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Assembly of the draft genome of buckwheat and its applications in identifying agronomically useful genes

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Abstract

Buckwheat (Fagopyrum esculentum Moench; 2n = 2x = 16) is a nutritionally dense annual crop widely grown in temperate zones. To accelerate molecular breeding programmes of this important crop, we generated a draft assembly of the buckwheat genome using short reads obtained by next-generation sequencing (NGS), and constructed the Buckwheat Genome DataBase. After assembling short reads, we determined 387,594 scaffolds as the draft genome sequence (FES_r1.0). The total length of FES_r1.0 was 1,177,687,305 bp, and the N50 of the scaffolds was 25,109 bp. Gene prediction analysis revealed 286,768 coding sequences (CDSs; FES_r1.0_cds) including those related to transposable elements. The total length of FES_r1.0_cds was 212,917,911 bp, and the N50 was 1,101 bp. Of these, the functions of 35,816 CDSs excluding those for transposable elements were annotated by BLAST analysis. To demonstrate the utility of the database, we conducted several test analyses using BLAST and keyword searches. Furthermore, we used the draft genome as a reference sequence for NGS-based markers, and successfully identified novel candidate genes controlling heteromorphic self-incompatibility of buckwheat. The database and draft genome sequence provide a valuable resource that can be used in efforts to develop buckwheat cultivars with superior agronomic traits.

Key words: buckwheat, draft sequence, database usage, GBS marker, heteromorphic self-incompatibility

1. Introduction

The genomes of model plants, such as Arabidopsis thaliana and Oryza sativa (rice), were fully sequenced by the start of the 21st century, and databases containing chromosomal pseudo-molecules and gene annotation information have subsequently been developed and are widely used as tools and resources for plant genomics and genetics studies. Recently, next-generation sequencing (NGS) has emerged as a powerful technique for analysing the genomes of non-model crops
in which few molecular genetic studies have been performed. Genome sequences obtained by NGS can be used to construct databases that contain information of genes inferred from available information of genes in model plants. These genome databases in non-model crops will pave the way for the rapid identification of useful genes for crop breeding, which have already been identified in model plants. These databases will also facilitate the construction of fine genetic maps [based on single nucleotide polymorphism, simple sequence repeat (SSR), and NGS-based markers], and make it possible to identify genomically important genes by map-based cloning. Thus, genome analyses in various non-model crops are underway, and the genomes of >50 non-model crops have already been sequenced (CoGePedia; https://genomeweb.org/). For example, NGS has been used to sequence the genomes of crops that produce beneficial secondary metabolites, such as flavonoid-producing Vipurnum tilobum (American cranberry) and capsaicin-producing Capsicum annuum (hot pepper), and of crops that are tolerant to environmental stress, such as Setaria italica (foxtail millet) and Cajanus cajan (pigeonpea), which grow in semi-arid regions. NGS technology has opened the door to elucidating the molecular mechanisms that control agriculturally important traits, and there is much interest in using this technology to analyse the genomes of non-model crops.

Buckwheat (Fagopyrum esculentum) is a widely cultivated annual crop in temperate zones. This nutritionally dense non-model crop contains high levels of starch, protein, flavonoids, and dietary fibre in the grain. Furthermore, buckwheat flour is gluten-free and can replace wheat flour in a coeliac diet. Buckwheat, however, has two major defects as a crop. First, its outcrossing nature, caused by heteromorphic self-incompatibility (SI), makes it difficult to produce pure cultivars of buckwheat and to fix useful traits. Second, buckwheat grains contain allergens, which induce anaphylactic reactions in some people. Improving the nutritional quality of the grain and removing genes responsible for SI and allergens are important breeding objectives in buckwheat, and various genetic molecular marker systems have been developed for this purpose (e.g., amplified fragment length polymorphism (AFLP) markers, SSR markers, expressed sequence tag (EST) markers, and array-based markers). However, AFLP markers have not yet been converted to single locus markers in the buckwheat genome. SSR markers have limited utility in buckwheat due to difficulty in amplifying specific loci because of the high level of genetic diversity between buckwheat cultivars, and EST marker systems do not span the entire genome. The newest genome map of buckwheat constructed using array-based markers has sufficient markers to cover the entire genome; however, it requires a specialized instrument to interpret the fluorescence signals of the arrays.

Recently, a versatile NGS-based genotyping method with a low-cost, genotyping-by-sequencing (GBS) marker system was developed. The GBS system utilizes redundant libraries constructed with PCR fragments that have recognition sites of two kinds of restriction enzymes on both ends. The PCR fragments sequenced using NGS technology are mapped to reference sequences for genome-wide genotyping. The GBS system has been used to genotype various crop species to date. A draft genome of buckwheat could be used as a reference sequence for developing GBS markers to identify genes that control desirable breeding traits.

Here, we used NGS-based technology to sequence the buckwheat genome, and constructed the Buckwheat Genome DataBase (BGDB; http://buckwheat.kazusa.or.jp). This database can be used for the rapid detection of homologues of genes previously identified in other plants, and we present three examples of buckwheat genes identified using this approach, i.e., genes controlling flavonoid biosynthesis and genes encoding 2S albumin-type allergens and granule-bound starch synthases (GBSSs). Furthermore, to illustrate that the draft genome can be used as a reference sequence for NGS-based genotyping, we used GBS technology to identify novel candidate genes for controlling heteromorphic SI of buckwheat.

2. Materials and methods

2.1. Plant material

A single buckwheat plant with short-styled flowers, a descendant of material used in a previous study to construct a buckwheat BAC library, was obtained from sib-crossing (BC1F2). Nuclei were extracted from leaf tissues of the single plant as described previously. Subsequently DNA was extracted from the nuclei according to a previously described method. To construct a training set for gene prediction using Augustus 3.0.3, total RNA was prepared from the anthers of short-styled and long-styled plants, cv ‘KOTON’ using a previously described method.

2.2. Genome sequencing of buckwheat

A paired-end (PE) library with insert sizes of 180–200 bp and a mate-pair (MP) library with insert sizes of 3, 5, 10, and 20 kb were constructed from nuclear DNA according to the manufacturer’s protocol (Illumina Inc., San Diego, CA, USA). A PE RNA-Seq library with insert sizes of ~275 bp was also constructed. Sequencing of genomic and RNA-Seq libraries using Illumina HiSeq 2000 was respectively carried out at Hokkaido System Science Co., Ltd and Beijing Genomics Institute. The PE and MP reads were subjected to quality trimming by PRINSEQ 0.20.4 and further to adaptor trimming by the fastx_clipper program in the FASTX-toolkit 0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit). The quality value threshold used for quality trimming was 10 from the 3′ terminal, and the adaptor sequence used was ‘AGATCGGAAGAGC’. Then, for the PE library with insert sizes of 180 bp, one base at the 3′ terminal was trimmed from all reads due to low quality, and PE reads shorter than 99 bp and including undetermined nucleotides (Ns) were excluded. For the MP library with insert sizes of 3, 5, and 10 kb, reads shorter than 49 bp and including Ns were excluded, and the 50 bp from the 5′ terminal were used for scaffolding. For the MP library with an insert size of 20 kb, reads shorter than 99 bp and including Ns were excluded, and the 50 bp from the 3′ terminal were used for scaffolding. For the PE RNA-Seq data, reads shorter than 89 bp and including Ns were excluded. The trimmed reads were used for further analyses.

2.3. Estimation of genome size

For genome size estimation, we used PE reads with a k-mer size of 17, as successfully used in a previous study. The k-mer distribution was investigated using Jellyfish 2.1.3. The genome size and coverage (i.e., the number of base pairs sequenced as a multiple of the number of base pairs present in the genome) were estimated using the peak at 47 on the k-mer frequency distribution curve (Supplementary Fig. S1) according to a previously described method.

2.4. Assembly of the buckwheat genome sequences

The trimmed PE reads were assembled using SOAPdenovo2 rev24020 with k-mer sizes of 61, 71, 81, and 91 nt. The option used was –RF –M 1–K [k-mer size]. After the assembly, gaps in scaffolds were closed using GapCloser 1.10 (http://soap.genomics.org.cn/soapdenovo.html) (P = 31). The trimmed MP reads were used for scaffolding by
labeled amplicons were sequenced by Illumina HiSeq 2000 at Barcode adaptors are listed in Supplementary Table S2. Barcode-25S rRNAs (accession number: X52320.1) and 18S rRNA (accession
wheat landraces from around the world (Supplementary Table S1) Total DNA extracted from 18 short-styled and 18 long-styled buck-
2.7. Genotyping-by-sequencing and detection
2.6. Database construction
The draft genome sequence (FES_r1.0), predicted gene sequences, deduced amino acid sequences, annotations derived from BLAST searches against the TAIR10 and NCBI’s NR database, and domains identified in the search against InterPro were included in the BGDB. In addition, local BLAST searches and keywords searches for gene names and their annotations were also implemented in the BGDB.

2.7. Genotyping-by-sequencing and detection of S-allele-specific sites
Total DNA extracted from 18 short-styled and 18 long-styled buck-
Hokkaido System Science Co., Ltd. The PE reads were subjected to quality and adaptor trimming by Trimomatic 0.3.2. The quality value threshold used for quality trimming was 25 with a window size of 5, and the adaptor sequences used were ‘CAGCGC GCTCTTCCGATCT’ and ‘ACCGCTCTTCCGATCTGTAA’. Then, PE reads longer than 39 bp were aligned to reference sequences (FES_r1.0) using BWA 0.7.9, and the mapping results were processed with SAMtools 0.1.18. To minimize mismatching bases across all the reads, local realignment procedure was carried out using RealignerTargetCreator and IndelRealigner in GATK 3.4.

All the sites on reference sequences that mapped with reads were extracted and combined in a variant call format file using the UnifiedGenotyper in GATK 3.4 with the option of -out_mode EMTT_ALL_CONFIDENT_SITES. Sites at which >50 reads were mapped in long-styled plants but not in short-styled plants were defined as ‘non-SS’. Likewise, sites at which >50 reads were mapped in short-styled plants but not in long-styled plants were defined as ‘non-LS’. Then, the number of short-styled plants with mapped reads at each non-LS site and the number of long-styled plants with mapped reads at each non-SS site were counted. Non-LS sites shared by >10 short-styled plants were regarded as S-allele-specific sites (see Results and discussion). Then, scaffolds harbouring >39 S-allele-specific sites were regarded as S-allelic scaffolds.

2.8. Phylogenetic analyses
Alignments of amino acid sequences were carried out using CLUS-
TALW, and the neighbor-joining (NJ) trees were obtained from pairwise distances corrected by the JTT model. These analyses were conducted using MEGA6.

3. Results and discussion
3.1. Genome assembly of buckwheat
The k-mer frequency distribution curve (k-mer = 17) using PEs with 180 and 200 bp is shown in Supplementary Fig. S1. Based on this curve, the genome size of buckwheat was estimated to be between 1,212,021,130 and 2,424,042,260 bp using peaks at a multiplicity of 94 (coverage = 111.9) and 47 (coverage = 56.0), respectively. The genomic DNA used in this study is expected to contain heterozygous genomic regions, due to the outcrossing nature of buckwheat; however, we used sib-mating descendant plants as material to reduce heterozygous genomic regions. The haploid genome size of 1.2 Gb calculated based on the major peak (multiplicity = 94) is almost the same as that estimated from cytometry analyses (1.34 Gb).

The numbers of raw and trimmed reads are summarized in Sup-
plementary Table S3. The trimmed reads with k-mer sizes of 61, 71, 81, and 91 nt were assembled using SOAPdenovo2. The N50 values of the assemblies using k-mer sizes of 61, 71, 81, and 91 nt were, respectively, 1,388, 1,419, 1,350, and 770 bp. The longest scaffolds, i.e. those assembled with a k-mer size of 71, were used for further analysis. Gaps in the contigs were closed using GapCloser 1.10 (http://soap.genomics.org.cn), and mate-pair reads were used for scaffolding in SPACe2.0. The 2,693,661 scaffolds that were shorter than 299 bp and the 1,908 scaffolds that exhibited signs of contamination (identified in a BLAST search) were excluded, and the remaining 387,594 scaffolds were designated as the draft genome sequence, FES_r1.0 (Table 1). The total length of FES_r1.0 was 1,177,687,305 bp, and the N50 length was 25,109 bp. The scaffolds were named ‘Fes_sc’ followed by a six-digit identifier and the sequence version (e.g. Fes_sc000001.1).
Considering the genome size, the total length of the assembled genome sequence was close to the estimated size; therefore, the draft genome sequence (FES_r1.0) was considered as the haploid genome sequence. The draft genome sequence spanned 98.3% of the genome (FES_r1.0) was considered as the haploid genome sequence was close to the estimated size; therefore, the draft genome sequence was spanned 98.3% of the genome (FES_r1.0) was considered as the haploid genome sequence.

### Table 1. Statistics of the draft genome sequence (FES_r1.0)

| Statistic                          | Value          |
|-----------------------------------|----------------|
| Number of sequences               | 387,594        |
| Cumulative length of sequences    | 1,177,687,305  |
| Average length of sequences per contig | 3,038         |
| Max length of sequences           | 1,053,114      |
| Min length of sequences           | 300            |
| N50 length (bases)                | 25,109         |
| Number of undetermined bases      | 309,030,247    |
| G ± C% (GC/ATGC)                  | 39.1           |

The draft genome sequence spanned 98.3% of the genome sequence (FES_r1.0) was considered as the haploid genome sequence. The draft genome sequence spanned 98.3% of the genome sequence (FES_r1.0) was considered as the haploid genome sequence. The draft genome sequence spanned 98.3% of the genome sequence (FES_r1.0) was considered as the haploid genome sequence.

#### 3.2. Gene prediction and annotation

Gene predictions were performed using Augustus 3.0.3 with the buckwheat training set (Method 1), Augustus 3.0.2 with the A. thaliana training set (Method 2), or geneid with the A. thaliana training set (Method 3), and the results obtained using the three methods (Methods 1–3) are summarized in Supplementary Table S4. If the genes were located at the same locus when using Methods 1–3, the longest gene was selected. After the results were integrated, the total length of the assembled genome sequence (FES_r1.0) was 2,126,917,911 bp composed of 286,768 CDSS, and N50 was 1,101 bp. The gene name was prefixed with a six-digit identifier followed by the prediction method and scaffold number (i.e. aug: Augustus 3.0.3, buckwheat training set, Method 1; aua: Augustus 3.0.2, A. thaliana training set, Method 2; and gai, geneid 1.4.4, A. thaliana training set, Method 3), as in the following example: Fes_sc0012271.1.g000001.aua.1.

Genes related to transposable elements (TEs) were inferred according to BLAST searches against the NCBI’s NR database (Supplementary Table S5). The total length of known repeats was 212,917,911 bp composed of 286,768 CDSS, and N50 was 1,101 bp. The gene name was prefixed with a six-digit identifier followed by the prediction method and scaffold number (i.e. aug: Augustus 3.0.3, buckwheat training set, Method 1; aua: Augustus 3.0.2, A. thaliana training set, Method 2; and gai, geneid 1.4.4, A. thaliana training set, Method 3), as in the following example: Fes_sc0012271.1.g000001.aua.1.

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### 3.2.1. Example 1: identifying buckwheat genes that regulate flavonoid biosynthesis

Buckwheat contains several kinds of flavonoids, such as flavonols, proanthocyanidins, and anthocyanins. The flavonol rutin is present at high levels in buckwheat seeds and seems to be beneficial for human health. Several genes encoding enzymes related to flavonoid biosynthesis in buckwheat have been reported and are presumed to be regulated by transcription factors (TFs), such as MYB, bHLH, and WD40, as in other plant species. However, little is known about such TFs in buckwheat. The R2R3-MYB TFs are thought to play central roles in plant-specific processes, based on their specific gene expression patterns. To provide an overview of genes that regulate plant-specific processes, including flavonoid synthesis in buckwheat, we searched for candidate genes encoding R2R3-MYB TFs using the BGDB.

By conducting a keyword search using the term ‘MYB’, we identified 274 genes predicted to encode MYB TFs. From these, we excluded partial sequences, pseudogenes, and genes that did not contain fully conserved R2R3 regions. The remaining 71 putative R2R3-MYB TFs obtained from the database are listed in Supplementary Table S9. Phylogenetic analyses based on the R2R3 domain often reveals functionally characterized groups that are present in a wide range of plant species. In the present study, six putative R2R3-MYBs were assigned within known functional groups consisting of representatives from other plant species (Supplementary Fig. S2). Though functional analyses would need to be conducted to determine the role of
each gene, this finding shows that R2R3-MYB genes, which likely have different roles, can successfully be obtained from the BGDB.

To initiate the transcription of genes encoding enzymes in the flavonoid biosynthetic pathway, a TF such as MYB or the MYB-bHLH-WD40 (MBW) complex must bind to TF binding sites (TFBSs) in the promoter region of each gene. Mutation of TFBSs alters the expression of genes. Therefore, MYB TFs as well as the TFBSs of target genes can be manipulated to improve flavonoid production. To identify promoter sequences in a non-model plant species, genome walking, which is time-consuming and expensive, would usually be performed. In this study, we tried to identify the promoter sequences of genes in the flavonoid biosynthetic pathway estimated the TFBSs using PLACE (http://www.dna.affrc.go.jp/PLACE/). cDNA sequences of nine genes in the pathway have already been registered in GenBank. For three of these genes (chalcone isomerase, CHL; flavonoid 3′-hydroxylase, F3′H; anthocyanidin synthase, ANS), we successfully determined the 1,000–2,000 bp upstream region after a BLASTN search against the BGDB. DNA motifs relating to the MYB or MBW complex predicted by PLACE analysis are shown in Supplementary Fig. S3. These results can be confirmed by molecular techniques such as gel-shift assays, as reported for other plant species (e.g. apple, persimmon, and soybean). The BGDB is thus a powerful tool for isolating promoter sequences and accelerating molecular-based analyses of TFs. In this study, however, we could not identify sufficiently long promoter regions (over 1,000 bp) for the remaining six genes, mainly because of gaps between contigs. Gap closing using long reads generated by a PacBio sequencer will greatly improve the ability to search for the promoter regions of target genes.

3.2.2. Example II: identifying a duplicate of a buckwheat allergen gene, Fag e 2

Buckwheat seeds contain allergens. For instance, Fag e 2 (16 kDa protein) is a pepsin-resistant 2S albumin that causes an immediate allergic reaction. Although Fag e 2 cDNA has been sequenced, no further genomic information is available. Efforts to develop hypoallergenic buckwheat and establish inspection techniques to minimize allergen contamination in food products require detailed genomic information of Fag e 2.

Figure 1. Assignment of proteins to KOG functional categories in the three plant species. Genes from F. esculentum (red), B. vulgaris (blue), and A. thaliana (green) were classified based on NCBi’s KOG database by performing BLAST searches with an E-value cut-off of 1E−4. KOGs were classified into functional categories. The percentage of KOGs in each functional category is plotted, and percentages are arranged in ascending order within each group. (A) RNA processing and modification; (B) chromatin structure and dynamics; (C) energy production and conversion; (D) cell cycle control, cell division, and chromosome partitioning; (E) amino acid transport and metabolism; (F) nucleotide transport and metabolism; (G) carbohydrate transport and metabolism; (H) coenzyme transport and metabolism; (I) lipid transport and metabolism; (J) translation, ribosomal structure, and biogenesis; (K) transcription; (L) replication, recombination, and repair; (M) cell wall/membrane/envelope biogenesis; (N) posttranslational modification, protein turnover, and chaperones; (P) inorganic ion transport and metabolism; (Q) secondary metabolites biosynthesis, transport, and catabolism; (R) general function prediction only; (S) function unknown; (T) signal transduction mechanisms; (U) intracellular trafficking, secretion, and vesicular transport; (V) defense mechanisms; (W) extracellular structures; (Y) nuclear structure; and (Z) cytoskeleton. Note that KOGs in Groups 1–3 are shown, and that fewer than 10 KOGs were assigned to category N (cell motility) in the three species and were excluded.

Table 2. Fag e 2 and its homologues obtained by BLASTP search for buckwheat genome database

| Gene ID of the BGDB | Scaffold ID | Similarity with reported allergen of F. esculentum | E-value |
|---------------------|------------|---------------------------------------------------|---------|
| Fes_sc0000087.1.g0000011.aua.1 | Fes_sc0000087.1 | 97% (BW 8 kDa allergen protein) | 8e−74 |
| Fes_sc0000087.1.g0000013.aua.1 | Fes_sc0000087.1 | 79% (Fag e 2) | 3e−26 |
| Fes_sc0000087.1.g0000014.aua.1 | Fes_sc0000087.1 | 100% (Fag e 2) | 7e−104 |
| Fes_sc0000087.1.g0000028.aua.1 | Fes_sc0000087.1 | 98% (BW 8 kDa allergen protein) | 5e−85 |
| Fes_sc0007211.1.g000003.aua.1 | Fes_sc0007211.1 | 43% (BW 8 kDa allergen protein) | 4e−21 |

Scaffold ID, ID number of the scaffold in which the predicted gene is situated; BW 8 kDa, buckwheat 8 kDa allergen of 2S albumin; Fag e 2, buckwheat 16 kDa allergen of 2S albumin.

The GenBank accession numbers of BW 8 kDa and Fag e 2 are AB055892 and DQ304682, respectively.
A BLAST search of Fag e 2 (accession number: DQ304682) among the predicted proteins in the BGDB yielded one identical gene (Fes_sc0000087.1.g0000014.aua.1) and four homologues (Fes_sc0000087.1.g0000087.1.g0000011.aua.1, Fes_sc0000087.1.g0000087.1.g0000028.aua.1, and Fes_sc0007211.1.g000003.aua.1) (Table 2). The results of a BLAST search against the NCBI nr database indicated high similarities of four homologues with previously reported allergens of buckwheat. As shown in Fig. 2, the predicted amino acid sequences of Fes_sc0000087.1.g0000011.aua.1 and Fes_sc0000087.1.g0000028.aua.1 showed high levels of similarity (97 and 98%, respectively), with the buckwheat 8 kDa allergen, which is an important part of the human diet and has industrial applications. Starch contains two types of glucose polymer, i.e. amylepectin and amylose.61 Amylopectin is the major component of starch (60–90%), whereas the amylose content varies among plant species.63 Since the amylose content affects the properties of starch, modulating the amylose content has been an important breeding objective in crops.64 GBSS catalyses amylose synthesis.65 Thus, we searched for GBSS genes encoding starch synthases (SSs) from the BGDB, and aimed to identify GBSS genes using phylogenetic approaches.

To identify buckwheat GBSS genes, we conducted a keyword search using ‘starch synthase’ and obtained 42 hits. We then filtered these hits using modified five conserved sequence motifs proposed by Cao et al.66 [i.e. P(2)K(1)GGL(1)D(4)L, VS(5)E, G(2)NG(7)P(2)D, R(3)QKG, D(5)S(2)EPC(1)L(1)Q(5)YG(8)GGL (numbers in parentheses represent numbers of amino acids)]. Of the eight resulting sequences, one was excluded, as it was annotated as a pseudogene. The seven remaining sequences were each derived from a different scaffold.

To assign these seven putative SSs of buckwheat to previously proposed phylogenetic groups,67 we performed NJ analysis using the deduced amino acid sequences of SSs from various plant species. A keyword search using ‘starch synthase’ was also conducted in Phytozome 10.3 (http://phytozome.jgi.doe.gov/) analysing 36 angiosperm species (Supplementary Table S10). Of the 27,852 sequences identified, 238 sequences remained after the same filtering procedure as mentioned earlier and were subjected to phylogenetic analysis. Two GBSS sequences of Fagopyrum species, one from F. esculentum deposited at the EMBL/GENBANK/DDBJ (accession number: HW041459) and the other from F. tataricum (AHA36967.1),68 were also included in the analyses. A NJ-tree based on the alignment was suggested to show that the deduced amino acid sequences of the buckwheat SSs formed two distinct phylogenetic groups, which can be distinguished by the number of cysteine residues. The first group contains the predicted proteins of Fes_sc0007211.1.g000003.aua.1, and the other from F. tataricum (AHA36967.1),68 which were also included in the analyses. A NJ-tree based on the alignment was suggested to
contain the following five known phylogenetic groups:SSI, SSII, SSIII, SSIV, and GBSS (Supplementary Fig. S4). The SS sequences obtained from the BGDB belonged to four of the five classes. This suggests that the BGDB can be used to identify agronomically important genes. However, the previously deposited GBSS (HW041459) sequence is not identical to any of the seven sequences identified in the BGDB, and a BLASTN search using the coding region of HW041459 as a query detected only partially identical sequences over 360-bp and three scaffolds (Fes_sc00195744.1, Fes_sc0059460.1, and Fes_sc0005470.1). This is a shortcoming of the short scaffold size of the assemblies in the BGDB.

The GBSS clade contained four phylogenetically distinguishable sequences in total: HW041459 and three from BGDB. To clarify the detailed phylogenetic relationship among these four GBSS genes, we performed NJ analysis based on 71 aligned amino acid sequences of GBSS including those from Physcomitrella patens as outgroup (Fig. 3). The copy number of GBSS genes varies in plants; two diverged groups exist in the rosids and several copies of GBSS in buckwheat seem to also have diverged, at least in two lineages. In the cladogram, a GBSS sequence, Fes_sc0004292.1.g000004, clustered with a GBSS sequence from F. tataricum, which belongs to the same genus. GBSS of F. tataricum was confirmed to be expressed in the endosperm, thus Fes_sc0004292.1.g000004 is likely to be expressed in the endosperm too. If more than one GBSS is active within endosperms, the amylose content of buckwheat flour can be controlled by altering their copy number. In hexaploid wheat, Yamamori and Quynh evaluated the dosage effects of three GBSS genes. Loss of function of these genes is expected to differentiate the starch properties of the grain; moreover, distinct proportions of amylose might be produced according to copy number of active GBSS genes. Studies are underway to examine the expression of the three buckwheat GBSS genes identified here, and also the previously identified one (HW041459).

3.2.4. Example IV: isolation of heteromorphic SI genes

Finally, we screened for candidate genes that control heteromorphic SI in buckwheat. Buckwheat is a heteromorphic self-incompatible crop with dimorphic flowers (i.e. short-styled and long-styled flowers). Short-styled flowers have short styles and long stamens, whereas long-styled flowers have long styles and short stamens. The SI response is expressed between plants bearing the same flower morph, but not between plants bearing different flower morphs. Flower morph and SI response are determined by a diallelic system at the SELF-INCOMPATIBILITY supergene complex locus (S locus); Ss heterozygotes and s/s recessive homozygotes bear short-styled and long-styled flowers, respectively. Recently, S-LOCUS EARLY FLOWERING 3 (S-ELF3), which controls the short-styled phenotype of buckwheat, was isolated. Furthermore, it was suggested that recombination is strongly suppressed around S-ELF3. Based on these findings, we predicted that S-allelic scaffolds exist in which heteromorphic SI-related genes other than S-ELF 3 are located. Thus, we tried to detect S-allelic scaffolds in the draft genome and to identify novel candidate genes involved in the SI response in these.

To obtain S-allele-linked scaffolds from the draft genome, we used GB reads obtained from each of 18 short-styled and 18 long-styled landraces of buckwheat originating from various countries (Supplementary Table S1). Briefly, GB reads from 36 plants were mapped to scaffolds of >1,000 bp, and we subsequently extracted the non-LS sites (i.e. sites not present in all the long-styled plants) in which no reads from all of the 18 long-styled plants were mapped. The number

Figure 3. NJ tree based on amino acid sequences of GBSS from buckwheat and other plant species. The bootstrap values (500 replicates) not<n<50 are shown next to the branches. The scale bar corresponds to 0.05 substitutions per site. Two GBSSs from Physcomitrella patens were used as outgroup sequences. Species names are coloured according to their order: Poales, grey; Ranunculales, cyan; Vitales, purple; Cucurbitales, blue grey; Fabales, green; Malpighiales, blue; Rosales, pink; Myrtales, teal; Brassicales, indigo; Malvales, brown; Sapindales, orange; Caryophyllales, red; Lamiales, yellow; and Solanales, lime. Four GBSSs from F. esculentum are indicated by red circles next to the sequence names. Sequences obtained from BGDB are abbreviated (sc00002521: Fes_sc00002521.1.g000004; sc0004292: Fes_sc0004292.1.g000004; and sc00005258: Fes_sc00005258.1.g000004). Two sequences of Fagopyrum species (AHA36967 and HW041459) were obtained from GenBank. Sequences excluding those from Fagopyrum species were obtained using Phytozome 10.3 and the accession numbers are in parentheses.
Plants were dehydrogenated with the restriction enzyme combination (EcoRI and Msel) to obtain the GBS reads. Sites at which >50 reads were mapped in long-styled plants but not in short-styled plants were defined as ‘non-LS’. Sites at which >50 reads were mapped in short-styled plants but not in long-styled plants were defined as ‘non-SS’. The number of non-LS (blue bar) and non-SS sites (red bar) was plotted against the number of short-styled plants sharing the non-LS sites and of long-styled plants sharing the non-SS sites, respectively.

Figure 4. The number of non-LS sites and non-SS sites identified in GBS reads. Sites at which >50 reads were mapped in long-styled plants but not in short-styled plants were defined as ‘non-SS’. Sites at which >50 reads were mapped in short-styled plants but not in long-styled plants were defined as ‘non-LS’. The number of non-LS (blue bar) and non-SS sites (red bar) was plotted against the number of short-styled plants sharing the non-LS sites and of long-styled plants sharing the non-SS sites, respectively.

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4. Conclusion and future perspective

The genome size of buckwheat is relatively large (~1.2 Gb), and some genomic regions are expected to be in the heterozygous state due to its outcrossing nature. These factors would reduce the lengths of our scaffolds in the buckwheat draft genome, which was assembled using only Illumina short reads. Though the draft genome was truncated and divided into a large number of scaffolds (387,594 scaffolds), we have successfully identified genes that control agronomically important traits using gene predictions and subsequent annotations in the BGDB. Furthermore, we also identified novel candidate genes involved in heteromorphic SI of buckwheat using the draft genome as a reference sequence for GBS mapping. Even if the scaffolds in a draft genome are truncated, they can nonetheless be used for database construction and as a reference sequence for NGS-based genetic markers. We are now preparing induced mutant pools of buckwheat using heavy-ion beams and chemicals such as ethyl methanesulfonate. Using NGS-based multi-dimensional screening, mutants of the genes identified in this study will be rapidly identified from the pool and will be used to develop superior varieties of buckwheat.
5. Data availability
The Illumina reads used in this study are available from DDBJ/EMBL/NCBI under the accession numbers listed in Supplementary Table S3. The BRAK data accession number of the Illumina reads used in GBS analysis is DRA004489. The scaffold sequences are available under the accession numbers BCYN01000001-BCYN01387594 (387,594 entries).

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Supplementary data
Supplementary data are available at www.dnaresearch.oxfordjournals.org.

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