Electronic Journal of Biotechnology 35 (2018) 57–62

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Genotyping-by-sequencing based single nucleotide polymorphisms enabled Kompetitive Allele Specific PCR marker development in mutant *Rubus* genotypes

Jaihyunk Ryu [a,c], Woon Ji Kim [a], Juhyun Im [a], Sang Hun Kim [a], Kang-Seop Lee [b], Han-Jig Jo [b], Ee-Youb Kim [b], Si-Yong Kang [c], Jeong-Hee Lee [d], Bo-Keun Ha [a,*]

[a] *Division of Plant Biotechnology, Chonnam National University, Gwangju 61186, Republic of Korea*
[b] *Bioplus Co., Wanju 55310, Republic of Korea*
[c] *Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongup 56212, Republic of Korea*
[d] *Seeders Inc., Daejeon 34015, Republic of Korea*

ARTICLE INFO

Article history:
Received 21 May 2018
Accepted 9 August 2018
Available online 30 August 2018

Keywords:
Blackberry
Boysenberry
Cultivar identification markers
Fruit crop
Gamma irradiation
Genotyping-by-sequencing
KASP methodoloy
Mutation breeding
Phylogenetic tree
Rubus
SNP

ABSTRACT

Background: Rubus is an economically important fruit crop across the globe. Recently, several *Rubus* mutant genotypes with improved agronomic traits have been developed using gamma ray irradiation. This study investigated genetic diversity and variations in *Rubus* mutant genotypes using single nucleotide polymorphism (SNP) markers generated from genotyping-by-sequencing (GBS) analysis. A GBS library of 14 *Rubus* genotypes, consisting of seven boysenberry mutant lines, four blackberry mutant lines, and three original varieties, were sequenced on the Illumina Hiseq2000 platform. A set of SNPs were analyzed by Kompetitive Allele Specific PCR (KASP) assay in order to discriminate the *Rubus* genotypes.

Results: A total of 50,831,040 (86.4%) reads of clean data were generated, and the trimmed length ranged from 116,380,840 to 509,806,521 bp, with an average of 228,087,333 bp per line. A total of 19,634 high-quality SNPs were detected, which contained 11,328 homozygous SNPs and 8306 heterozygous SNPs. A set of 1504 SNPs was used to perform a phylogenetic analysis, which showed that there were clear differences among the *Rubus* genotypes based on their origin. A total of 25 SNPs were used for the KASP assays, of which six KASP primer sets were successfully distinguished among the *Rubus* genotypes.

Conclusions: This study demonstrated that the SNP and KASP method is an economically efficient tool for mutant screening in *Rubus* breeding programs.

How to cite: Ryu J, Kim WJ, Im J, et al. Genotyping-by-sequencing based single nucleotide polymorphisms enabled kompetitive allele specific PCR marker development in mutant Rubus genotypes. Electron J Biotechnol 2018;35. https://doi.org/10.1016/j.ejbt.2018.08.001.

1. Introduction

The genus *Rubus* consists of 900–1000 species worldwide, of which the most important fruits are blackberry, boysenberry, and raspberry [1,2]. *Rubus* fruits are considered to be a good source of phenolic compounds, such as anthocyanins, ellagic acid, quercetin, and phenolic acid. The antioxidant activities of these compounds have accelerated the development of new *Rubus* varieties that produce higher quality and healthier fruits [3,4,5].

Generally, *Rubus* species breeding progress has been limited by a lack of genetic variation in the germplasm for important agronomic traits [5,6]. Therefore, interspecific hybridization and mutation breeding are important breeding techniques for *Rubus* species [1,5]. The interspecific hybridization between red raspberry (*Rubus idaeus* L.) and blackberry (*Rubus fructicosus* L.) has created a number of new varieties, such as boysenberry, loganberry, and nessberry [5,6,7]. Boysenberry (*Rubus ursinus* Chamisso & Schlenhtendal) is a hybrid *Rubus* berry derived from a cross between loganberry (*Rubus loganobaccus* Bailey) and trailing blackberry (*Rubus fruticosus* L.) [1]. Mutation breeding has been used to improve specific agronomic traits, such as larger fruit sizes, early maturation, greater disease resistance, and higher anthocyanin content in *Rubus* fruits [3,4,5,6,7]. Recently, novel *Rubus* genotypes that have improved agronomic characteristics and high

* Corresponding author.
E-mail address: bkha@jnu.ac.kr (B.-K. Ha).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2018.08.001

levels of functional compounds (minerals, anthocyanins and ellagic acid) have been developed using mutation breeding techniques [3,4,5].

Recent advances in next generation sequencing (NGS) technology has led to an efficient and cost-effective re-sequencing of the plant genome and could potentially be used to directly detect single nucleotide polymorphisms (SNPs) in the genome [8,9,10]. SNPs have molecular genetic marker applications in many crops, including marker-trait association, high-resolution genetic map construction, linkage disequilibrium-based association mapping, genetic diagnostics, genetic diversity analysis, cultivar identification, phylogenetic analysis, and plant breeding applications [8,10]. Genotyping-by-sequencing (GBS) is a new approach to sequence-based genotyping. These methods detect and identify mutations using a condensed description of the genome and GBS has been widely applied in plant genetics and breeding [11,12,13,14,15]. In *Rubus* species, NGS technology has been used to sequence the whole genome sequence, genetically map and *de novo* RNA-seq red raspberry (*R. idaeus*), black raspberries (*R. occidentalis*), and blackberries (*Rubus* spp.) [9,13,16].

Advances in next generation sequencing and high-throughput SNP-based genotyping technologies have revolutionized plant genomic studies that led to the faster development of markers linked to traits of interest in plant breeding [17,18]. Kompetitive Allele Specific PCR (KASP) is a novel competitive allele specific PCR for SNP genotyping, where the sample DNA is amplified with a thermal cycler and allele specific primers [19,20]. When an allele-specific primer is hybridized with the target DNA, the fluorescent dye and the quencher are separated based on allele-specific oligo extension and fluorescence resonance energy transfer resulting in the emission of the corresponding fluorescence [19,20,21]. It has become a marker system of choice for various crops due to its low cost, locus specificity, and efficiency [19,21,22].

The objectives in this study were to investigate genetic diversity and variations in *Rubus* mutant genotypes using SNPs detected by GBS analysis, and to develop a KASP assay for a set of SNPs that will improve variety identification in *Rubus* mutant genotypes.

2. Materials and methods

2.1. Plant materials

xFourteen *Rubus* genotypes were used in this study (Table 1). The BS_PI genotype has thorns and was introduced from Japan. The others included stabilized lines from advanced generations, and all of them are thornless. The BS_Hybrid was developed from a cross between the thornless blackberry (*R. fruticosus* L. 'V3') and BS_PI [4,5]. Six mutant lines (BSA036 to BSA144) were developed by subjecting hybrid boysenberry explants to 20 Gy gamma-ray treatments, and the BSB032 line was developed after hybrid boysenberry had been subjected to 40 Gy gamma-ray treatments. V3 is the mother variety for four blackberry mutant lines, which were derived from gamma-ray (80 Gy) irradiation of tissue culture material. The mutant lines had improved agronomic characteristics, including higher fruit yields and sugar contents, than the original parent [3,4]. In this study, fresh young leaves from each genotype were harvested for DNA sampling. The DNA was extracted using a DNeasy plant mini kit (Qiagen, Hilden, Germany) and quantified using a NanoDrop 2000 (Thermo Scientific, DE, USA).

2.2. Preparation of the libraries for next-generation sequencing

The GBS libraries were constructed using the restriction enzyme ApeKI (GCWGC) and a protocol modified from Elshire et al. [11]. The DNA samples (100 ng/μL) with adapters were digested overnight at 75°C by 3.6 U ApeKI (New England Biolabs, Ipswich, MA). Sets of digested DNA samples, each with a different barcode adapter, were combined and purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Restriction fragments from each library were then amplified in 50 μL volumes that contained 2 μL of pooled DNA fragments, HerculaseII Fusion DNA Polymerase (Agilent, CA, USA), and 25 pmol each of the following primers: (A) 5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T-3′ and (B) 5′-CAA GCA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T-3′. These amplified sample pools constituted a sequencing "library". The library was sequenced on the Illumina Hiseq 2000 platform by the Seeders Co. (Daejeon, Korea).

2.3. Sequence pre-processing and de novo assembly

Demultiplexing was performed using barcode sequencing, and adapter sequence removal and sequence quality trimming were also undertaken. Adapter trimming was performed using cut adapt (version 1.8.3) [23], and sequence quality trimming was undertaken using the DynamicTrim and LengthSort programs in the SolexaQA (v.1.13) package: The DynamicTrim phred score was ≥20, and the LengthSort process used a short read length of ≥25 bp [24]. The cleaned reads were *de novo* assembled using SOAPdenovo2 (Ver. 2.04) [25]. A k-mer of 31 produced the largest contigs assembly size and this was used as the reference sequence. The BWA (0.6.1-r104) program [26] generated cleaned reads that passed the preprocessing process and the reads were aligned to the boysenberry assembled contig. A SAM format file was created using the default values, except for the following options: a seed length (-l) of 30, a maximum difference in the seed (-k) of 1, number of threads (-t) of 16, a mismatch penalty (-M) of six, a gap open penalty (-O) of 15, and a gap extension penalty (-E) of eight.

Table 1
Origins of the *Rubus* genotypes used in this study.

No.	Line	Origin	Treatment	Section
1	BS_PI	Boysenberry from Japan		*Rubus ursinus*
2	BS_Hybrid	Cross breeding	Blackberry(V3)′BS_PI	*Rubus* genotypes
3	BSA036	BS_Hybrid	Gamma-ray 20 Gy	*Rubus* genotypes
4	BSA065	BS_Hybrid	Gamma-ray 20 Gy	*Rubus* genotypes
5	BSA078	BS_Hybrid	Gamma-ray 20 Gy	*Rubus* genotypes
6	BSA101	BS_Hybrid	Gamma-ray 20 Gy	*Rubus* genotypes
7	BSA119	BS_Hybrid	Gamma-ray 20 Gy	*Rubus* genotypes
8	BSA144	BS_Hybrid	Gamma-ray 20 Gy	*Rubus* genotypes
9	BSB032	BS_Hybrid	Gamma-ray 40 Gy	*Rubus* genotypes
10	V3	Blackberry from New Zealand	Somaclonal variation	*Rubus fruticosus*
11	V7	V3	Gamma-ray 80 Gy	*Rubus fruticosus*
12	Maple	V3	Gamma-ray 80 Gy	*Rubus fruticosus*
13	Heukjinju	V3	Gamma-ray 80 Gy	*Rubus fruticosus*
14	Heukgwang	V3	Gamma-ray 80 Gy	*Rubus fruticosus*

2.4. Raw SNP (InDel) detection and consensus sequence extraction

The generated SAM format files were used to detect the raw SNP (InDel) using the SAMtools (0.1.16) program [27], which also extracted consensus sequences. Then SNP validation was performed using the Seeders Co. in-house script [28] and the raw SNPs (InDels) were detected. The default values were used, except for the following options: a minimum mapping quality for SNPs (-Q) of 30, a minimum mapping quality for gaps (-q) of 15, a minimum read depth (-d) of 3, a min InDel score for nearby SNP filtering (-G) of 30, an SNP within INT bp around a gap to be filtered (-w) of 15, a window size for filtering dense SNPs (-W) of 30, and a maximum read depth (-D) of 238.

2.5. Generation of the SNP (InDel) matrix and polymorphic SNP (InDel) detection

An integrated SNP matrix between samples was created in order to analyze the SNPs (InDels) between the analysis objects. A list of unions was constructed using the raw SNP (InDel) positions obtained by comparing each sample with a standard dielectric, and non-SNP loci were filled in from the consensus sequence of the sample. Then, the final SNP matrix was created by filtering the mis-called SNP (InDel) positions using an SNP comparison between samples. The SNPs (InDel) were divided into homozygous SNPs where the SNP read depth was ≥90%, and heterozygous SNPs, which ranged between: 40% ≤ SNP read depth ≤ 60% based on their position.

2.6. Phylogenetic analysis using polymorphic SNPs

The evolutionary history was inferred using the Neighbor-Joining method [29]. The percentage number of replicate trees in the associated taxa that clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree was drawn to scale, and the branch lengths and evolutionary distances used to infer the phylogenetic tree had the same units. A total of 16,726 union SNP matrix loci were generated using the SNPs for the 14 *Rubus* genotypes (Table S1). All ambiguous positions were removed for each sequence pair. The SNP datasets were filtered for SNPs using a missing rate of <30 %. There was a total of 1504 positions in the final data set (Fig. S1). A pair wise distance analysis was undertaken using the Poisson correction model [30] and evolutionary analyses were performed by MEGA7 [31].

2.7. Development of the KASP primers

First, the selected 25 SNPs positions were transformed into functional genotyping SNP assays using the KASP method (LGC Genomics, Beverly, MA, USA). Then, primers for the KASP SNP assays were designed using the LGC primer picker software (LGC Genomics). The KASP genotyping assays were based on competitive allele-specific PCR and were used to bi-allelically score the SNPs at specific loci. The KASP primer sequences are listed in Table S2.

2.8. Validation of SNPs

The newly designed KASP primers were initially used in a molecular survey of the 14 *Rubus* genotypes. The PCR reactions were performed in a total volume of 10 μL, which contained DNA (50 ng/5 μL), KASP Assay Mix (0.14 μL), and KASP Master Mix. Genotyping reactions were performed using ABI Step one plus (Applied Biosystems, CA, USA). The following cycling conditions were followed: pre-reading at 30°C for 1 min (1 cycle), holding at 94°C for 15 min (1 cycle), and ten touchdown cycles (94°C for 20 s; touchdown 65°C, -1°C per cycle, 60 s). There was also further cycling at 94°C for 20 s and 55°C for 60 s (26 cycles), followed by a post-read stage at 30°C for 1 min. The end-point fluorescence data were analyzed using StepOne software V.2.1 (Applied Biosystems, CA, USA).

3. Results

3.1. Sequencing and reads

The 14 *Rubus* genotypes, which consisted of three original varieties (boysenberry; BS_PI, blackberry; V3, and *Rubus* hybrid; BS_Hybrid), seven *Rubus* hybrid mutant lines, and four blackberry mutant lines, were subjected to GBS analysis. A summary of the sequence data generated from the 14 *Rubus* genotypes is included in Table 2 and Table S3. A total of 58.8 million reads with a total read length of 5.9G bp were generated after sequencing using the Illumina Hiseq2000 platform for *Rubus* mutant lines. There was an average of 3.7 million reads per line. After removing the adapter sequences, low-quality reads, and unique barcodes, 50,831,040 (86.4%) reads of clean data were generated for the *Rubus* genotypes. The total trimmed length was approximately 3.7 Gbp and the individual trimmed lengths ranged from 116,380,840 to 509,806,521 bp with an average of 228,087,333 bp per plant sample. *De novo* assembly was then conducted using the cleaned reads and boysenberry assembled contigs. A total length of 27,382,832 nucleotides was generated. The cleaned reads were mapped using BWA (0.6.1-r104) and the *de novo* assembled contigs as a reference. The total numbers of mapped reads ranged from 263,102 to 997,835 with an average of 457,373. An average of 12.7% of the filtered reads was mapped to the assembled contigs.

3.2. Identified SNPs and InDels

To identify mutations, the common SNPs (InDels) for each genotype were first selected from the union SNPs (InDels) in the matrix position between samples. Subsequently, they were identified as filtered homozygous and heterozygous types. A summary of these SNPs and InDels is shown in Table 3. A total of 19,634SNPs were identified for all lines, of which 11,328 SNPs were homozygous and 8306 were heterozygous variants. The largest number of SNPs was recorded for BSA144 and the lowest number was for Heukgwang. The homozygous SNPs ranged from 359 to 1362 with an average of 809.1 SNPs per plant sample. The heterozygous SNPs ranged from 143 to 1824 with an average of 593.3 SNPs per plant sample. The SNPs for the varieties were more homozygous than heterozygous, with the exception of BSA078 and BSA144.

A total of 389 InDels were identified for all *Rubus* genotypes, of which 97 InDels were heterozygous and 292 were homozygous variants. The highest number of total insertions and deletions were detected in BSA144 and Heukgwang had the lowest number.

3.3. Genetic relationship analysis

The pairwise distance matrix obtained from 1504 SNPs is shown in Table S4. The genetic distances ranged from 0.010 to 0.375 with an average of 0.141. BSA101 and BSA144 were the most closely related. The most distant genetic distance was V3 and BSA036. The genetic

Table 2
Overview of the GBS sequence data and alignment to the reference sequence.

	Total	Average/Plant
Total raw reads	58,819,606	3,676,225
Trimmed reads	50,831,040	3,176,940
Total length of raw reads (bp)	5,940,780,206	371,298,763
Total length of trimmed reads (bp)	3,649,397,325	228,087,333
Mapped reads on assembled contigs	6,403,221	457,373
No. of mapped regions	260,548	16,284
Total length of mapped regions (bp)	26,917,524	1,682,345

Table 3
Summary of SNPs and InDels detected among the *Rubus* genotypes.

Line name	Total nos. of detected SNPs	No. of homozygous SNPs*	No. of heterozygous SNPs**	Total detected InDels	No. of homozygous InDels*	No. of heterozygous InDels**
BS_PI	1607	994	613	36	24	12
BS_Hybrid	1895	1190	705	51	43	8
BSA036	1024	706	318	23	18	5
BSA065	1282	807	475	18	12	6
BSA078	1663	692	971	22	19	3
BSA101	1759	1033	726	41	25	16
BSA119	1268	704	564	27	22	5
BSA144	3186	1362	1824	75	50	25
BSB032	838	545	293	19	15	4
V3	1378	891	487	19	15	4
V7	551	387	164	7	6	1
Maple	1554	884	670	24	20	4
Heukjinju	1127	774	353	21	17	4
Heukgwang	502	359	143	6	6	0
Total	19,634	11,328	8306	389	292	97
Average/plant	1402.4	809.1	593.3	27.8	20.9	6.9

* Read depth ≥ 90%.
** 40% ≤ read depth ≤ 60%.

distance of *Rubus* hybrid populations was much higher (0.111) than blackberry population (0.029) in within population.

A cluster analysis was carried out, based on the maximum composite likelihood method between *Rubus* genotypes, and a dendrogram was generated with 1504 SNPs using the Neighbor-joining method for genetic relationships (Fig. 1). The cluster analysis suggested that the *Rubus* genotypes could be divided into three related groups and two independent groups. Group I consisted of blackberry original genotypes (V3) and four blackberry mutant lines (Heukjinju, Maple, V7, and Heukgwang); Group II contained four *Rubus* hybrid mutant lines (BSB032, BSA119, BSA101, and BSA144); and Group III contained an original boysenberry variety (BS_PI), BS_Hybrid, and BSA036. Two *Rubus* hybrid mutant lines (BSA065 and BSA078) did not belong to any groups.

3.4. Validation of SNPs markers using KASP

Twenty five candidate SNPs were used for the KASP assays (Table S2). The newly developed KASP primers were initially applied to the 14 *Rubus* genotypes. Six KASP primer sets were successfully distinguished among the *Rubus* genotypes. They were clearly separated and the dots on each line were close together (Table 4 and Fig. 2). The 442,304 marker set were identified in the blackberry mutant lines. Furthermore, 442,304 and 427,156 marker sets were used to distinguish BSA036, and 536,540 marker set were used to distinguish the spiny BS_PI variety.

4. Discussion

Rubus varieties have limited genetic diversity because there are only limited genetic resources available for *Rubus* [1,6]. This has limited the application of cross-breeding to generate new cultivars with important agronomical traits in *Rubus*. One of the most important methods used to create genetic resources in crops is naturally or artificially induced mutation [32]. Mutagenesis has already been used in *Rubus* plants to introduce many useful traits, such as improvements in fruit size, time to maturity, seed color, and resistance to pathogens [3,4,5]. The BS_Hybrid genotype, which was obtained from crosses between a thornless blackberry (*R. fruticosus* L. cv. V3) and boysenberry, has unfavorable fruit characteristics, such as a malformed fruit and relatively low sugar content. In contrast, the *Rubus* mutant lines developed by gamma irradiating the BS_Hybrid showed improved fruit yield and sugar contents [5]. The five blackberry lines derived from gamma-ray mutation had improved fruit characteristics, such as larger size (Maple, V7, Heukjinju, and Heukgwang), increased disease resistance (all lines), and greater

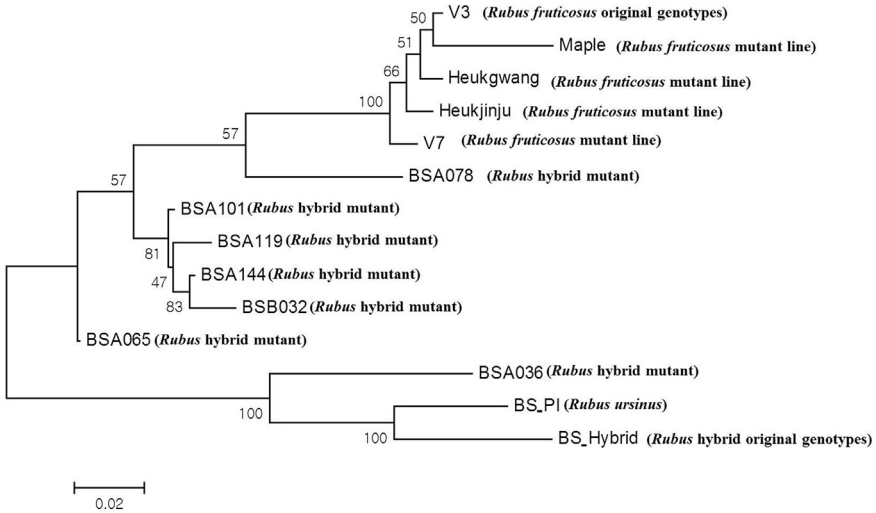

Fig. 1. Neighbor-joining dendrogram based on a pairwise distance matrix, which shows how the 14 *Rubus* line accessions obtained from 1504 SNPs were grouped.

Table 4
New, validated KASP markers for the *Rubus* genotypes used in this study.

No.	ID	Reference allele	Expected alleles												
			1**	2	3	4	5	6	7	8	9	10	11	12	13	14
1	354992*	A	W***	W	T	T	W	T	T	T	T	T	T	T	T	T
2	366,042	T	Y	T	Y	T	T	T	T	T	T	T	T	T	T	T
3	426,936	C	C	C	C	C	C	C	C	C	C	Y	Y	Y	Y	C
4	427,156	A	A	A	G	A	A	A	A	A	A	A	A	A	A	A
5	442,304	C	Y	Y	T	Y	Y	Y	Y	Y	Y	C	C	C	C	C
6	536,540	G	C	S	G	G	S	G	G	G	G	G	G	G	G	G

* Primer sequence listed in Table S2.
** Line numbers listed in Table 1.
*** W: A or T; Y: C or T; S: G or C.

cyanidin-3-*O*-glucoside contents (Maple), compared to the original genotype (V3) [3].

In this study, 14 *Rubus* genotypes were analyzed. These were three original lines and 11 mutagenesis progeny of *Rubus* lines derived from gamma-ray irradiation. Unfortunately, whole genome sequencing and re-sequencing of *Rubus ursinus* and *Rubus fruticosus* have not been under taken. Therefore, *de novo* assembled contigs were generated from the 3.7 Gbp of trimmed sequence data used in the GBS analysis and 19,634 SNPs were identified in the 14 *Rubus* genotypes. The GBS-based SNP frequency in the 14 *Rubus* genotypes was 1/145,554 bp. The GBS data showed that the SNP frequency was much lower than that recorded by Garcia-Seco et al. [9] who reported a value of 1/552 bp for blackberry (*Rubus* sp. Var. Lochness) after whole genome resequencing. The GBS-based SNPs have been detected in various crops, such as soybean, and cabbage (*Brassica oleracea* L.) and the data showed that the SNPs had a lower frequency than the re-sequencing analysis results suggested [10,14]. However, GBS technology is now used as a cost-effective molecular tool for the routine breeding and screening of many mutants [12,18].

The boysenberry genotypes and blackberry genotypes used in this study were hardly congruent; with 4044 (24.2%) union SNPs mapped in both *Rubus* species (Table S1). The map rates were low because, even though boysenberry and blackberry belong to the genus *Rubus*, they are inter-specific hybridization species. A previous study identified and mapped a total of 6,912 SNPs in a *Rubus* hybrid and developed a comprehensive SNP reference map for red raspberry [13]. The multiple origins of polyploidy *Rubus* species might also be the main contributor to genetic diversity in *Rubus* [2].

The phylogenetic tree revealed a clear pattern of division among the *Rubus* genotypes, and the boysenberry genotypes could be divided into a gamma-ray treated group and a non-treatment group, except for BSA036. Previous studies have also attempted to phylogenetically analyze the same boysenberry genotypes using 103 simple sequence repeat (SSR) markers and, again, the genotypes could be divided into a gamma-ray treated group and a non-treatment group [5]. Similarly, the amplified fragment length polymorphism (AFLP) analysis results revealed that the gamma-ray treated mutants and the cross-bred (*R. fructicosus* × *R. parvifolius*) line could be separated into independent groups [7].

This study identified numerous SNP markers based on the GBS method and a diverse group of *Rubus* mutants. Furthermore, a set of SNPs was validated by converting them into a KASP assay, which is a

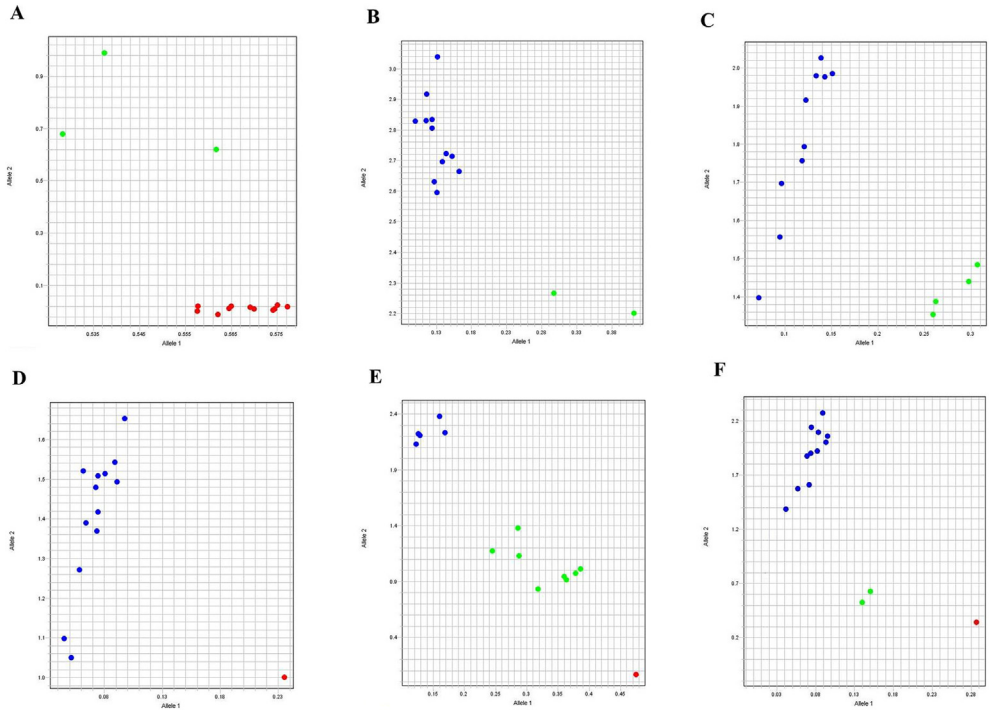

Fig. 2. Genotyping results for the *Rubus* mutant lines after using the KASP assay technique. The scatter plots with axes x and y represent allele discrimination of the genotypes. The red and blue dots represent the homozygous alleles and the green dots represent the heterozygous alleles. A: 354992, B: 366042 C: 426936, D: 427156, E: 442304, and F: 536540. Expected alleles are listed in Table 4.

uniplex SNP genotyping platform that uses real-time PCR. This study attempted to convert 25 SNPs into KASP assays. However, only six markers were successfully converted and this was probably due to the presence of duplicate loci, incorrect primer design near the SNP, or the need to optimize PCR conditions [19,22]. Therefore, the further optimization of primer design and amplification conditions could improve the development of successful KASP assay techniques. In this study, the six KASP assays were successfully used to distinguish among the original genotypes and mutant genotypes in *Rubus* hybrid. Ryu et al. [4] reported that the BSA078 genotype had the highest total anthocyanin concentration and the soluble solids content were higher in the BSA119 and BSA144 mutants than in the original genotype. Especially, two (442,304 and 427,156) KASP marker sets were used to distinguish BSA036. The stearic acid concentration of BSA036 mutant genotype was significantly higher than that of the original genotype [4]. Therefore, these results demonstrated that the developed KASP markers could detect the elite genotypes from mutant lines. There have been previous reports on the development of KASP markers in various crop plants, such as *indica* rice, tomato, *Gossypium hirsutum*, and peanut [20,21,22,33]. This is the first report on developed KASP markers in *Rubus* genotypes and these results showed that this study had successfully developed a KASP assay method that efficiently and accurately distinguished *Rubus* mutant genotypes.

Conflict of Interest Statement

The authors report no relationships that could be construed as a conflict of interest.

Financial support

This work was supported by grants from the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (IPET) through the Agri-Bio Industry Technology Development Program funded by the Ministry of Agriculture, Food, and Rural Affairs (Grant No. 315025-03-1-HD060) and the National Research Foundation of Korea (NRF) (NRF-2018M2A2A6A05057264), Republic of Korea.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2018.08.001

References

[1] Wood GA, Andersen MT, Forster RLS, et al. History of Boysenberry and Youngberry in New Zealand in relation to their problems with Boysenberry decline, the association of a fungal pathogen, and possibly a phytoplasma, with this disease. N Z J Crop Hortic Sci 1999;27:281–95. https://doi.org/10.1080/01140671.1999.9514108.
[2] Zafra-Stone S, Yasmin T, Bagchi M, et al. Berry anthocyanins as novel antioxidants in human health and disease prevention. Mol Nutr Food Res 2007;51:675–83. https://doi.org/10.1002/mnfr.200700002.
[3] Ryu J, Kwon SJ, Jo YD, et al. Comparison of phytochemicals and antioxidant activity in blackberry (*Rubus fruticosus* L.) fruits of mutant lines at the different harvest time. Plant Breed Biotechnol 2016;4:242–51. https://doi.org/10.9787/PBB.2016.4.2.242.
[4] Ryu J, Kwon SJ, Jo YD, et al. Fruit quality and chemical contents of hybrid boysenberry (*Rubus ursinus*) lines developed by hybridization and gamma irradiation. Plant Breed Biotechnol 2017;5:228–36. https://doi.org/10.9787/PBB.2017.5.3.228.
[5] Ryu J, Kim WJ, Im J, et al. Study of transferability of *Rubus* microsatellite markers to hybrid boysenberry. Plant Breed Biotechnol 2017;5:253–60. https://doi.org/10.9787/PBB.2017.5.4.253.
[6] Knight VH. *Rubus* breeding worldwide and the raspberry breeding programme at Horticultural Research International, East Malling Jugosl Vocar, 2004;38:23–28. [Cited August 3, 2018]. http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.474.8790&rep=rep1&type=pdf.
[7] Ryu J, Ha BK, Ds Kim, et al. Genetic diversity and relationship assessment based on AFLP analysis in blackberry (*Rubus fructicosus* L.) mutant lines. Plant Breed Biotechnol 2014;2:386–95. https://doi.org/10.9787/PBB.2014.2.4.386.
[8] Kumar S, Banks TW, Cloutier S. SNP discovery through next generation sequencing and its applications. Int J Plant Genomics 2012:831460. https://doi.org/10.1155/2012/831460.
[9] Garcia-Seco D, Zhang Y, Gutierrez-Manero FJ, et al. RNA-Seq analysis and transcriptome assembly for blackberry (*Rubus* sp. Var. Lochness) fruit. BMC Genomics 2015;16:5. https://doi.org/10.1186/s12864-014-1198-1.
[10] Lee KJ, Kim DS, Kim JB, et al. Identification of candidate genes for an early-maturing soybean mutant by genome resequencing analysis. Mol Genet Genomics 2016;291:1561–71. https://doi.org/10.1007/s00438-016-1183-2.
[11] Elshire RJ, Glaubitz JC, Sun Q, et al. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS One 2011;6:e19379. https://doi.org/10.1371/journal.pone.0019379.
[12] Poland JA, Rife TW. Genotyping-by-sequencing for plant breeding and genetics. Plant Genome 2012;5:92–102. https://doi.org/https://doi.org/10.3835/plantgenome2012.05.0005.
[13] Ward JA, Bhangoo J, Fernandez-Fernandez F, et al. Saturated linkage map construction in *Rubus idaeus* using genotyping by sequencing and genome-independent imputation. BMC Genomics 2013;14:2. https://doi.org/10.1186/1471-2164-14-2.
[14] Lee J, Izzah NK, Choi BS, et al. Genotyping-by-sequencing map permits identification of clubroot resistance QTLs and revision of the reference genome assembly in cabbage (*Brassica oleracea* L.). DNA Res 2016;23:29–41. https://doi.org/10.1093/dnares/dsv034.
[15] Nguyen TK, Yu J, Choi HW, et al. Optimization of genotyping-by-sequencing (GBS) in *Chrysanthemums*: Selecting proper restriction enzymes for GBS library construction. Hortic Sci Technol 2018;36:108–14. https://doi.org/10.12972/kjhst.20180012.
[16] Van Buren R, Bryant D, Bushakra JM, et al. The genome of black raspberry (*Rubus occidentalis*). Plant J 2016;87:535–47. https://doi.org/10.1111/tpj.13215.
[17] Deschamps S, Llaca V, May GD. Genotyping-by-sequencing in plants. Biology 2012;1:460–83. https://doi.org/10.3390/biology1030460.
[18] He J, Zhao X, Laroche A, et al. Genotyping-by-sequencing (GBS), an ultimate marker-assisted selection (MAS) tool to accelerate plant breeding. Front Plant Sci 2014;5(484). https://doi.org/10.3389/fpls.2014.00484.
[19] Semagn K, Babu R, Hearne S, et al. Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): Over view of the technology and its application in crop improvement. Mol Breed 2014;33:1–14. https://doi.org/10.1007/s11032-013-9917-x.
[20] Devran Z, Goknur A, Mesci L. Development of molecular markers for the *Mi-1*Gene in tomato using the KASP genotyping assay. Hortic Environ Biotechnol 2016;57:156–60. https://doi.org/10.1007/s13580-016-0028-6.
[21] Islam MS, Thyssen GN, Jenkins JN, et al. Detection, validation, and application of genotyping-by-sequencing based single nucleotide polymorphisms in upland Cotton. Plant Genome 2015;8:1–10. https://doi.org/10.3835/plantgenome2014.07.0034.
[22] Steele KA, Quinton-Tulloch MJ, Amgai RB, et al. Accelerating public sector rice breeding with high-density KASP markers derived from whole genome sequencing of *indica* rice. Mol Breed 2018;38(38). https://doi.org/10.1007/s11032-018-0777-2.
[23] Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J 2011;17:10–2. https://doi.org/10.14806/ej.17.1.200.
[24] Cox MP, Peterson DA, Biggs PJ, et al. At-a-glance quality assessment of Illumina second-generation sequencing data. BMC Bioinf 2010;11:485. PMid: 20875133.
[25] Luo R, Liu B, Xie Y, et al. SOAPdenovo2: An empirically improved memory-efficient short-read de novo assembler. Giga Sci 2012;1:1–6. https://doi.org/10.1186/2047-217X-1-18.
[26] Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 2009;25:1754–60. https://doi.org/10.1093/bioinformatics/btp324.
[27] Li H, Handsaker B, Wysoker A, et al. The sequence alignment/map format and SAMtools. Bioinformatics 2009;25:2078–9. https://doi.org/10.1093/bioinformatics/btp352.
[28] Kim JE, Oh SK, Lee JH, et al. Genome-wide SNP calling using next generation sequencing data in tomato. Mol Cell 2014;37:36–42. https://doi.org/10.14348/molcells.2014.2241.
[29] Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. PNAS 2004;101:11030–5. https://doi.org/10.1073/pnas.0404206101.
[30] Zuckerkandl E, Pauling L. Evolutionary divergence and convergence in proteins. In: Bryson V, Vogel HJ, editors. Evolving Genes and Proteins. New York: Academic Press; 1965. p. 97–166. https://doi.org/10.1016/B978-1-4832-2734-4.50017-6.
[31] Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 2016;33:1870–4. https://doi.org/10.1093/molbev/msw054.
[32] FAO/IAEA. Mutant Variety Database; 2017 [Cited August 3,2018]. https://mvd.iaea.org/.
[33] Zhao S, Li A, Li C, et al. Development and application of KASP marker for high throughput detection of AhFAD2 mutation in peanut. Electron J Biotechnol 2017;25:9–12. https://doi.org/10.1016/j.ejbt.2016.10.010.