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ORIGINAL PAPER

RNA-seq analysis of *Rubus idaeus* cv. Nova: transcriptome sequencing and de novo assembly for subsequent functional genomics approaches

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Abstract

Key message **Using Illumina sequencing technology, we have generated the large-scale transcriptome sequencing data containing abundant information on genes involved in the metabolic pathways in *R. idaeus* cv. Nova fruits.**

Abstract *Rubus idaeus* (Red raspberry) is one of the important economical crops that possess numerous nutrients, micronutrients and phytochemicals with essential health benefits to human. The molecular mechanism underlying the ripening process and phytochemical biosynthesis in red raspberry is attributed to the changes in gene expression, but very limited transcriptomic and genomic information in public databases is available. To address this issue, we generated more than 51 million sequencing reads from *R. idaeus* cv. Nova fruit using Illumina RNA-Seq technology. After de novo assembly, we obtained 42,604 unigenes with an average length of 812 bp. At the protein level, Nova fruit transcriptome showed 77 and 68 % sequence similarities with *Rubus coreanus* and *Fragaria versa*, respectively, indicating the evolutionary relationship between them. In addition, 69 % of assembled unigenes were annotated using public databases including NCBI non-redundant, Cluster of Orthologous Groups and Gene ontology database, suggesting that our transcriptome dataset provides a valuable resource for investigating metabolic processes in red raspberry. To analyze the relationship between several novel transcripts and the amounts of metabolites such as γ-aminobutyric acid and anthocyanins, real-time PCR and target metabolite analysis were performed on two different ripening stages of Nova. This is the first attempt using Illumina sequencing platform for RNA sequencing and de novo assembly of Nova fruit without reference genome. Our data provide the most comprehensive transcriptome resource available for *Rubus* fruits, and will be useful for understanding the ripening process and for breeding *R. idaeus* cultivars with improved fruit quality.

Keywords Transcriptome · De novo assembly · GABA · Anthocyanin · *Rubus idaeus* cv. Nova

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Introduction

Of the widely cultivated fruit crops, raspberry is one of the most diverse genera comprising 12 subgenera. However, most of the berries cultivated in the world derive from just two of them, red raspberry (*Rubus idaeus*) and black raspberry (*R. occidentalis*) (Deighton et al. 2000; Yousefi et al. 2013). According to Food Agricultural Organization of United Nations (FAO), the total production of

raspberries in the world was 462,389 tons in 2010 (Yousefi et al. 2013), indicating the economic importance of raspberries. Recently, there have been numerous studies on the development and maturation of raspberries because of the uniqueness of this process in fruit biology and a desire to understand the physiological, biochemical, and molecular characteristics that determine fruit quality. A number of studies have revealed that the fruits of raspberry species have essential positive effects on human diet and health, which could be mainly due to the medicinally active phytochemicals such as polyphenols including various flavonoids (such as anthocyanins and flavonols), condensed and hydrolysable tannins and phenolic acid derivatives (Kahkonen et al. 2001). The major polyphenols in raspberries are anthocyanins and ellagitannins (Dobson et al. 2012; McDougall et al. 2008; Mullen et al. 2002; Ross et al. 2007), which make up almost >90 % of the total phenolic contents. Since anthocyanins are responsible for the deep red coloration, raspberry breeders consider anthocyanins as important targets to improve and maintain consumer quality perception (Dobson et al. 2012). Likewise, ellagitannins imparting astringency and flavor of raspberries have also been taken into account in breeding efforts (Dobson et al. 2012). In addition, raspberry fruits contain various amino acids including serine, asparagine, glutamic acid, valine and γ-aminobutyric acid (GABA) (Dincheva et al. 2013). Among the amino acid found in raspberry, GABA serves as a major inhibitory neurotransmitter, and dietary intake of GABA as food component leads to immunity enhancement under stress conditions, a hypotensive effect and relaxation. Therefore, GABA has been suggested as a valuable food component (Hyun et al. 2013a).

The global changes in the biosynthetic pathways of these phytochemicals reflect the transcriptional modulation of many genes, but very little is known about these transcriptional changes and their regulation in red raspberry. The analysis of the genetic linkage map and quantitative traits loci from a segregating red raspberry population after a cross between two strongly differentiated parents has provided a first picture of transcriptome dynamics during anthocyanin biosynthesis by mapping two potential candidate genes, a structural gene, PKS1 and a transcription factor, bHLH (Kassim et al. 2009). However, these approaches have suffered from a number of drawbacks and the data are quite limited. Recent findings suggest that the primary and secondary metabolites of berries can act as functional foods for the consumers in food industry and medicine, and so the manipulation of fruit metabolites linked to fruit quality parameters has become a legitimate objective of crop improvement. It clearly indicates a need to provide new strategies analyzing the functional complexity of transcriptomes. Next-generation sequencing (NGS) has already revolutionized the way we study genomes, and the quantity and quality of sequencing data derived from NGS continue to improve at a rapid pace. Most importantly, RNA-seq has also become a standard analysis method in model organisms where the phenotypes are linked to the changes in gene expression and splicing events derived from both forward and reverse genetics (Aanes et al. 2011; Hyun et al. 2012; Rosel et al. 2011; Vesterlund et al. 2011). Recently, a genome-wide expression study involving comparative RNA-seq has thrown light on genes involved in the possible mechanisms of resistance to Phytophthora root rot (caused by *Phytophthora rubi*) in red raspberry 'Latham' (Ward and Weber 2012), indicating the value of the transcriptome analysis using de novo assembly in non-model organisms.

In this study, using Illumina paired-end sequencing technology, we have generated 51,186,030 sequence reads that were assembled into 42,604 unigenes to characterize the fruit transcriptome of *R. idaeus* cv. Nova. Based on BLASTX against public database, we found that 68.86 % of all unigenes are matched to known genes in different plants. In addition, 51 unigenes encoding 38 proteins were identified as genes involved in γ-aminobutyric acid (GABA) shunt or anthocyanin biosynthesis. To further validate these findings, the expression pattern of each gene was compared with the variation of GABA and anthocyanins in different ripening stages of Nova fruits. This trasncriptome dataset might serve as an important public information platform to identify the genes that can be used for in-depth transcriptome-wide species comparisons.

Materials and methods

Sample collection and RNA isolation

Nova (*R. idaeus* cv. Nova) fruits were collected from Gochang Black Raspberry Research Institute, Republic of Korea. The fruit sampling involved two different ripening stages of Nova, the fruits turning red (20 days post-anthesis, stage 1) and the red fruits attached to the crown (25 days post-anthesis, stage 2). After harvesting, samples were immediately frozen in liquid nitrogen until further processing.

Total RNA from a pool of stage 1 fruits (~20 fruits) was isolated using RNeasy Plant Mini kit (Qiagen, USA) according to the manufacturer's instructions. After treatment of RNase-free DNase I (Promega, USA), total RNA content, purity and degradation were assessed by Nanodrop 2000C spectrophotometer (Thermo scientific, USA) before proceeding to the next step.

Illumina sequencing and sequence assembly

The poly-A mRNA was purified from total RNA using the oligo(dT) magnetic beads, and digested into short fragments (about 200 bp) using a fragmentation buffer. Taking these short fragments as templates, the first-strand cDNA was synthesized using random hexamer primers, and the second-strand cDNA was further synthesized using DNA polymerase I and RNase H. Then, these cDNA fragments were purified with a QiaQuick PCR extraction kit (Qiagen, USA), and ligated to sequencing adaptors. The products from this ligation reaction were purified by agarose gel electrophoresis, and enriched by PCR to create the final cDNA library. The cDNA library was sequenced using Illumina HiSeq^{TM} 2000. The Illumina reads generated in this study are available at the website (http://kimjy.gnu.ac.kr/DB_Nova.files/slide0003.htm) and National Agricultural Biotechnology Information Center (NABIC, http://nabic.rda.go.kr) with the accession number NN-0869-0000001. Quality control of raw reads was done by removing adapter sequences, empty reads, low-quality reads and the reads with more than 10 % $Q < 20$ bases through the standard Illumina pipeline. Transcriptome de novo assembly was performed with short reads (90 nt) assembling program, Trinity (assembly parameter, k-mer value = 25, CPU = 25) (Grabherr et al. 2011). BLASTX alignment (E value $<10^{-5}$) between unigenes and protein databases including NCBI non-redundant protein (Nr) database (http://www.ncbi.nlm.nih.gov/), Swiss-Prot protein database (http://www.expasy.ch/sprot), Cluster of Orthologous Groups (COG) database (http://www.ncbi.nlm.nih.gov/COG), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg) was performed, and the best aligning results were used to decide the sequence direction of unigenes. Blast2GO was used to obtain Gene ontology (GO) annotation of unigenes based on BLASTX hit against NR database with a cut-off E value of 10^{-5}.

Construction of phylogenomic tree and analysis of sequence similarity

To construct a phylogenomic tree with Nova transcriptome, we used 25 plant genomes and transcriptome, *Cucumis sativus*, *Medicago truncatula*, *Lotus japonicus*, *Glycine max*, *Carica papaya*, *Vitis vinifera*, *Populus trichocarpa*, *Ricinus communis*, *Manihot esculenta*, *Prunus persica*, *Malus domestica*, *Fragaria vesca*, *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Mimulus guttatus*, *Aquilegia coerulea*, *Brachypodium distachyon*, *Orayza sativa japonica*, *Orayza sativa indica*, *Zea mays*, *Sorghum bicolor*, *Setaria italic*, *Selaginella moellendorffii Physcomitrella patens*, and *Rubus coreanus* (Korean black raspberry; NCBI accession number, SRX347804; Hyun et al. 2014). Based on 979,357 proteins including Nova transcriptome, we clustered them using Tribe-MCL (inflation values 2.0) resulting in 1,755 clusters. Phylogenomic tree was drawn using Neighbor-joining phylogenetic tree with 1,000 bootstrap repeats using MEGA5 (Tamura et al. 2011) as described by (Hyun et al. 2012).

For analyzing sequence similarity, we downloaded the proteome and transcriptome data sets for *F. vesca*, *R. coreanus*, *V. vinifera*, *P. trichocarpa*, *M. domestica*, *G. max*, *C. sativus*, *A. thaliana*, *S. bicolor*, *O. sativa japonica*, *Z. mays*, from their respective genome and transcriptome project websites. The Nova transcripts were searched against proteome sequences using BLASTX program.

Quantitative real-time PCR

Total RNA from three independent pools of each stage (5 fruits of the same maturation degree) was isolated using RNeasy Plant Mini kit (Qiagen, USA) according to the manufacturer's instructions. Then, total RNA was reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen, USA). Gene-specific primers were designed using GenScript Real-time PCR Primer Design tool (https://www.genscript.com/ssl-bin/app/primer). Primer sequences are listed in Supplementary Table 1. Real-time PCR was carried out in triplicate using the ECO^{TM} Real-time PCR system (Illumina, USA) with the SsoFast^{TM} EvaGreen supermix (Bio-Rad, USA) according to the conditions described previously (Hyun et al. 2013b). The expression levels of different genes were normalized to the constitutive expression level of histone H3 (AF304365).

Determination of GABA, glutamate and anthocyanins

Nova fruits were freeze-dried over 5 days and stored at below −70 °C before extraction. Freeze-dried samples were homogenized by mortar and pestle. Each powdery sample (20 mg) was extracted with 1 mL of 80 % methanol using Mixer mill (Retsch MM400, Germany) at 30 HZ s^{-1} for 20 min, and centrifuged at 4 °C and 13,000g for 5 min. The supernatant ($\sim$100 μL) was completely dried using a speed vacuum concentrator (Biotron, Seoul, Korea). Analysis of GABA and glutamate in different stage fruits was determined by GC–TOF–MS analysis. For the GC–TOF–MS analysis, two derivatization steps were performed. First, oximation was carried out by dissolving the dried extracts in 50 μL of methoxyamine hydrochloride (20 mg mL^{-1} in pyridine) and it was allowed to react at 30 °C for 90 min. Then, the samples were silylated with 50 μL of MSTFA at 37 °C for 30 min. Anthocyanin analysis of nova fruits was performed using UPLC–Q-TOF–MS. Supernatant of dried

Table 1 Overview of the sequencing and assembly

	R. idaeus cv. Nova
Total number of reads	51,186,030
Total nucleotides (nt)	4,606,742,700
GC percentage	47.76
Q20 percentage	96.20
Step-wise assembly	
Contig	
Total number of contig	44,339
Length of all contig (nt)	36,172,264
Average sequence size of contig (nt)	816
Contig N50 (nt)	1,323
Unigene	
Total number of unigenes	42,604
Length of all unigenes (nt)	34,589,862
Average sequence size of unigenes (nt)	812
Unigenes N50 (nt)	1,320

extracts was resolved with 80 % methanol and filtered through a 0.2 μm PTFE filter.

GC–TOF–MS and UPLC–Q-TOF–MS analyses

An Agilent 6890N GC system (Palo Alto, CA) equipped with an Agilent 7683 autosampler was coupled to a time-of-flight Pegasus III mass spectrometer (Leco, St. Joseph, MI, USA), operating in electron ionization (EI) mode (70 eV). A DB-5MS column (30 m length × 0.25 mm i.d. × 0.25 m film thickness, J & W Scientific, Folsom, CA, USA) was used with helium at a constant flow of 1.5 mL min^{-1}. 1 μL of the derivatized sample was injected with a split ratio 5:1 mode. The oven temperature was maintained at 75 °C for 2 min, increased to 300 °C at 15 °C min^{-1}, and then held at 300 °C for 3 min. The acquisition rate was set to 20 scans s^{-1} with a mass scan range of $m/z = 45$–1,000. The injector and ion-source temperatures were 250 and 230 °C, respectively.

UPLC was performed on a Waters ACQUITY UPLC system (Waters Corp., Milford, MA, USA) equipped with a binary solvent delivery system, an autosampler, and a UV detector. Chromatographic separation was performed on a Waters Acquity high performance liquid chromatography (HPLC) BEH C_{18} column (100 × 2.1 mm i.d. × 1.7 μm particle size). The elution was performed with an acetonitrile/water gradient containing 0.1 % formic acid. The gradient was linearly increased from 5 to 100 % acetonitrile in 10 min, and then decreased to 5 % over 2 min. Total run time, including re-equilibration of the column to the initial conditions, was 12 min. The injection volume was 5 μL, and the flow rate was 0.3 mL min^{-1}. The Waters Q-TOF Premier (Micromass MS Technologies, Manchester, UK) was operated in a wide pass quadrupole mode for the MS experiments, and the TOF data were collected between m/z 100 and 1,000 in negative (−) and positive (+) ion modes. The desolvation gas (nitrogen) was set to 650 L h^{-1} at a temperature of 300 °C; the cone gas (nitrogen) was set to 50 L h^{-1}, and the source temperature was 80 °C. The capillary and cone voltages were set to 2.3 kV and 30 V, respectively. Data were collected in the centroid mode, with a scan accumulation time of 0.2 s. All analyses were acquired using an independent reference spray via the LockSpray interference to ensure accuracy and reproducibility; leucine enkephalin ions were used as the lock mass (m/z 554.2615 (−) and 556.2771 (+)) at a flow rate of 10 μL min^{-1}. The accurate masses and compositions for the precursor ions and for the product ions were calculated using the MassLynx software (Waters Corp.) incorporated in the instrument. The MassLynx software has a feature that calculates all possible elemental compositions from the accurate mass. The MS^n analysis was performed using 212-LC Binary solvent delivery system equipped with a Prostar 410 Autosampler, and a Prostar 335 photodiode array detector (PDA), which was coupled to the Varian 500-MS ion-trap mass spectrometer equipped with an electrospray interface (Varian Tech., Palo Alto, CA, USA). 10 μL of each samples was injected into PurSuit XRs C_{18} column (100 mm × 2.0 mm i.d., 3 μm; Varian, Lake Forest, CA, USA) with a MetaGuard 2.0 PurSuit XRs C_{18} guard column (Varian, Lake Forest, CA, USA). The column temperature was set at 40 °C and flow rate was 0.2 mL min^{-1}. Mobile phases and mass ranges were carried out under the same Q-TOF conditions. The gradient applied was as follows: from 0 to 2 min 10 % B, from 2 to 10 min to 40 % B, from 10 to 20 min 70 % B, from 20 to 25 min 90 % B, from 25 to 30 min 90 % B, from 30.6 min 10 % B, and held for 40 min 10 % B before the next sample injection. The parameters were as follows: drying temperature, 300 °C; capillary voltage, 70 V; needle voltage, 5 kV; drying gas pressure (nitrogen), 20 psi; and nebulizer gas pressure (air), 40 psi. MS^n analysis was performed using Turbo data-dependent scanning (DDS) under the same conditions used for full scanning. The PDA was set the absorbance from 200 to 600 nm and managed by Polyview 2000 (version 6.9) (Varian, Walnut Creek, CA).

Identification and quantification of GABA, glutamate, and anthocyanins

γ-Aminobutyric acid (GABA), glutamate, and anthocyanins were mainly identified using standard compounds by comparing both the mass spectra and retention time. In UPLC–Q-TOF–MS analysis, the accurate masses and elemental compositions were calculated using the MassLynx software

(Waters Corp.). MS^n fragmentation patterns and UV spectrum were also supported to identify anthocyanins using MS workstation software (version 6.9, Varian, USA) in LC–IT–MS/MS. When standard compounds were not available, a tentative identification was performed based on the MS spectra using references, and an in-house library (Bradish et al. 2012). GABA, glutamate and cyanidin-3-rutinoside were quantified by external standards. Cyanidin-3-2^G-glucosylrutinoside and pelargonidin-3-rutinoside were relatively quantified by their peak area. Nine biological and three analytical replicates from each different samples were used for both GC–TOF–MS and UPLC–Q-TOF–MS analyses.

Results

De novo assembly and phylogenomic analysis of Nova fruit transcriptome

Based on pair-end reads from the Illumina platform, we generated 51,186,030 reads from the total RNA of Nova fruit (stage 1). These reads contained a total of 4,606,742,700 nucleotides and 96 % G20 bases (base quality more than 20). The raw reads were assembled into 44,339 contigs by Trinity program (Grabherr et al. 2011) with an average length of 816 bp and half of the total assembly length (N50) in contigs >1.3 kb (Table 1). The sequences were determined to represent 42,604 (34,589,862 nt) unigenes with an average length of 812 bp. In addition, we were able to obtain 24,323 unigenes (57 % of unigenes) which are longer than 500 bp in length (Supplementary Fig. 1). Large-scale transcriptome data have already been suggested as a potential resource for multigene phylogenomic analysis (Hyun et al. 2012; Logacheva et al. 2011). Therefore, to statistically evaluate the variability between Nova and other plants, Nova fruit transcriptome was clustered with 25 plant genomes, including *R. coreanus* transcriptome. Using Tribe-MCL, we selected 1,755 clusters for generation of phylogenetic tree using Neighbor-joining method in rectangular cladogram. As shown in Fig. 1a, Nova fruit transcriptome was found to be closely related to *R. coreanus* transcriptome and *Fragaria versa* genome, which belong to the sub-family *Rosoideae*. In addition, Nova fruit transcriptome exhibited 77 and 68 % similarities with *R. coreanus* and *F. versa* at protein level, respectively, whereas the monocot proteomes have about 55 % of similarity with Nova fruit transcriptome (Fig. 1b). These findings were consistent with the phylogenetic analysis between the *Rosaceae* family, where nucleotide sequence data from six nuclear and four chloroplast regions were used (Potter et al. 2007).

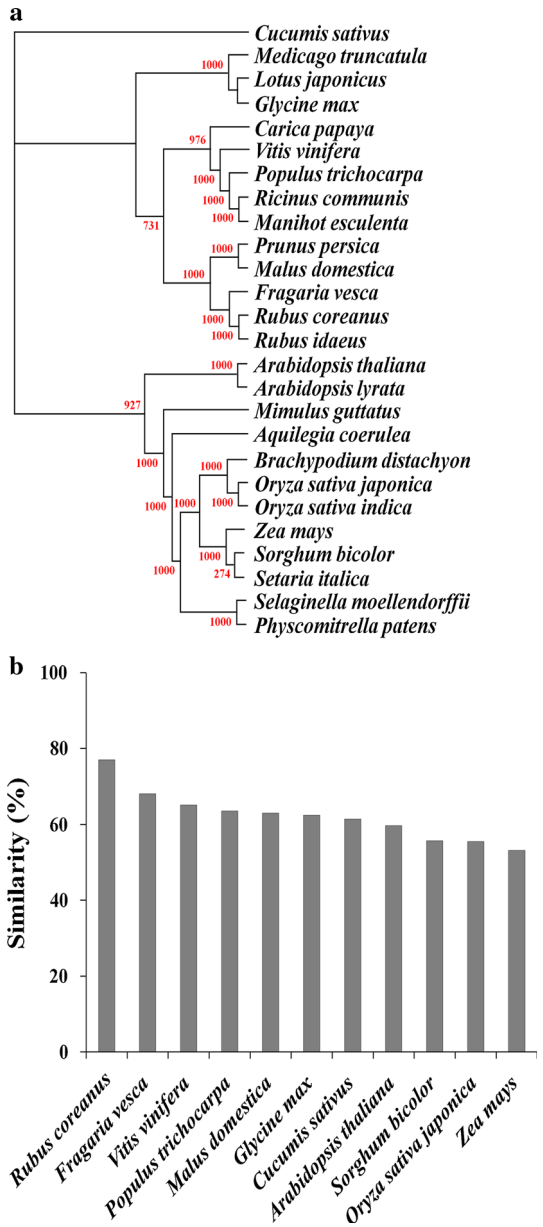


Fig. 1 Phylogenomic tree and sequence conservation of Nova fruit transcripts with other plant species. **a** Phylogenomic tree of Nova fruit transcriptome with different plants species. Phylogenomic analysis was carried out using the neighbor-joining method with 1,000 bootstrap repeats using MEGA5. **b** Sequence conservation of Nova fruit transcripts with annotated proteins of plant species. The Nova transcripts (coverage ≥70 %) were searched against proteome sequences using BLASTX program. The percentage of transcripts showing significant similarity (*E* value $<10^{-5}$) in BLASTX search is shown

Functional annotation of Nova fruit transcriptome

For annotation of genes, coding sequence and predicted proteins, all assembled sequences were searched using BLASTX against NCBI non-redundant (NR) database, and

29,068 unigenes that showed significant similarity (cut-off *E* value of 10^{-5}) were annotated (Table 2 and Supplementary Table 2). The protein data of Swiss-Prot database were used for additional alignments to produce more definitive annotations. Among them, 21,979 (52 % of all unigenes) had BLAST hits to known proteins in Swiss-Prot database (Table 2). Based on sequence homology, 11,396 unigenes were assigned at least with one GO term. Function of the majority of proteins was assigned to the cellular components followed by biological process and molecular function (Supplementary Fig. 2). 4,484 unigenes that are related to biological processes were assigned to the metabolic processes including primary and secondary metabolic processes, thus providing abundant regulatory information on metabolic genes, which can regulate the nutritional quality of fruits. To analyze the phylogenetically widespread domain families, the assembled unigenes were compared against Cluster of Orthologous Groups (COG). 11,162 of the assembled sequences were assigned to the COG classification. Among the 25 COG categories, the cluster for "general function only" represents the largest group (3,570 unigenes) followed by "Transcription" (2,078 ungenes) and "Replication, recombination and repair" (1,823 unigenes), whereas "Extracellular structures" and "Nuclear structure" are among the smallest groups (Supplementary Fig. 3). Furthermore, to identify the active biochemical pathways in the fruit of Nova, the annotated sequences were mapped against the reference canonical pathways of Kyoto Encyclopedia of Genes and Genomes (KEGG). A total of 16,255 unigenes were annotated to be involved in 125 biosynthesis pathways in KEGG database (Table 2 and Supplementary Table 3). The metabolic pathway was the largest group (3,803 unigenes) followed by "biosynthesis of secondary metabolites (1,653 unigenes). Other pathways included "plant-pathogen interaction (1,234 unigenes)", "plant hormone signal transduction (871 unigenes)" and "spliceosome (831 unigenes)". Taken together, this broad coverage of our transcriptome library provides a valuable resource for investigating the biological system in *R. idaeus*.

Table 2 Summary of annotations of the *R. idaeus* cv. Nova unigenes in public protein databases

Public protein database	*R. idaeus* cv. Nova	
	No. of unigene hit	Percentage
NR	29,068	68.2
KEGG	16,225	38.1
COG	11,162	26.2
Swiss-prot	21,979	51.6
GO	11,396	26.8
Total	29,334	68.9

Expression pattern of transcripts related to the GABA shunt pathway

GABA, a non-protein amino acid found in all living organisms, is synthesized by the pathway known as GABA shunt, consisting of three enzymes: glutamate decarboxylase (GAD; EC 4.1.1.15), catalyzing the decarboxylation of glutamate to GABA and CO_2; GABA transaminase (GABA-T; EC 2.6.1.19), which converts GABA and pyruvate to succinic semialdehyde; and succinic semialdehyde dehydrogenase (SSADH; EC 1.2.1.16), catalyzing the NAD^+-coupled oxidation of succinic semialdehyde to succinate (Fig. 2a). Since GABA has been identified as one of the most important neurotransmitters, it has been suggested as a key factor in regulating the intracellular activity and growth of polarized cells of mammals (Abdou et al. 2006; Ageta-Ishihara et al. 2009; Oláh et al. 2009). In higher plants, differential levels of GABA have been determined during the response to abiotic and biotic stresses, seed germination and fruit ripening process (Bouche and Fromm 2004; Cercós et al. 2006; Fortes et al. 2011; Hyun et al. 2013a). Similarly, the variation of GABA content in Nova fruits at the different ripening stages was also determined. As shown in Fig. 2b, stage 2 fruits contained the higher level of GABA, together with glutamate, compared to stage 1 fruits. During grape ripening process, the accumulation of GABA has been suggested as a result of decreasing *SSADH1* transcription (Fortes et al. 2011). Therefore, we hypothesized that the variation of GABA contents in Nova ripening process is due to the alteration of catabolism. To test this hypothesis, we analyzed the transcription level of genes involved in GABA shunt. Based on the KEGG pathway assignment, we found 9 unigenes encoding 9 putative enzymes involved in GABA shunt pathway from Nova transcriptome library (Table 3). The expression properties of putative GABA shunt genes in fruit were analyzed with quantitative real-time PCR using unigene-specific primer pairs. Although all *GAD* genes were expressed in stage 1 and stage 2 fruits, they exhibited differential expression patterns in stage 2 fruits (Fig. 2c). The changes in *Nova GAD1*, *3*, *4* and *6* gene expression were in good accordance with the increase of GABA contents in stage 2 fruits. In addition, the expression of *Nova GABA-T* and *SSADH* was observed in fruits; however, they exhibited decreased expression pattern in stage 2 fruits. This suggests that the accumulation of GABA in red-colored fruits is due to the increasing expression of *Nova GAD1*, *3*, *4* and *6* as well as the reduction of GABA-degrading enzymes.

Analysis of anthocyanin biosynthesis-related genes in Nova fruit

Anthocyanin is a major class of flavonoids synthesized via the flavonoid biosynthetic pathway, and known as a strong

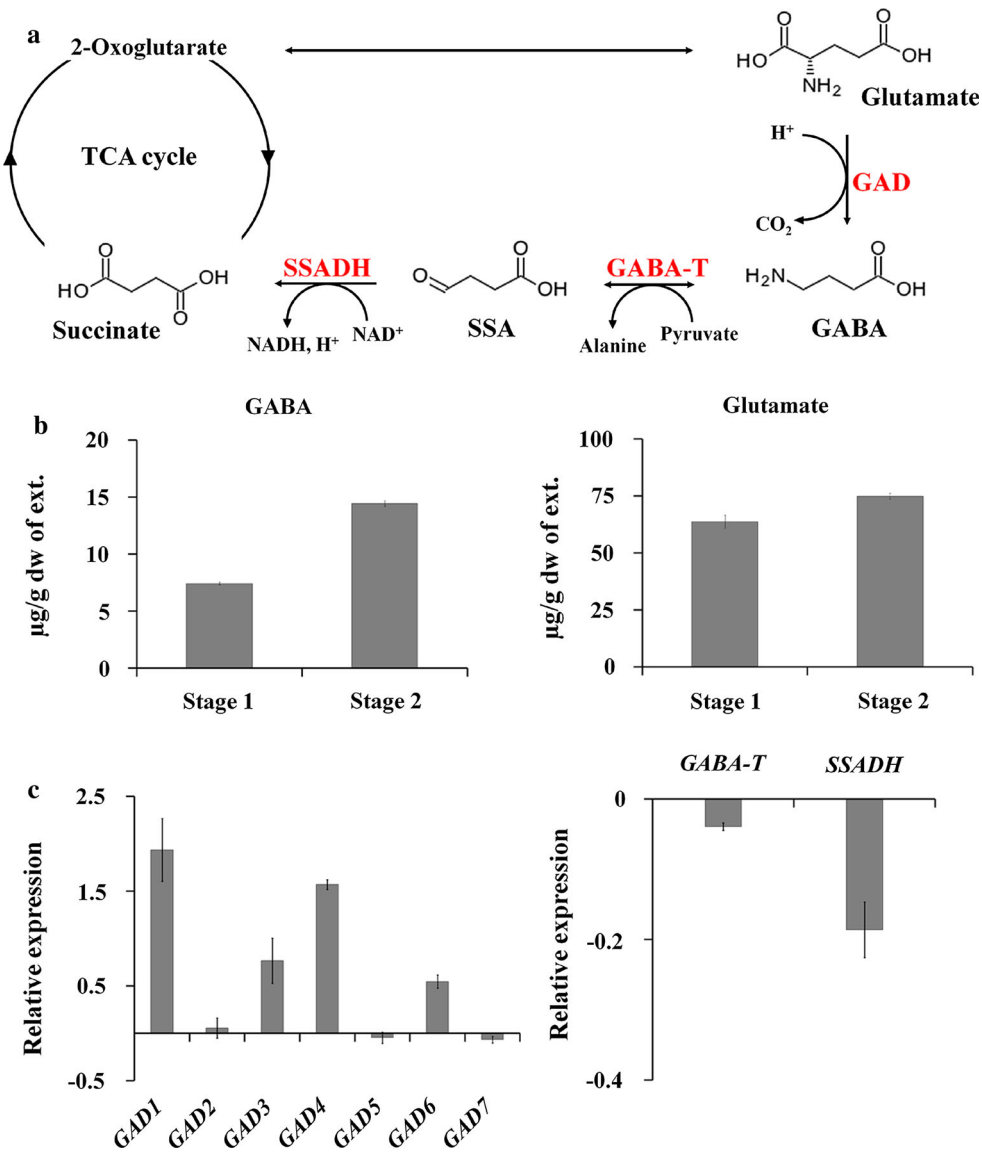


Fig. 2 Alteration of GABA shunt during the ripening process. **a** GABA shunt pathway. Enzyme names were abbreviated as follows: Glutamate decarboxylase (GAD), GABA transaminase (GABA-T) and Succinic semiadehyde dehydrogenase (SSADH). **b** The amount of GABA and its precursor, glutamate, was analyzed by GC–TOF–MS. The quantification was performed by peak integration using the external standard method. The amount was normalized to the dry weight of extract in each stage sample (μg/g dw of ext.). *Error bars* are the standard deviations from replication measurements. **c** Expression pattern of genes involved in GABA shunt pathway. Expression levels of genes from ripening stage 2 were compared to stage 1. The *Y-axis* represents normalized relative expression values ($\log_{10}$). The means and standard errors were calculated from three independent measurements

antioxidant that contributes to health benefits of red raspberry (Grotewold 2006; Kassim et al. 2009). Briefly, the biosynthesis of anthocyanin begins with chalcone synthase (CHS) catalyzing the stepwise condensation of malonyl-CoA with 4-coumaroyl-CoA to form naringenin chalcone, which is rapidly converted into the colorless naringenin by chalcone flavanone isomerase (CHI). Naringenin is then hydroxylated to produce dihydrokaempferol by flavanone 3-hydroxylase (F3H). Dihydrokaempferol can be further hydroxylated at the 3′ position or at both 3′ and 5′ positions of the B-ring by flavonoid 3′-hydroxylase (F3′H) to produce dihydroquercetin or by flavonoid 3′5′-hydroxylase (F3′5′H) to form dihydromyricetin. By the actions of dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX) and UDP-glucose:anthocyanidin 3-O-glucosyltransferase (3GT), dihydrokaempferol, dihydroquercetin and dihydromyricetin lead to the production of the pelargonidin-, cyanidin- and delphinidin-based pigments, respectively (Grotewold 2006). Based on Nova transcriptome, we identified 42 unigenes that encode 29 enzymes involved in anthocyanin biosynthesis (Table 3). More than one unique sequence was assigned to the same enzyme, indicating that such unigene sequences can either represent different fragments of a same transcript, or different members of a gene family, or both. To identify the relationship between these anthocyanin biosynthesis genes and anthocyanin amounts, we analyzed the difference in gene expression profile between stage 1 and stage 2 fruits. In case of early flavonoid biosynthetic genes required for the flavonol production, *CHI* and *F3H* unigenes were highly expressed in stage 2 fruits compared to stage 1 (Fig. 3a). Although Nova transcriptome library

Table 3 Unigenes potentially related to GABA shunt and anthocyanin biosynthesis pathway

Pathway	Gene	EC number	Gene name[a]	*R. idaeus* cv. Nova			*Fragaria vesca*
				NU	MNPU	NGSN	
GABA shunt	Glutamate decarboxylase	EC 4.1.1.15	GAD	7	7	XM_004299440	FV5G15870
							FV1G29220
							FV4G24590
	GABA transaminase	EC 2.6.1.19	GABA-T	1	1		FV6G36690
	Succinic semiadehyde dehydrogenase	EC 1.2.1.16	SSADH	1	1		FV3G12610
Anthocyanin biosynthesis	Chalcone synthase	EC 2.3.1.74	CHS	9	3	AF400566	FV7G02590
						AF400565	FV7G02600
	Chalcone isomerase	EC 5.5.1.6	CHI	3	3	–	FV2G25040
							FV7G25290
							FV7G31110
	Flavanone 3-hydroxylase	EC 1.14.11.9	F3H	4	3	–	FV1G13680
							FV6G51040
	Dihydroflavonol-4-reductase	EC 1.1.1.219	DFR	4	4	–	FV2G34030
							FV2G50590
							FV2G50610
							FV7G11200
	Flavonoid 3′-hydroxylase	EC 1.14.13.21	F3′H	6	4	–	FV5G00700
							FV5G00610
							FV5G12250
							FV0G15960
	Flavonoid 3′,5′-hydroxylase	EC 1.14.13.88	F3′5′H	2	1	–	FV3G27120
	Leucoanthocyanidin dioxygenase	EC 1.14.11.19	LDOX	3	2	–	FV5G01390
							FV7G29560
	Anthocyanidin 3-O-glucosyltransferase	EC 2.4.1.115	3GT	11	9	–	FV5G37930
							FV5G37980
							FV5G38000
							FV7G06660
							FV7G33970
							FV0G29830
Total				51	38	3	

NU number of unigenes, *MNPU* maximum number of proteins encoded by unigenes, *NGSN* number of genes stored in NCBI

[a] Following the KEGG nomenclature

contained 9 unigenes encoding 3 CHS proteins (Table 3), it was challenging to differentiate their expression level because of high sequence identities between them. In addition, most of genes, except *F3′5′H*, mainly involved in late biosynthetic pathway exhibited the decreased level of expression in stage 2 fruits (Fig. 3a), whereas the accumulation of cyanidin and pelargonidin derivatives were found in stage 2 fruits (Fig. 3b and Supplementary Table 4). This indicates a non-correlation between its transcription pattern and anthocyanin levels. One possibility is that the increased anthocyanin accumulation during the ripening process is due to the expression of genes, which do not present in our transcriptome library. Other possibilities might be the changes in anthocyanin degradation as suggested by (Lijavetzky et al. 2012).

Discussion

Red raspberry is an economically important berry crop with high antioxidant activity. It provides nutrients and micronutrients essential for human health, and contains numerous bioactive compounds with potential health benefits including anthocyanins, ellagic acid, vitamins and GABA with potential health benefits (Beekwilder et al. 2005; de Ancos et al. 2000; Kim et al. 2009; Mullen et al.

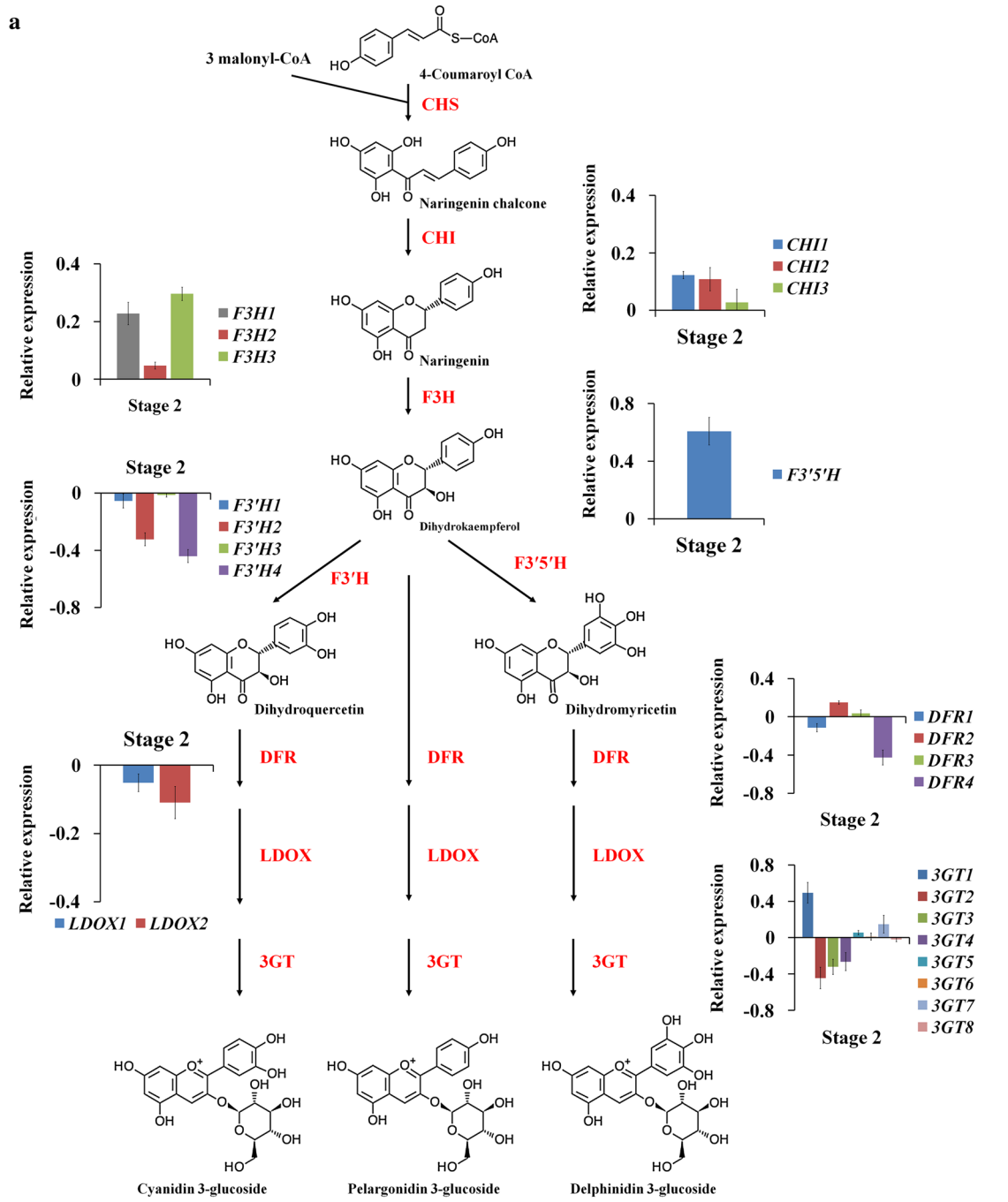
2002; Tamura et al. 2011). Though several Red raspberry cultivars have been generated, Nova has been suggested as an alternative early-season floricane-fruiting type because of its hardiness, productivity, disease resistance and anthocyanin contents (Anttonen and Karjalainen 2005; Hanson et al. 2005). This indicates that Nova could be a suitable model red raspberry for investigating anthocyanin biosynthetic pathway and pathogen resistance mechanism.

In this study, based on de novo transcriptome sequencing and assembly, 51,186,030 sequence reads assembled into 42,604 unigenes were obtained from Nova fruit (Table 1). Based on the phylogenomic tree and sequence similarity analyses, it could be found that Nova belongs to the subfamily *Rosoideae* (Fig. 1a), indicating that the transcriptome sequence of nova fruit are correctly assembled. Comparison of the assembled transcripts to gene catalogs of other plants, 29,334 unigenes (69 % of total unigenes) were annotated by BLAST analysis against public database including the NR, Swiss-Prot, GO, COG and KEGG databases (Table 2). Although no genomic information for *R. idaeus* and little genomic information of the *Rosaceae* family are available, this functional annotation and classification predict that the Nova fruit transcriptome contains an extensive and diverse expressed gene catalog, and also suggest that Nova transcriptome library will provide a solid foundation for further investigation and identification of functional genes in the genus *Rubus*.

In higher plants, GABA has been suggested as a signal molecule, which regulates the expression of *14-3-3* gene family members and nitrate transporter (Beuve et al. 2004; Lancien and Roberts 2006; Shelp et al. 2012). In tomato, the maximum level of GABA has been determined at the mature-green stage, whereas it rapidly decreases during the ripening process (Akihiro et al. 2008; Rolin et al. 2000). However, the increasing level of GABA has been analyzed in grape (Fortes et al. 2011) and Nova ripening process (Fig. 2b). The function of GABA in higher plants seems to be mediated by ethylene and abscisic acid (ABA). A loss or an excess of ethylene, a key regulator of the ripening process in climacteric fruits (Alexander and Grierson 2002), results in a failure to respond to GABA (Lancien and Roberts 2006). In addition, ABA-insensitive mutants, *abi1* and *abi2*, do not respond to GABA, indicating that ABA, a regulator of non-climacteric fruits ripening (Jia et al. 2011), is required for the induction of response to GABA (Lancien and Roberts 2006). Although the relationship between both hormones and GABA biosynthesis is not understood, the variation in GABA levels during the ripening process of tomato, grape and Nova might be mediated by the physiological difference between climacteric and non-climacteric fruits. In our Nova transcriptome library, we identified nine putative enzymes involved in GABA shunt pathway (Table 3). It is known that GAD is the primary factor for GABA accumulation (Shelp et al. 1999) and its activity is mediated by the concentration of glutamate, Ca^{+}/calmodulin (CaM) and H^{+} (Shelp et al. 2012). In our study, the higher expression levels of *GAD*s were observed in stage 2 fruits compared to stage 1 fruits, whereas *GABA-T* and *SSADH* were highly expressed in stage 1 fruits (Fig. 2c). This finding indicates that GABA accumulation in Nova fruits is not only due to the upregulation of *GADs*, but also due to the downregulation of GABA-degrading enzymes.

The production of anthocyanins is the most important indicator of maturity and quality in various fruit species. Anthocyanin accumulation during the ripening process is strongly correlated with the expression of flavonoid pathway genes, together with R2R3 MYB transcription factors (Jaakola et al. 2002; Li et al. 2012; Lin-Wang et al. 2010; Niu et al. 2010; Palapol et al. 2009; Zifkin et al. 2012). However, the decreased levels of *F3′Hs* and *LDOXs* expression were observed in stage 2 fruits, whereas these fruits contained higher level of cyanidin derivatives than stage 1 fruits (Fig. 3). Similarly, non-correlation between gene expressions and anthocyanin productions has also been observed through the transcriptional profiling during flesh and skin ripening of grape (Lijavetzky et al. 2012). Although further investigations are required, one possible explanation of anthocyanin accumulation in transcription-independent manner could be due to the alteration of degradation rate of anthocyanins or transferring rate to vacuole (Lijavetzky et al. 2012).

In conclusion, using Illumina sequencing technology, we have generated the large-scale transcriptome sequencing data in Nova fruits, one of the most economically important plants. Based on de novo transcriptome analysis, we have found that Nova fruit transcriptome contains abundant information on genes involved in the various metabolic pathways including GABA shunt and anthocyanin biosynthetic pathway. In addition, the analysis of the relationship between several novel transcripts and the amounts of metabolites suggests that anthocyanins were accumulated in transcription-independent manner, whereas the accumulation of GABA was mediated by differentially expressed genes involved in GABA shunt pathway. Further investigation on the variation of anthocyanin degradation and stability during fruit ripening process will be an exciting and important endeavor that will continue to disclose the mechanism of fruit ripening. The availability of Nova fruit transcriptome will be a step forward for the understanding of the fruit ripening processes in non-model plants. This transcriptome dataset will act as a valuable resource for further molecular genetic studies and breeding in *R. idaeus*, and will also provide a solid foundation for the isolation and characterization of functional genes involved in different metabolic pathways.


a
3 malonyl-CoA
4-Coumaroyl CoA
CHS
Naringenin chalcone
CHI
Naringenin
F3H
Dihydrokaempferol
F3′H
F3′5′H
Dihydroquercetin
Dihydromyricetin
DFR
LDOX
3GT
Cyanidin 3-glucoside
Pelargonidin 3-glucoside
Delphinidin 3-glucoside
Relative expression
Stage 2
F3H1
F3H2
F3H3
CHI1
CHI2
CHI3
F3′5′H
F3′H1
F3′H2
F3′H3
F3′H4
LDOX1
LDOX2
DFR1
DFR2
DFR3
DFR4
3GT1
3GT2
3GT3
3GT4
3GT5
3GT6
3GT7
3GT8
b
Cyanidin-3-rutinoside *
Cyanidin-3-2G-glucosylrutinoside
Pelargonidin-3-rutinoside
µg/g dw ext.
Relative peak area
Stage 1
Stage 2

◀**Fig. 3** Changes in the level of genes expression in the anthocyanin biosynthesis pathway and anthocyanins during ripening process. **a** Real-time PCR analysis of genes involved in anthocyanin biosynthesis pathway. The expression levels for each gene in stage 2 were calculated relative to its expression in stage 1. The *Y-axis* represents normalized relative expression values ($\log_{10}$). The means and standard errors were calculated from three independent measurements. Enzyme names were abbreviated as follows: chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxilase (F3′H), flavonoid 3′5′-hydroxylase (F3′5′H), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX) and anthocyanidin 3-O-glucosyltransferase (3GT). **b** Anthocyanin contents determined by UPLC–Q-TOF–MS were represented by box–whisker plots. The relative contents of stage1 and 2 were expressed by their peak area using MassLynx software. The *error bars* are the standard deviations from replication measurements. *Asterisk* the quantification of cyaniding-3-rutinoside was performed by peak integration using the external standard method. The amount was normalized to the dry weight of extract in each stage sample (μg/g dw of ext.)

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Conflict of interest The authors declare that they have no conflict of interest.

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