugar metabolism pathway enzymes from some related species (*Malus domestica*, *Fragaria vesca* sub species *vesca*, *Prunus persica* and *Vitis vinifera*) were used as query sequences for tBLASTn searches against GenBank Transcriptome Shotgun Assembly Sequence (TSA) database of *V. virgatum*. Genes for enzymes Cell-wall Invertase(CwINV), Vacuolar Invertase (VINV), Neutral Invertase (NINV), Hexokinase (HK), Fructokinase (FK), Sucrose Phosphate Phosphatase (SPP), Sucrose Phosphate Synthase(SPS) and Sucrose Synthase(SS) were searched. In the second step, these identified virgatum genes were used as query sequences for the Sequence Read Archive (SRA) nucleotide BLAST searches against GenBank Sequence Read Archive (SRA) (SRX3387852 and SRX3387853) transcriptome to identify highly similar bilberry (*V. myrtillus*) transcripts.

Altogether 32 sugar metabolism pathway genes were identified; Four isoforms of CwINV, three isoforms of VINV, five isoforms of NINV, five isoforms of HK, five isoforms of FK, two isoforms of SPP, four isoforms of SPS and four isoforms of SS. All the identified *V. virgatum* sequences, with their matching sequences from TSA database and corresponding bilberry sequences from SRA database are presented in Table 1. Similar sequences were identified from SRA database. Only sequence of more than 100 bp were included in the result.Only invertases were used for further studies in this thesis as they are one of the key enzymes involved in sugar metabolism.As the identified bilberry sequences were only short transcripts and the identified *V. virgatum* sequences were of full length and are highly similar to bilberry sequences (Table 1), *V.virgatum* genes were used for the study of phylogenetic relationship and sub cellular localization of the enzymes whereas the sequences of bilberry from SRA database were used to design primers.

Table 1 Identified sugar metabolism genes of V.virgatum and their TSA (Transcriptome Shotgun Assembly) IDs and homologues gene with the highest similarity and corresponding V. myrtillus genes SRA(Sequence Read Archive) IDs.

Gene Name	Sequence ID in TSA Database (*V. virgatum*)	Matching sequence Details			
		GenBank ID (Species)	% Identity	SRA ID (*V. myrtillus*)	% Identity
CwINV1	GGAB01072872	*NC034011(P. persica)*	66	729392	97
CwINV2	GGAE01109889	*NW021025375(C. sinensis)*	82	976747	97
CwINV3	GGAE01121540	*NC012010(V. vinifera)*	64	1364501	99
CwINV4	GGAE01124826	*JQ412748(P. persica)*	68	–	–
VINV1	GGAE01006295	*KU884473(C. sinensis)*	76	505506	97
VINV2	GGAB01063390	*NC012022.3(V. vinifera)*	67	1675652	98
VINV3	GGAE01077959	*NC041794(M. domestica)*	60	–	–
NINV1	GGAE01022874	*KF718860(C. sinensis)*	84	1523998	99
NINV2	GGAE01119103	*CM014052(M. domestica)*	76	783733	95
NINV3	GGAE01011826	*NC_012012(V. vinifera)*	79	1386518	99
NINV4	GGAE01022873	*KP053405(C. sinensis*	88	1278054	98
NINV5	GGAE01030565	*NC034016(P. persica)*	86	1057615	99
HK1	GGAE01107266	*NC034015(P. persica)*	83	1301214	100
HK2	GGAE01118471	*AM456450.2(V. vinifera)*	83	1529550	99
HK3	GGAE01031242	*JN118545.1(V. vinifera)*	82	–	–
HK4	GGAE01000009	*NW021027372(C. sinensis)*	75	–	–
HK5	GGAB01088020	*NC041799(M. domestica)*	72	1137583	99
FK1	GGAE01074415	*NW021026114(C. sinensis)*	78	1269317	100
FK2	GGAB01032707	*XM008378279(M. domestica)*	84	1052446	98
FK3	GGAB01048669	*JX067537(A. chinensis)*	85	232383	97
FK4	GGAB01032403	*JX067535(A. chinensis)*	90	1177935	99
FK5	GGAB01084714	*NC012011(V. vinifera)*	86	1423238	96
SPP1	GGAE01112488	*AY509994(A. chinensis)*	87	664663	99
SPP2	GGAB01063835	*NC012014.3(V. vinifera)*	68	1470994	96
SPS1	GGAE01109516	*AF318949(A. chinensis)*	85	554516	99
SPS2	GGAB01078977	*NC012017(V. vinifera)*	78	324571	99
SPS3	GGAE01036876	*ONI28760.1(P. persica)*	82	1146340	97
SPS4	GGAE01005886	*NC012024.3(V. vinifera)*	77	–	–
SS1	GGAB01025193	*JX067545(A. chinensis)*	93	279328	98
SS2	GGAB01053670	*NC012013(V. vinifera)*	85	808357	96
SS3	GGAE01025208	*NW021027954(C. sinensis)*	87	–	–
SS4	GGAE01053339	*NC041805(M. domestica)*	77	826437	97

3.2 Predicted Localization of Invertasesof *V. virgatum*

As different subcellular localization is one of the differentiating factor of invertases, to check the precision of identified genes subcellular localization of invertases was predicted using three different web-based software. All the findings from localization prediction have been summarised in the Table 2 . According to Deep Loc, CwINV1, CwINV2 and CwINV4 were predicted to localize in Endoplasmic Reticulum, CwINV3 was predicted to be extracellular. PSI tool predicted that all the isoforms of cell wall invertase to be to be extracellular whereas Plant-mPloc tool predicted them to be in cell-wall. All the three prediction tools predicted all the isoforms of vacuolar invertase to localize in vacuole. According to Deep Loc, NINV1 was predicted to be in mitochondrion, NINV2, NINV3 and NINV4 were predicted to localize in plastid. Deep Loc also predicted NINV5 to localize in cytoplasm. PSI tool predicted them to be in plastid whereas it predicted NINV 5 to be in cytoplasm. Plant-mPloc tool predicted all the isoforms of neutral invertase to be localize in chloroplast..

Table 2 Predicted subcellular location of isoforms of invertases of V. virgatum.

Gene	**Length**	Localization prediction Tool				
		Deep Loc		Plant-mPloc	PSI	
		Localization	Likelihood	Localization	Localization	Likelihood
CwINV1	573	ER[1]	0.48	Cell-wall	Extracellular	0.76
CwINV2	576	ER[1]	0.43	Cell-wall	Extracellular	0.75
CwINV3	583	Extracellular	0.42	Cell-wall	Extracellular	0.62
CwINV4	577	Extracellular	0.48	Cell-wall	Extracellular	0.49
NINV1	676	Mitochondria	0.51	Chloroplast	Plastid	0.90
NINV2	664	Plastid	0.60	Chloroplast	Plastid	0.83
NINV3	641	Plastid	1.00	Chloroplast	Plastid	0.76
NINV4	665	Plastid	0.68	Chloroplast	Plastid	0.52
NINV5	562	Cytoplasm	0.57	Chloroplast	Cytoplasm	0.50
VINV1	652	Lysosome/ Vacuole	0.80	Vacuole	Vacuole	0.65
VINV2	657	Lysosome/ Vacuole	0.79	Vacuole	Vacuole	0.67
VINV3	674	Lysosome/ Vacuole	0.70	Vacuole	Vacuole	0.61[1]

Using the Plant-mPloc software the length of the translated amino acid sequences was also predicted. The amino acid sequences of CwINV1, CwINV2, CwINV3 and CwINV4 were

[1] ER: Endoplasmic Reticulum

predicted to contain 573, 576, 583 and 577 amino acid residues. The amino acid sequences of VINV1, VINV2 and VINV3 were predicted to contain 652, 657 and 674 amino acid residues. The amino acid sequences of NINV1, NINV2, NINV3, NINV4 and NINV5 were predicted to contain 676, 664, 641, 665 and 562 amino acid residues, respectively.

3.3 Phylogeny analysis of invertases

To analyse the relationship among different invertases genesphylogenetic analysiswas done. An unrooted phylogenetic tree was constructed using deduced amino acid sequences of *V. virgatum* along with invertases of some other species. Phylogenetic analysis of the invertases showed that the three invertases can be divided into two branches; acid invertase branch (CwINVs and VINVs) and neutral/alkaline invertase branch (NINVs). Both CwINVs and VINVs subgroup of acid invertase branch could be further divided into two subgroups.

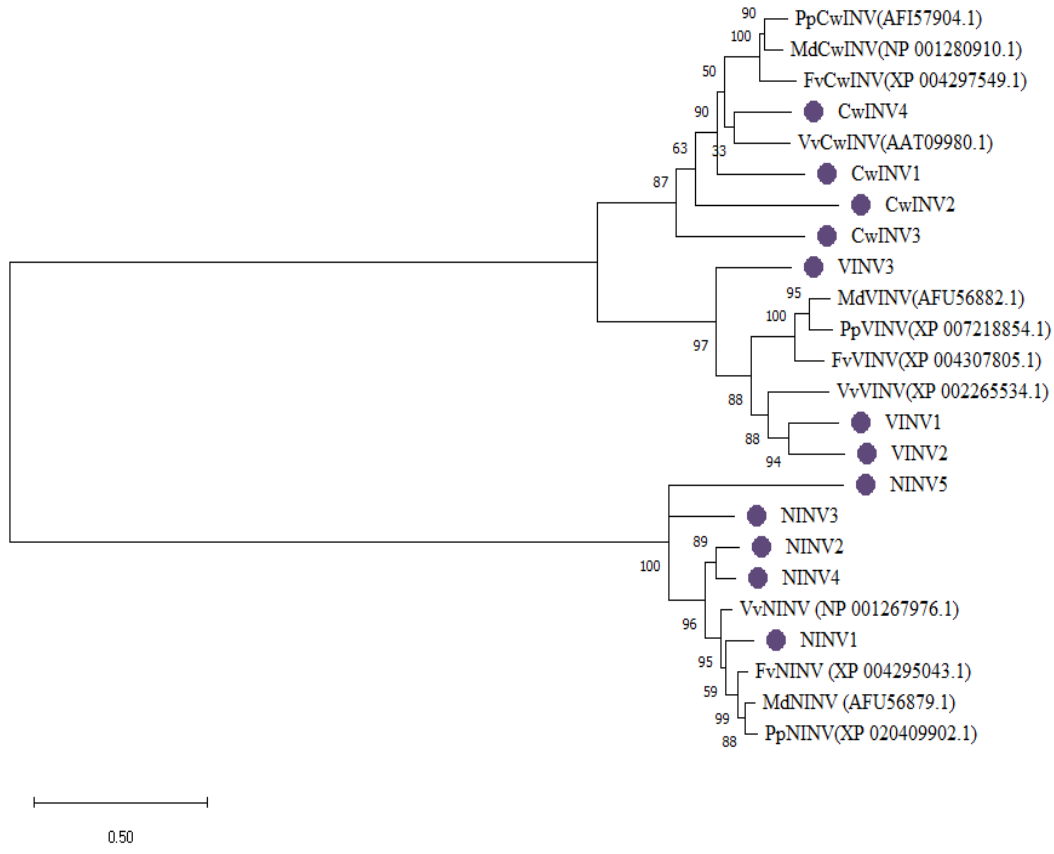

Figure 4 Maximum likelihood phylogeny tree of invertases. The tree represents phylogenetic relation of Cell wall invertase (CwINVs), Vacuolar Invertases(VINVs), and Neutral Invertases(NINVs) genes encoding enzymes in V. virgatum with those from M. domestica, V. vinifera, P. persica or F. vesca. The tree was produced using Whelan And Goldman model and a discrete gamma model with 5 categories. Bootstrapping was performed with1000 replicates in MEGA X (Kumar et al., 2018)

3.4 Multiple Sequence Alignment

To gain further insights into the characteristics of identified genes, CWINVs, VINVs, and NINVs among the related plant species, amino acid conservation of invertases were examined by multiple sequence alignment analysis of deduced amino acid sequences. Both cell wall invertases and vacuolar invertases contained two key motifs essential for the catalytic activity of acid invertases; β-fructosidase motif (NDPNG) and cysteine catalytic domain (WECP/VDF) as well as four amino acids residues(DECD) that are proposed to be essential for recognition and binding of sucrose by Wan (2018). FvVINV and VINV2 contained 'I' and 'L' instead of 'P' in WECPDF motif respectively (Figure 5 and Figure 6).

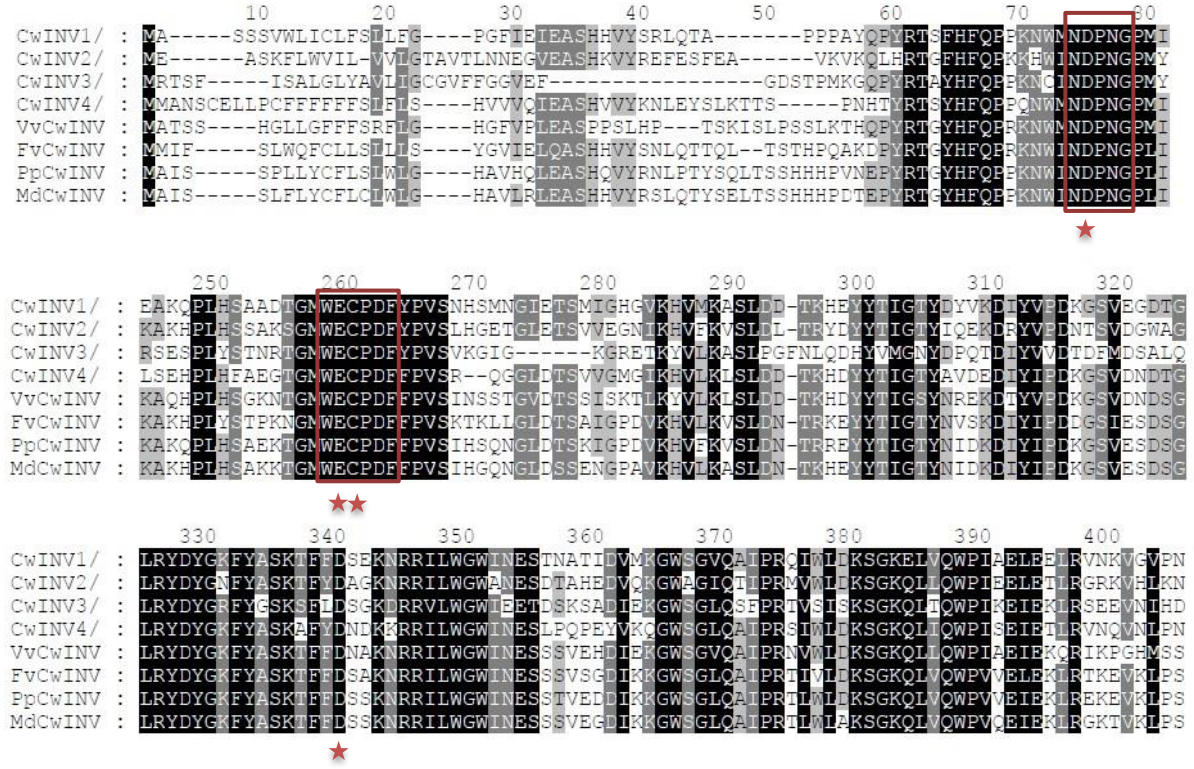

Figure 5 Amino acid sequence alignment of CwINVs from V. virgatum with those from M. domestica, V. vinifera, P. persica or F. vesca. Red box indicate β-fructosidase motif (NDPNG) and cysteine catalytic domain (WECPDF), Red stars indicate four amino acids residues(DECD) that are proposed to be essential for recognition and binding of sucrose (Wan, Wu, Yang, Zhou, & Ruan, 2018).

All the identified neutral invertases were lacked acid invertase specific NDPNG and WECPDF motifs. The neutral invertases contain two amino acids that corresponds to active residues of neutral/alkaline invertases mentioned by Wan (2018).

Figure 6 Amino acid sequence alignment of VINVs from V. virgatum with those from M. domestica, V. vinifera, P. persica or F. vesca. Red box indicate β-fructosidase motif (NDPNG) and cysteine catalytic domain (WECPDF), Red stars indicate four amino acids residues(DECD) that are proposed to be essential for recognition and binding of sucrose (Wan, Wu, Yang, Zhou, & Ruan, 2018

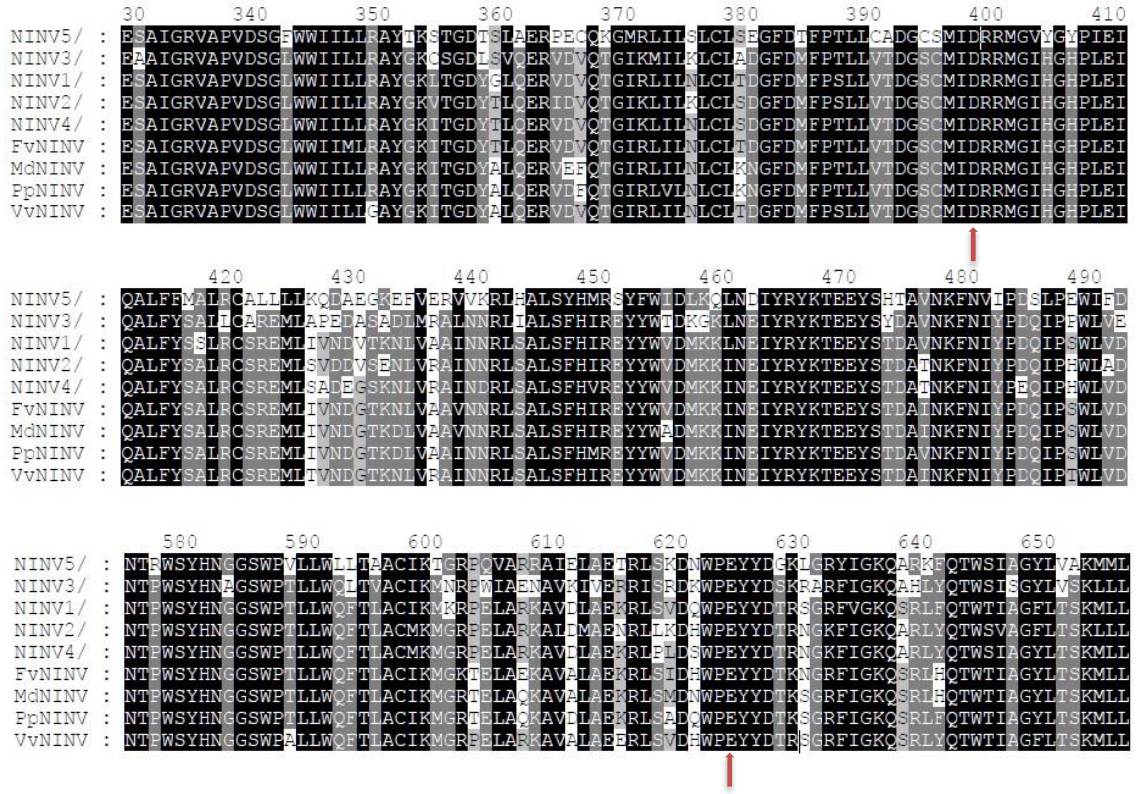

Figure 7 Amino acid sequence alignment of NINVs from V. virgatum with those from M. domestica, V. vinifera, P. persica or F. vesca.The red arrow indicate amino acids correspond to active residues for neutral/alkaline invertases.

For all the invertases sequence comparison was done at amino acid level with *M. domestica*, *F. vesca* sub species *vesca*, *P. persica* and *V. vinifera*. It revealed that these genes coding CwINVs shared sequence identity from 58% to 88% and VINVs sequence identity from 58 % to 71 % shared (Appendix 3). For neutral invertases, it was revealed that the genes share high sequence identity at the amino acid level (50.55 % to 88.68%). NINV2 and NINV4 shared homology of 78.48 %. NINV1 shared higher homology (79.1 %) with *VvNINV*. The highest similarity shared by NINV5 was with *MdNINV* (53.53%) and NINV3 was with *PpNINV* (60.41%) (Appendix 3).

3.5 The concentration and purity of isolated RNA

RNA was extracted from different fruit development stages of bilberry. The concentration and purity of isolated RNA samples were determined by spectrophotometric analysis and the result from the analysis is presented in Table 3.

Table 3 Concentration and purity of isolated RNA samples.

Sample	Replicates	Concentration (ng/µl)	260/280 ratio	260/230 ratio
 **S2**[2]	**1**	867.6	2.03	2.29
	2	884.4	2.02	2.28
	3	578.2	1.93	2.25
	4	1140	2.03	2.31
 **S3**[2]	**1**	983.5	2.05	2.3
	2	1221.7	2.05	2.31
	3	678.9	1.99	2.21
	4	886.8	2.08	2.24
 **S4**[2]	**1**	346.8	2.05	1.99
	2	450.1	2.1	2.06
	3	303.3	2.08	2.03
	4	475.7	2.08	2.07
 **S5**[2]	**1**	176.9	2.11	1.82
	2	178	2.01	1.86
	3	162.1	2.09	2.03
	4	145.1	2.04	1.93[2]

[2] S2 is small unripe green fruit; S3 is large unripe green fruit; S4 is ripening purple fruit, and S5 is fully ripe blue fruit.

The concentration of the sample ranges from 145 ng/µl to 1222 ng/µl. The RNA concentration in unripe fruits was found to higher than ripe fruits which could be due to the abundance of polysachharides, phenolics and other metabolites in ripe fruits than unripe fruits. The absorbance ratios of 260/280 and 260/230 were used to check the purity of the isolated RNA samples. RNA having 260/280 ratio ~2 and 260/230 ratio in the range of 2.0-2.2 is generally accepted as pure. For isolated RNA samples, the absorbance ratios of 260/280 and 260/230 were found to be in the range of 1.9 - 2.11 and 1.8 - 2.3 which indicates that the isolated RNA samples were pure and free from protein and other organic contaminants. .

The integrity of the isolated RNA was analysed by gel electrophoresis. The images from gel electrophoresis is presented below in Figure 8. An intense upper band indicating 28S RNA and a faint lower band indicating 18S RNA band was clearly visible for all the isolated RNA samples, which indicates that the isolated RNA samples were intact and could be used for further experiments.

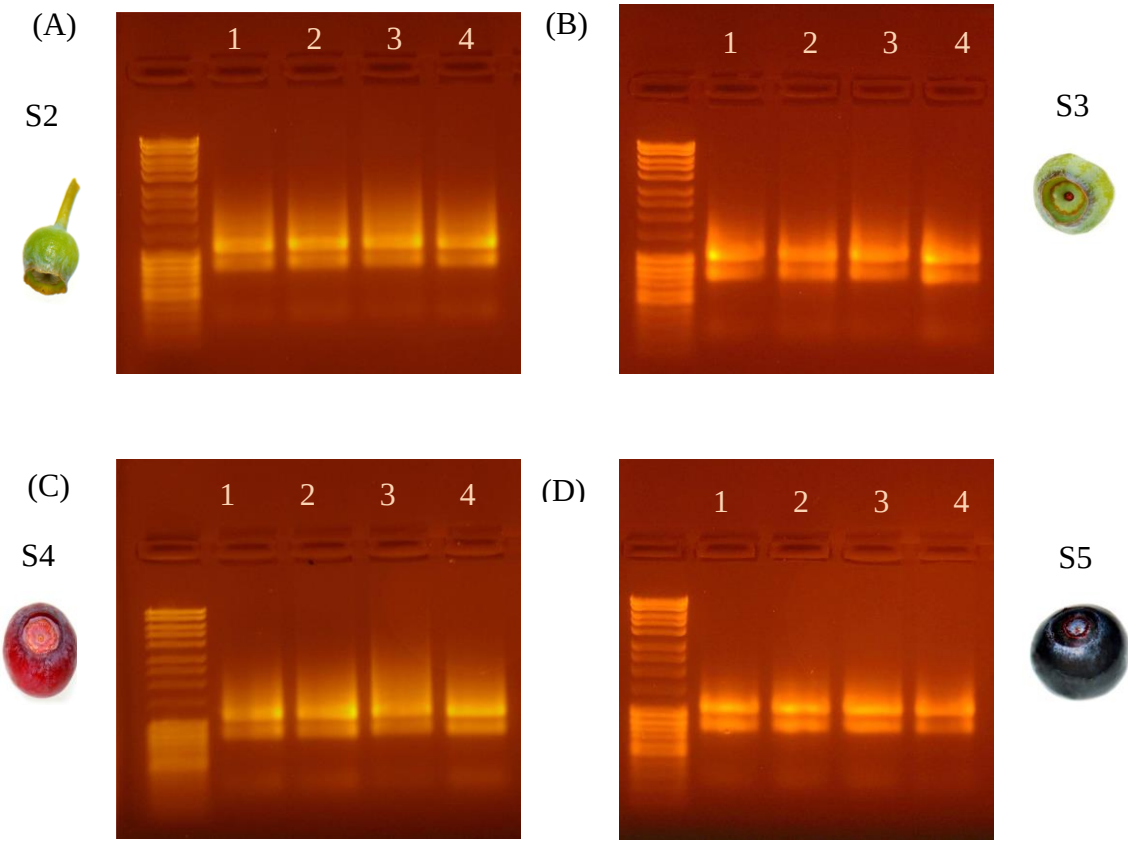

Figure 8 RNA samples visualized in Ethidium Bromide stained 1% Agarose gel electrophoresis. The first clear band indicates the 28S and the second clear band indicates the 18S RNA. A. RNA samples of small unripe green fruit(S2); B. RNA samples of large unripe green fruit(S3); C. RNA samples of ripening purple fruit(S4); D. RNA samples of fully ripe blue fruit(S5). In each figure lane 1 is molecular ladder (Appendix 2) and lane 2, 3, 4, and 5 is replicates of samples from each developmental and ripening stages.

3.6 Expression of Invertases during bilberry fruit development

The gene expression levels of the genes encoding invertases were determined by using qRT-PCR. A relative expression abundance among fruits of different fruit developmental and ripening stages; small green unripe fruit (S2), large unripe green fruit (S3), ripening purple fruit (S4) and fully ripe blue fruit (S5) was analysed. The identified transcripts for *VmCwINV4* and *VmVINV3* was less than 100 bp so primers couldn't be designed for them. Thus, in this study the expression analysis was done only for three isoforms of cell-wall invertases (*VmCwINV1*, *VmCwINV2* and *VmCwINV3*), two isoforms of vacuolar invertases (*VmVINV1* and *VmVINV2*) and five isoforms of neutral invertases (*VmNINV1*, *VmNINV2*, *VmNINV3*, *VmNINV4*, and *VINV5*).

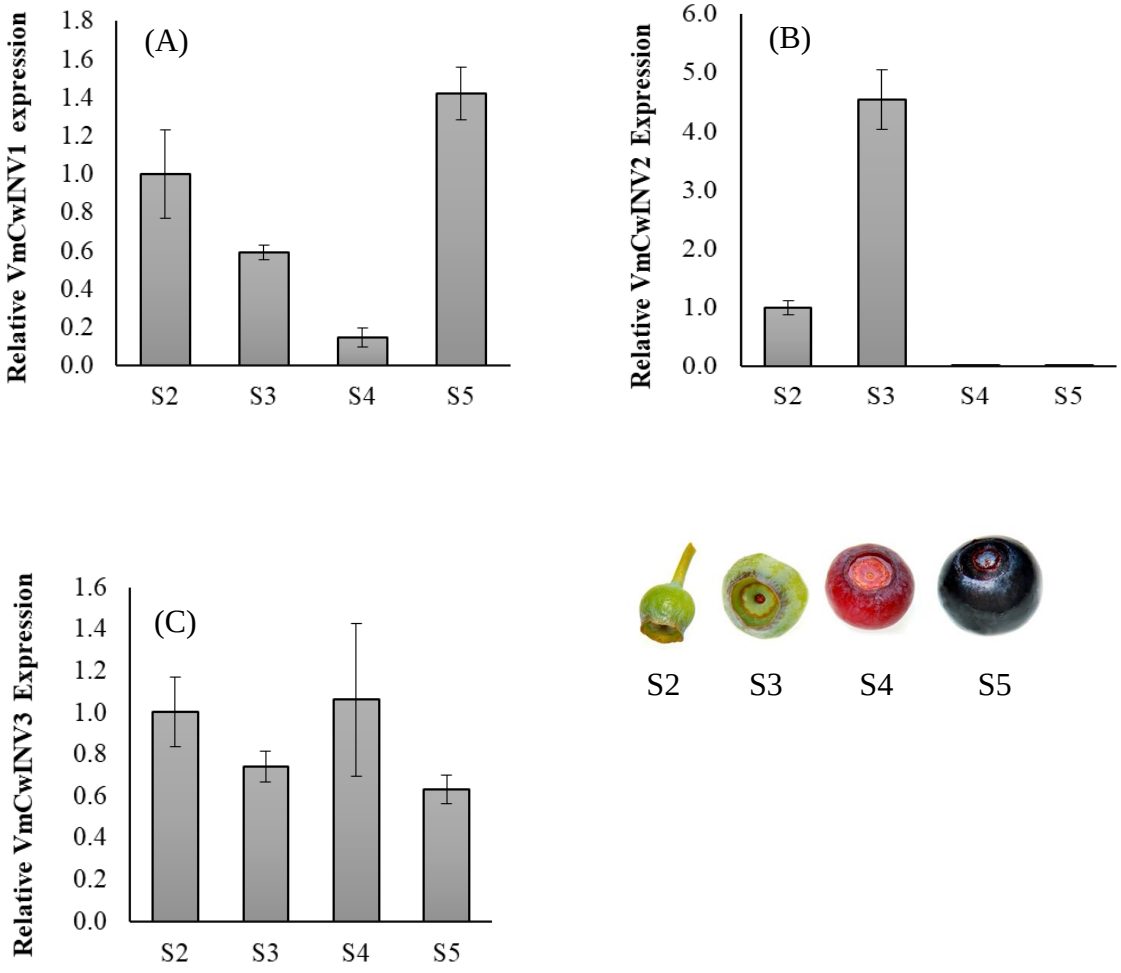

Figure 9 Relative expression for cell wall invertase genes during bilberry fruit ripening (A) VmCwINV1, (B) VmCwINV2, (C) VmCwINV3. The y-axis represents the relative expression levels of each gene quantified by qRT-PCR and analysed using the ΔΔCq method. The expression level of each gene was normalized to the level of both VmGAPDH and VmActin taking expression at S2 stage as control. The x-axis indicates different stages of fruit development and ripening S2, small unripe green fruit; S3, large unripe green fruit; S4, ripening purple fruit; S5, fully ripe blue fruit. Values are means of three replicates ± SEM.

The expression pattern of all three isoforms of cell wall invertases is different from one another (Figure 9). *VmCwINV1* have been expressed low in stage 3 and stage 4 but have slightly higher expression in the ripening stage S5, whereas *VmCwINV2* showed 4 times higher expression in developmental stage three (S3) than in small green unripe fruit (S2). In later stages of ripening (S4 and S5), there was no expression of the *VmCwINV2*. There was no significant variation in the expression of *VmCwINV3* among the different fruit development and ripening stages. The *VmCwINV3* showed slightly low expression in S3 than in S2, whereas it showed similar expression in stage 4 and again decreased expression in fully ripe fruit (Figure 9C).

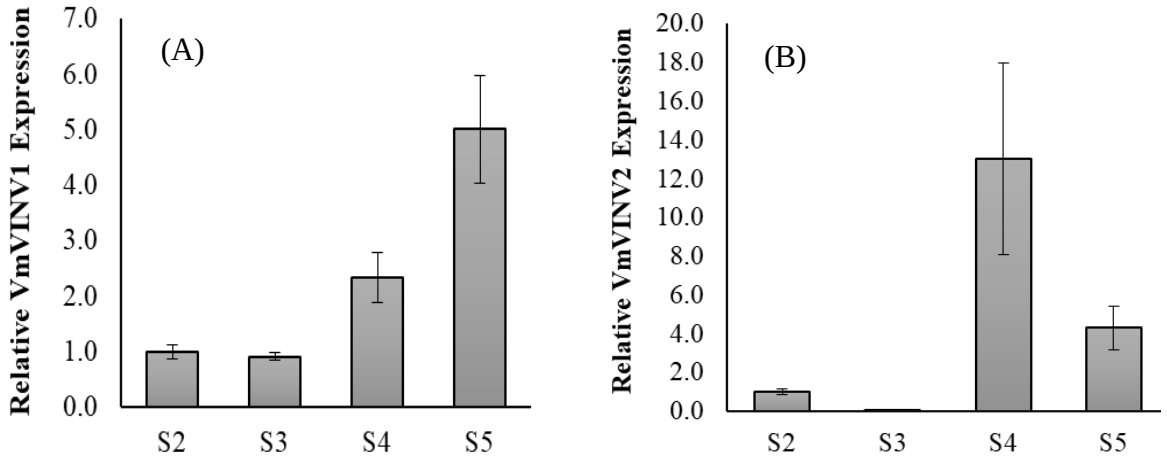

Figure 10 Relative expression for vacuolar invertase genes during bilberry fruit ripening (A) VmVINV1 and (B) VmVINV2 The y-axis represents the relative expression levels of each gene quantified by qRT-PCR and analysed using the ΔΔCq method. The expression level of each gene was normalized to the level of both VmGAPDH and VmActin taking expression at S2 stage as control. The x-axis indicates different stages of fruit development and ripening S2, small unripe green fruit; S3, large unripe green fruit; S4, ripening purple fruit; S5, fully ripe blue fruit. Values are means of three replicates ± SEM.

The expression of *VmVINV1* shows increased expression from S3 to S5 (Figure 10A). *VmVINV1* has the highest expression in fully ripe blue fruit (S5). *VmVINV2* is 13 times more expressed ripening purple fruit whereas the expression of *VmVINV2* decreased significantly in fully ripe blue fruit. The expression of *VmVINV2* is negligible in large green unripe fruit (Figure 10B). The expression pattern of three isoforms of neutral invertases (*VmNINV1, VmNINV2* and *VmNINV3*) are similar among small green unripe fruit and large green unripe fruit (Figure 11). All three genes are slightly down expressed in large green unripe fruit than in small green unripe fruit. *VmNINV1* has highest expression in ripening purple fruit(S4), *VmNINV2* has the highest expression in fully ripe blue fruit (S5). *VmNINV3* has low expression in ripening purple fruit (S4), expression of *VmNINV3* in other three development and ripening stages is similar. *VmNINV4* has a similar expression in large green unripe fruit (S3) and fully ripe blue fruit (S5) and *VmNINV4* showed higher expression in these two stages than the small unripe green fruit

(S2). *VmNINV5* has much lower transcript levels in ripening purple fruit (S4) and fully ripe blue fruit (S5).

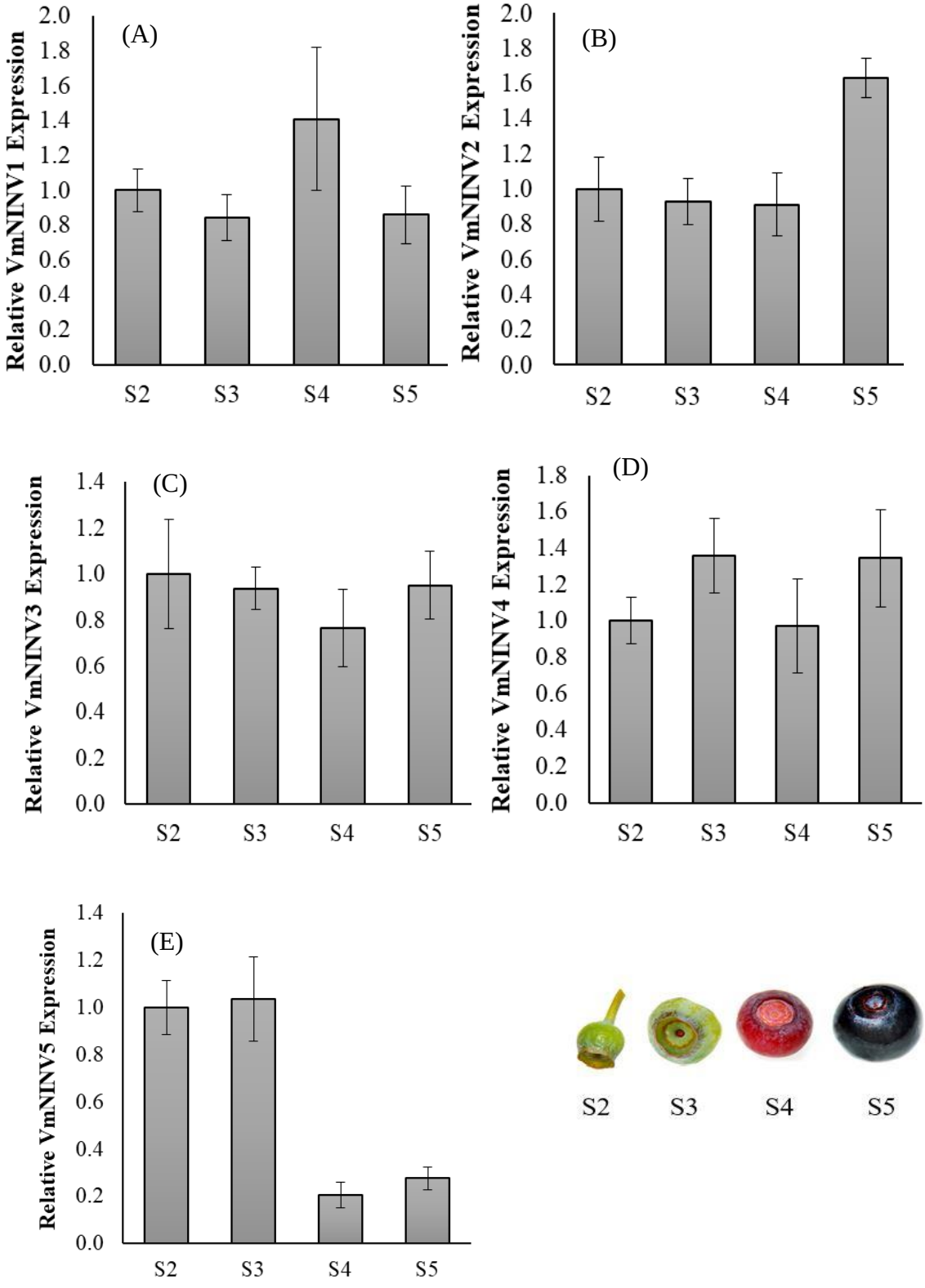

Figure 11 Relative expression for Neutral invertase genes during bilberry fruit ripening (A) VmNINV1, (B) VmNINV2, (C) VmNINV3, (D) VmNINV4 and (E) VmNINV5. The y-axis represents the relative expression levels of each gene quantified by qRT-PCR and analyzed using the ΔΔCq method. The expression level of each gene was normalized to the level of both VmGAPDH and VmActin taking expression at S2 stage as control. The x-axis indicates different stages of fruit development and ripening S2, small unripe green fruit; S3, large unripe green fruit; S4, ripening purple fruit; S5, fully ripe blue fruit. Values are means of three replicates ± SEM.

3.7 Concentrations of sugars during fruit development

The concentrations of fructose, glucose and sucrose were determined during fruit development for the better understanding of sugar metabolism. The analysis of sugar concentration was done using two methods. The total sugars concentration measured by phenol-sulfuric acid method in Table 4 showed highest sugar concentration in fully ripe berries. The sugar concentration decreased in S3 and increased in S4 and S5.

Table 4 Total sugar content (mean ± SE in mg g−1 dry weight) in different ripening stages of bilberry fruit analyzed using Phenol Sulfuric Method (Nielsen, 2010). Result represent the mean of four replicates.

Fruit Ripening Stages	Total Sugar Content
S2	135.63 ± 6.73
S3	87.56 ± 2.73
S4	214.55 ± 13.84
S5	333.36 ± 13.78

On the results (Table 5) from the assay kit, the fructose concentration is slightly higher than glucose concentrations and the sucrose concentration is relatively low than both fructose and sucrose. The fructose and glucose showed similar trend of change in concentration with respect to the development and ripening stages. In small unripe green fruit(S2) fructose and glucose concentration is 80 mg/g and 70 mg/g respectively, before decreasing nearly by two-fold at S3 stage, and then increasing by approximately 1.5 times at S4 stage. In final maturation stage (S5) the fructose concentration reached to 225 mg/g and glucose concentration reached to 161 mg/g. The sucrose concentration was about 4 mg/g in S2 which increased slightly in later stages of fruit development and ripening.

Table 5 Concentration of individual and total sugars (mean ± SE in mg g−1 in different ripening stages of bilberry fruit analyzed using Sucrose/D-Glucose/ D-Fructose assay kit. Result represent the mean of three replicates.

Fruit Ripening Stages	Glucose	Fructose	Sucrose	Total
S2	72.21 ± 2.24	80.28 ± 2.43	4.43 ± 0.46	156.92 ± 4.22
S3	36.29 ± 0.46	47.65 ± 1.79	7.55 ± 0.67	91.49 ± 2.80
S4	104.39 ± 2.86	128.56 ± 3.59	7.19 ± 1.39	240.14 ± 5.01
S5	161.17 ± 3.41	225.46 ± 0.90	8.34 ± 1.72	394.96 ± 5.96

4 Discussion

This study examined the sugar content and expression of invertases in four distinct bilberry development and ripening stages.

4.1 Sugar accumulation during bilberry fruit development

Sugars are one of the most important quality traits in fruits. Accumulation of sugars is closely related to the process of fruit development. Various soluble sugars are found in fruits such as glucose, fructose, sucrose, inositol, raffinose, galactose, arabinose and xylose (Yahia, 2019). The composition of these sugars varies among species, varieties, cultivar and even developmental stages.

There are few data available on sugar contents in bilberries. Previously, in most of the studies sugar content have been analysed only for the ripe berries (Viljakainen, Visti, & Laakso, 2002; Milivojević et al., 2012; Uleberg et al., 2012; Mikulic-Petkovsek et al., 2015), only one study is so far known for studying the sugar content in different developmental stages (Ayaz et al., 2001). The characterization of maturity as well as the methods of analysis between these studies are different, so direct comparison of the sugar content to those studies is difficult. However, all of these previous studies reported the dominance of fructose over other sugars in bilberry and reported fructose, glucose and sucrose as main sugars of bilberry fruit. So in this study, content of these sugars was analysed. The results of this study also revealed that the fructose is the predominant sugar in bilberry fruit followed by glucose and then sucrose (with relatively low amount than fructose and glucose) (Table 5). Similarly, Fructose dominance over other sugars was reported in some other vaccinium berries; *V. corymbosum* (X. Li et al., 2020), *V. angustifolium* (Gibson et al., 2013) and *V. arctostaphylos* (Ayaz et al., 2001). Some other fruits like apple, pear and strawberries were also reported to have fructose as dominant sugar (Li et al., 2012) but some fruits like peach and litchi were reported to have sucrose as dominant sugar (Yang et al., 2013; Desnoues et al., 2014; Aslam et al., 2019).

In this study, sugar content analysis was done using two methods. A preliminary assay to know about the total sugar content and a more detailed assay which give information about the individual sugars. The results from both assays (Table 4 and Table 5) revealed that there is rapid decrease in total sugar concentration on S3 (large green unripe fruit) stage of the fruit development. When looking at the concentration of individual sugars, fructose and glucose concentration also showed decreasing pattern in S3, but sucrose showed increase in the

concentration. As the S3 developmental stage is characterized by increased berry size, so this rapid decrease in accumulated sugar may indicate that the accumulated sugars were utilized for cell expansion, growth and other energy requirements. The total sugar content was the highest in ripe berries which implies that the ripe berries are sweetest. The maximum sugar concentration of 333-394 mg g^{-1} dry weight measured from ripe berries which is consistent with the previous study of sugar content in bilberry (Mikulic-Petkovsek et al., 2015). The total sugars concentration calculated using assay kit by R-biopharm have a little higher value of total sugar content (Table 5) but showed similar trend of sugar concentration measured by phenol-sulfuric acid method in Table 4.

4.2 Gene families of sugar metabolism pathway

Previous studies have reported that the enzymes involved in sugar metabolism pathway are coded by multiple gene isoforms and have identified 2-3 VINVs, 3-4 NINVs, 3-4 CwINVs, 3-6HKs, 3-4 FKs, 3-5 SuSys, 2-6 SPSs and 2 SPPs during their studies (Dai et al., 2011; Hu et al., 2016; M. Li et al., 2012). Similarly, in this study the key sugar metabolism genes enzymes (CwINV, VINV, NINV, HK, FK, SPS, SPP and SS) were found to be coded by multiple isoforms. Five isoforms of the enzymes hexokinase (HK) and fructokinase (FK) and neutral invertase (NINV), four isoforms of cell-wall invertase(CwINV), Sucrose Synthase(SS), and Sucrose Phosphate Synthase(SPS), and two isoforms of Vacuolar Invertase (VINV) and Sucrose Phosphate Phosphatase (SPP) were identified. These genes were first identified in *V. virgatum* and corresponding genes were identified in *V. myrtillus*. The only available *V. myrtillus* sequences were small transcripts fron SRA database. Bioinformatic analysis using the available bilberry sequence couldn't be done. Thus, for all the bioinformatic analysis done to check the precision of the identified genes, *V. virgatum* gene sequences were used assuming that the *V. virgatum* genes represents the characteristics of *V. myrtillus* genes. The 95-100 % percentage identity (Table 1) between *V. virgatum* and *V. myrtillus* gene sequences supports this assumption.

All the CWINVs identified in this study were predicted to either be localized extracellularly or in endoplasmic reticulum (ER). Enzymes like cell wall invertases is transported to apoplast through ER, golgi apparatus and the subsequent endomembrane system (Chung et.al, 2017). Thus, the predicted localization of CWINVs in ER is justified. The vacuolar invertases were predicted to be localized in vacuole, this implies that the identified gene are the correct form of invertase isozymes. Neutral invertases have been reported to be present in different subcellular

compartments like chloroplasts, mitochondria and nuclei (Vargas & Salerno, 2010). The result of this study also predicted neutral invertase to be localized different subcellular compartments (Table 2).

Pairwise comparison, phylogeny analysis and multiple sequence alignment was done to check whether the identified gene belongs to correct invertase isoenzyme or not. The results from pairwise comparison (Appendixes 3) indicated that the identified invertases shares 58 -90% sequence similarity with respective invertases from apple, grape, peach and strawberry. The results from the phylogenetic analysis (Figure 4) clearly indicated that the identified CwINVs, VINVs and NINVs of *V. virgatum* were grouped in the same branch respectively with CwINVs, VINVs and NINVs from apple, grape, peach and strawberry. Considering these results, it can be claimed that the identified invertase genes belongs to the correct invertase isoenzymes.

Some previous studies reported that cell wall invertase and vacuolar invertase can be distinguished based on P/V substitution in one of the conserved motifs (WEC(P/V)DF) of these genes (Chen, Gao, Su, Rao, & An, 2015; Cho et al., 2005; Goetz & Roitsch, 1999). In this study also, results from the multiple sequence alignment (Figure 5 and Figure 6) of CwINVs and VINVs of *V. virgatum* revealed this difference between CwINVs and VINVs. This finding further supports the claim of this study.The absence and presence of NDPNG motifs has been reported as one of the distinctions between soluble acid invertases (CwINVs and VINVs) and neutral invertases (Bosch, Grof, & Botha, 2004). The multiple sequence alignment results for NINVs revealed that all the identified NINV genes lacks the characteristic pentapeptide NDPNG sequence of acid invertases(CwINVs and VINVs). Based on that it can be concluded that all the NINVs genes identified here are of the NINV type.

4.3 Differential expression pattern of invertases during fruit development

The developmental stage specific expression could provide a basis for understanding the functions of invertases in bilberry fruit development. The expression pattern of all three invertases during different developmental stages were analysed.

The three isoforms of CwINVs showed differential expression patterns which suggests that each isoform of CwINVs has distinctive role in sugar metabolism and accumulation during fruit development and ripening. Among three CwINVs, *VmCwINV2* showed higher expression in unripe stage (S2 and S3) and negligible expression in ripening(S4) and fully ripe(S5) stage

which suggests that *VmCWINV2* may be mainly responsible for the apoplastic cleavage of sucrose at the early stage of the fruit development. The other two isoform *VmCwINV1* and *VmCwINV3* showed expression S4 and S5 stage, which suggests that these two isoforms may be responsible for sugar accumulation to ripe berries. Expression analysis of CwINVs done in apple has reported high expression of CwINV in young fruit compared to mature fruit (Li, 2012).

VINVs are known to have major role in cell expansion and sugar accumulation (Wan et al., 2018). Thus, it can be assumed that the expression of VINVs be upregulated in mature fruits. In line with the assumption, the two isoforms of VINVs, *VmVINV1* and *VmVINV2* showed increased expression in fully ripe (S5) stage and ripening stage (S4). This explains the increased concentration of fructose and glucose in ripening stage (S4) and fully ripe stage (S5). The high expression VINVs in mature fruit could be specific to hexose dominant fruit. In line with the finding of this study, upregulated acid invertase expression in mature litchi arils from hexose dominant cultivars, pear and blueberry fruit has been reported (Yang et al., 2013; Kou et al., 2018; X.Li et al., 2020) but these study has not specified the type of acid invertase. Whereas, the study of Hu et al., (2016) in jackfruit and Li et al., (2012) in apple fruit reported high expression of acid invertase (VINV) in young fruit compared tomature fruit.

The study of NINVs has been limited than other two invertase isoenzymes because of it's low enzyme activity and instability but their multiple localization makes them excellent candidates for the coordination of metabolic processes that take place in the different compartments (Vargas & Salerno, 2010; Wan et al., 2018). All the isoform of NINVs showed significant expression in all the developmental stages except *VmNINV5*. Similar expression pattern of NINVs has been reported in melon fruit (Dai et al., 2011). The *VmNINV5* is expressed highly in S2 and S3 stage than later stages of ripening which suggests that *VmNINV5* may not have role in symplasmic cleavage of the sucrose during ripening and fully ripe stages.

5 Conclusions

This thesis is the first work regarding sugar metabolism during bilberry fruit development. The results of this thesis demonstrate that the fructose is dominant sugar in bilberry and sugar content is higher in fully ripe berries. The high expression of *VmCwINVs* and *VmNINVs* in unripe green fruits and high expression of *VmVINVs* in ripe fruits suggests that the accumulation of fructose and glucose in early stage of fruit development is regulated mainly by *VmCwINVs* and *VmNINVs* and in later stages it is regulated mainly by *VmVINVs*.

It is beyond the scope of this study to analyse the enzymatic activity during fruit development, so this limits this study to draw the strong conclusions regarding the primary function and role of invertases in bilberry fruit development but it deepens the understandings of sugar metabolism as well as provides a basis for the further research in sugar metabolism during bilberry fruit development. The activity of other enzymes as well as the activity of inhibitors and sugar transporters regulates the overall sugar metabolism pathway. Thus, a detailed study of the activity of other enzymes, inhibitors and sugar transporters as well as expression profiling of genes encoding other enzymes, inhibitors and sugar transporters is recommended for the better understanding of the sugar metabolism during biberry fruit development.

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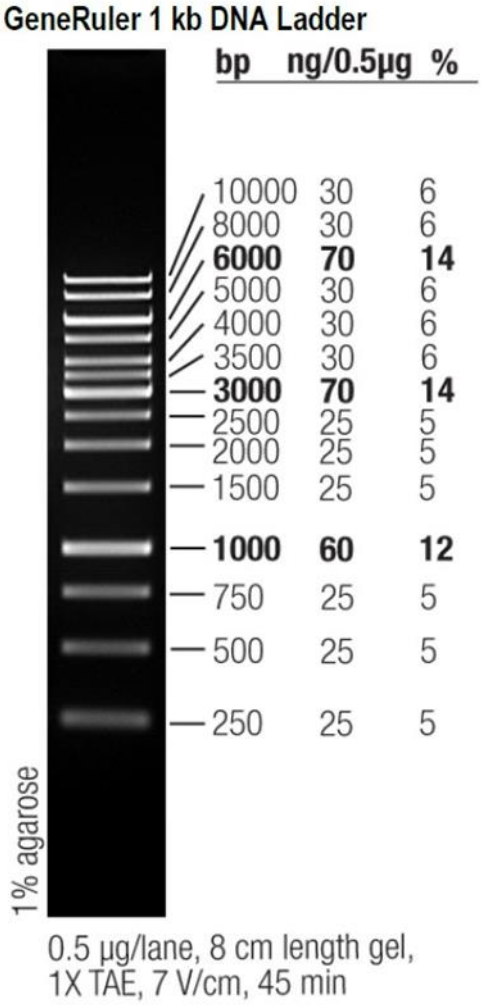
Appendixes

Appendix 1 : **Primers used in qRT-PCR**

Gene Name	Short Form	Primers
Cell wall Invertase1	VmCWINV1	5' GTGGACCGAAGCCAAACAACCT 3' 3' GCCCAGTCTTCGATGTCTCGAT 5'
Cell wall Invertase2	VmCWINV2	5' CTCTGATGCAACCAGTTCCTCT 3' 3' CTCCTGAGGGAAAGCTTCTTGT 5'
Cell wall Invertase3	VmCWINV3	5' GGCTTCAGTCGTTTCCAAGMC 3' 3' CCACCCTTGAGCTCTGTATCAT 5'
Vacuolar Invertase1	VmVINV1	5'TGATGACCTCTTGAAGGGTTGG 3' 3' AGCAGGGAATTCAGTACCGTTC 5'
Vacuolar Invertase2	VmVINV2	5' TTGCTCAAGGAGGGAGAACAGT 3' 3' TATAAGCCGAGTCCATCGACCA 5'
Neutral Invetase 1	VmNINV1	5' GGCCTGCATTAAGATGAAGAGG 3' 3' TTCCCTACAAATCTCCCACTGC 5'
Neutral Invertase 2	VmNINV2	5' GAAGTCCTAGACCCAGATTTCG 3' 3' CCAGTTACCTTCCCATAAGCAC 5'
Neutral Invertase 3	VmNINV3	5' GAAGGCGAGGAGTGGAGAAITA 3' 3' TGCGTCTCTCGGCTATCTTAAC 5'
Neutral Invetase 4	VmNINV4	5' CCGTTAGACAGTTGGCCTGAAT 3' 3' TCCTCCCAGTACAGCAAAGAAG
Neutral Invetase 5	VmNINV5	5' GAGTGGTTAAGCGTCTTCATGC 3' 3' TCCATTCAGGGAGAGAATCAGG 5'

Appendix 2 : **Gel Electrophoresis Mass Ruler**



Appendix 3 : **Pair wise comparison matrixes of invertases**

	CwINV3	CwINV2	CwINV1	CwINV4	VvCwINV (AAT09980.1)	FvCwINV (XP004297549.1)	PpCwINV (AFI57904.1)	MdCwINV (NP001280910.1)
CwINV3	100	50.36	55.86	58.35	58.51	58.36	56.55	58.36
CwINV2	50.36	100	59.44	58.87	58.51	57.6	59.54	59.19
CwINV1	55.86	59.44	100	66.78	67.49	67.61	69.72	70.07
CwINV4	58.35	58.87	66.78	100	70.47	67.96	70.53	71.05
VvCwINV (AAT09980.1)	58.51	58.51	67.49	70.47	100	68.18	72.13	72.47
FvCwINV (XP004297549.1)	58.36	57.6	67.61	67.96	68.18	100	79.83	79.83
PpCwINV (AFI57904.1)	56.55	59.54	69.72	70.53	72.13	79.83	100	88.04
MdCwINV (NP001280910.1)	58.36	59.19	70.07	71.05	72.47	79.83	88.04	100

	VINV3	MdVINV (AFU56882.1)	FvVINV (XP004307805.1)	VvVINV (XP002265534.1)	VINV1	VINV2
VINV3	100	58.37	59.42	58.46	58.33	58.03
MdVINV (AFU56882.1)	58.37	100	77.81	67.15	64.2	62.99
FvVINV (XP004307805.1)	59.42	77.81	100	65.28	63.07	62.82
VvVINV (XP002265534.1)	58.46	67.15	65.28	100	67.24	67.87
VINV1	58.33	64.2	63.07	67.24	100	71.17
VINV2	58.03	62.99	62.82	67.87	71.17	100

	NINV5	NINV3	NINV2	NINV4	FvNINV (XP004295043.1)	MdNINV (AFU56879.1)	PpNINV (XP020409902.1)	NINV1	VvNINV (NP001267976.1)
NINV5	100	50.55	52.37	52.1	52.93	53.53	52.9	51.64	52.65
NINV3	50.55	100	59.61	58.8	60.29	60.22	60.41	59.08	60.25
NINV2	52.37	59.61	100	78.48	68.98	70.29	71.58	71.04	73.62
NINV4	52.1	58.8	78.48	100	70.48	71.76	72.59	70.36	73.09
FvNINV (XP004295043.1)	52.93	60.29	68.98	70.48	100	84.95	85.8	75.72	78.05
MdNINV (AFU56879.1)	53.53	60.22	70.29	71.76	84.95	100	88.68	74.96	77.26
PpNINV (XP020409902.1)	52.9	60.41	71.58	72.59	85.8	88.68	100	76.84	78.7
NINV1	51.64	59.08	71.04	70.36	75.72	74.96	76.84	100	79.1
VvNINV (NP001267976.1)	52.65	60.25	73.62	73.09	78.05	77.26	78.7	79.1	100

Appendix 4 : **Pipetting scheme for determination of fructose, glucose and sucrose**

Pipette into cuvettes	Blank sucrose sample	Sucrose sample	Blank D-glucose/ D-fructose sample	D-Glucose/ D-fructose sample
solution 1* sample solution**	0.200 ml -	0.200 ml 0.100 ml	- -	- 0.100 ml
Mix*, incubate for 15 min at 20-25°C or for 5 min at 37°C (before pipetting, warm up solution 1 to 37°C). Addition of:				
solution 2 redist. water	1.000 ml 1.800 ml	1.000 ml 1.700 ml	1.000 ml 2.000 ml	1.000 ml 1.900 ml
Mix***, read absorbances of the solutions after approx. 3 min (A_1). Start reaction by addition of:				
suspension 3	0.020 ml	0.020 ml	0.020 ml	0.020 ml
Mix***, wait for completion of the reaction (approx. 10-15 min) and read absorbances of the solutions (A_2). If the reaction has not stopped after 15 min, continue to read the absorbances at 2 min intervals until the absorbance increases constantly over 2 min. Addition of:				
suspension 4	-	-	0.020 ml	0.020 ml
Mix***, read absorbances of the solutions after 10-15 min (A_3).				

Determine the absorbance differences (A_2-A_1) for both, blanks and samples. Subtract the absorbance difference of the blank from the absorbance difference of the corresponding sample.

$$\Delta A = (A_2\text{-}A_1)_{sample}\text{-}(A_2\text{-}A_1)_{blank}$$

The difference between $\Delta A_{total\ D\text{-}glucose}$ (from the sucrose sample) and $\Delta A_{D\text{-}glucose}$ (from the D-glucose sample) yields $\Delta A_{sucrose}$.

It follows for the determination of D-fructose:

Determine the absorbance differences (A_3-A_2) for both, blank and sample (D-glucose/D-fructose sample). Subtract the absorbance difference of the blank from the absorbance difference of the sample. This results in $\Delta A_{D\text{-}fructose}$.