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Research article

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

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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.

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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

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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*