Breeding Science 70: 13–18 (2020)
doi: 10.1270/jsbbs.19075

Invited Review

History of the progressive development of genetic marker systems for common buckwheat

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Genotyping is an essential procedure for identifying agronomically useful genes and analyzing population structure. Various types of genetic marker systems have been developed in common buckwheat (*Fagopyrum esculentum* Moench). In the 1980s, morphological and allozyme markers were used to construct linkage maps. Until the early 2000s, allozyme markers were widely used in population genetics studies. Such studies demonstrated that cultivated common buckwheat likely originated in the Sanjiang area of China. In the late 1990s and early 2000s, advances in PCR technology led to the development of various DNA marker systems for use in linkage mapping. However, PCR-based markers did not completely cover the genome, making genetic analysis of buckwheat challenging. The subsequent development of next generation sequencing, a game-changing technology, has allowed genome-wide analysis to be performed for many species. Indeed, 8,884 markers spanning 756 loci were recently mapped onto eight linkage groups of common buckwheat; these markers were successfully used for genomic selection to increase yield. Furthermore, draft genome sequences are now available in the Buckwheat Genome DataBase (BGDB). In this review, I summarize advances in the breeding and genetic analysis of common buckwheat based on contemporary genetic marker systems.

Key Words: AFLP, allozyme, common buckwheat, GBS, genetic marker, RAPD, SSR.

Introduction

Since genetic markers were first used for linkage mapping of Drosophila (Sturtevant 1913), advanced linkage analysis has been performed in many species based on genes for morphological traits, confirming that genes are arranged linearly on chromosomes. Allozyme analysis subsequently became possible due to the development of electrophoresis technology and revealed that allozyme polymorphisms are maintained in many plant species (Gottlieb 1981). Morphological traits and allozyme polymorphisms were successfully used to construct chromosome maps and to analyze population structure in common buckwheat (*Fagopyrum esculentum* Moench: $2n = 2x = 16$), a traditionally important pseudo cereal in Asian and European countries in the temperate zone (Ohnishi and Ohta 1987 for chromosome mapping; Ohnishi 1988, 1993a, 1993b, 2009 for population structure analysis). Common buckwheat is an outcrossing species with heteromorphic self-incompatibility due to its dimorphic (i.e., short- and long-styled) flowers and intramorph infertility (reviewed by Matsui and Yasui 2019). Thus, Ohnishi and his colleague used sib-crosses to elucidate the linkage relationships among morphological and allozyme markers.

In the late 1990s and early 2000s, DNA polymorphism analysis using Polymerase Chain Reaction (PCR) was commonly performed. Random Amplification of Polymorphic DNA (RAPD) (Williams *et al.* 1990) and Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.* 1995) were used as molecular markers in common buckwheat, and high-density maps and DNA markers linked to agronomically important genes were developed (Aii *et al.* 1998 for RAPD markers linked to a self-pollinating gene; Matsui *et al.* 2004 for AFLP markers linked to a shattering gene; Yasui *et al.* 2004 for genome-wide AFLP markers). Simple Sequence Repeat (SSR) (Litt and Luty 1989) and Expression Sequence Tag (EST) markers were subsequently developed for many organisms. SSR markers were used for population genetic analysis and the assessment of genetic diversity in common buckwheat (Iwata *et al.* 2005, Konishi and Ohnishi 2007, Ma *et al.* 2009), and EST markers were used to identify candidate photoperiod-sensitivity genes in this crop (Hara *et al.* 2011). As for other crops, PCR-based markers have been developed for common buckwheat. However, genotyping polymorphisms using PCR techniques requires labor-intensive electrophoresis: only a few hundred such markers have been developed for each marker system,

Communicated by Ryo Ohsawa
Received May 10, 2019. Accepted October 14, 2019.
First Published Online in J-STAGE on January 30, 2020.
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which suggests the difficulty in covering entire genome.

From 2010 onwards, Next Generation Sequencing (NGS) has been used in breeding and genetics studies of common buckwheat, and a DNA microarray marker system has been constructed based on NGS technology. DNA microarray markers designed from genome sequences obtained by NGS were used for quantitative trait locus (QTL) analysis and genomic selection in common buckwheat (Yabe *et al.* 2014, 2018). In addition, a draft genome of common buckwheat was published in 2016, and genome-wide Genotyping By Sequencing (GBS, Elshire *et al.* 2011) analysis was carried out using this draft genome sequence as a reference (Yasui *et al.* 2016). Therefore, genome-wide markers for common buckwheat can now be used. The price of NGS is expected to greatly decrease, allowing genome-wide genotyping to easily be performed via genome re-sequencing and/or RNA sequencing (RNA-seq) based on NGS technology. Scientists will simply need to extract DNA from plants for genotyping in plant breeding and genetics studies.

In the near future, there will be no need to construct a marker system, as genome re-sequencing and/or RNA-seq will permit identification of genome-wide markers at low cost with little labor. Thus, we anticipate that a new era will emerge in which phenotypic assessments will be the key procedure in breeding and genetic studies. This will clearly benefit the breeding and genetic analysis of under-studied crops for which molecular marker systems have not been established. In this review, I summarize the major results of breeding and genetics studies of common buckwheat using each marker system developed to date. I hope this information will facilitate the breeding of common buckwheat in this new era.

Morphological markers

Using sib-crosses, recessive morphological mutants of common buckwheat have been identified and used as genetic markers, primarily by Ohnishi and colleagues (Ohnishi and Nagakubo 1982, Ohnishi and Ohta 1987, Ohnishi 1990a). These authors confirmed 37 morphological traits controlled by single genes and revealed linkage relationships among 22 genes in this crop (reviewed in Ohnishi 1990a). Although these morphological markers may be expected to be outdated and obsolete, they have useful applications in buckwheat breeding. For example, these morphological trait markers include seven dwarf trait genes. Due to climate change, typhoons have caused serious damage in regions that previously experienced little damage, such as the northern part of Japan, including the Hokkaido area. Introducing lodging resistance is an important goal of common buckwheat breeding, and these dwarf genes are thought to represent a useful resource (Morishita *et al.* 2015). Moreover, four independent genes controlling the green stem trait have been mapped in common buckwheat using allozyme linkage analysis (Ohnishi 1990a). Mutants with defects in these genes are thought to be unable to accumulate anthocyanidin in the lower part of the stem. Due to the health-promoting properties of flavonoids such as anthocyanidin, which has antioxidant activity, these compounds have been a focus of study for common buckwheat breeding (Matsui *et al.* 2008, 2016). Mutants with defects in these compounds can be used to study the flavonoid biosynthetic pathway in common buckwheat.

Allozyme markers

In addition to morphological markers, Ohnishi and colleagues identified various allozyme variants in common buckwheat. Based on F_2 segregation data obtained from more than 100 pairwise sib-crosses, Ohnishi and Ohta (1987) constructed the first linkage map of common buckwheat, containing six allozyme and 15 morphological markers. Furthermore, Ohnishi (1993b) clarified the worldwide population structure of common buckwheat using 64 populations primarily obtained from Asian countries, such as Japan, China, Nepal, and Korea. In this study, 19 allozyme loci were examined in 200 individuals per population. The average heterozygosity of an allozyme locus in a cultivated population was found to be fairly high (ranging from 0.110 to 0.138) (Ohnishi 1993b). This value is higher than that of other outcrossing plants, including wild plant species (0.086 ± 0.017, average heterozygosity value for 21 species) (Gottlieb 1981). Ohnishi did not detect notable population differentiation among cultivated buckwheat populations worldwide, although decreased variation was observed in the outer edges of cultivated areas, such as in parts of India and Europe (reviewed in Ohnishi 1993b). High levels of heterozygosity and low levels of population differentiation coincide with the outcrossing nature of cultivated common buckwheat, pointing to the large population size and high migration rate among populations.

Allozyme markers have also been used to help clarify the origin of cultivated common buckwheat. In 1991, Ohnishi discovered the first wild ancestral species of cultivated common buckwheat (*F. esculentum* ssp. *ancestrale*), in Yunnan Province, China (Ohnishi 1990b). After an extensive investigation by Ohnishi (2009), new wild populations were discovered in Sichuan Province and the Tibet Autonomous Region of China. An analysis using 19 allozyme markers for wild and cultivated populations from Southern China (Yunnan, Sichuan, and Tibet) revealed that the wild ancestor is genetically more differentiated among populations than cultivated buckwheat and that cultivated populations are closely related to the wild population in the Sanjiang area, where the Yangtze River, Mekong River, and Salween River flow in parallel, forming steep-walled valleys (Ohnishi 2009). The extensive allozyme marker system established by Ohnishi and colleagues was successfully used for population genetics analysis, which suggested that the origin of cultivated common buckwheat is the Sanjiang area of China.

Breeding Science
Vol. 70 No. 1 BS

PCR-based DNA markers I: RAPD and AFLP markers

In the RAPD method, a specific DNA region as short as 10 bp is amplified by PCR using random primers at a relatively low annealing temperature. Using one random primer, several sizes of DNA fragments amplified by PCR are detected on an agarose gel and are generally scored as dominant markers. This low-cost method is not labor intensive. However, due to the use of a short primer with low annealing temperatures, the reproducibility of DNA amplification (i.e., genotyping) using this method is poor. To improve the stability of PCR amplification, the use of Sequence Characterized Amplified Regions (SCAR) markers (Paran and Michelmore 1993) has been proposed. SCAR markers are DNA fragments amplified by specific 15–30 bp primers. The primers are designed based on the nucleotide sequences of RAPD fragments. Using longer PCR primers, SCAR markers can be reproducibly amplified. Various RAPD and RAPD-derived SCAR markers have been used as linkage markers in buckwheat. For instance, RAPD markers linked to the self-pollination gene in the buckwheat species *F. homotropicum* were identified and converted into highly reproducible SCAR markers (Aii *et al.* 1998). Although RAPD markers lack reproducibility, they have been used in phylogenetic studies of common buckwheat, as they are cheap and easy to produce. For example, a study using RAPD markers revealed that *F. himalianum*, a buckwheat species found in the Himalayan region of India, should be considered a race of *F. esculentum* (Rout and Chrungoo 2007). In addition, an analysis of the diffusion pathway of common buckwheat using RAPD markers suggested that Japanese common buckwheat cultivars were transmitted from China to Japan via Korea (Murai and Ohnishi 1996). However, since the authors did not use samples from eastern China and only 40 RAPD primers were used, this possibility requires further verification.

To overcome the low reproducibility of RAPD markers, a novel DNA fingerprinting technique called AFLP was developed (Vos *et al.* 1995). Using this technique, restriction enzyme-digested genomic DNA fragments are ligated to oligonucleotide adapters and amplified using PCR primers designed based on the adapters. Typically, 50–100 restriction fragments are visualized on an acrylamide gel and are generally scored as dominant markers. The AFLP technique was used to develop the first chromosome maps of common buckwheat using genome-wide markers (Yasui *et al.* 2004). The authors succeeded in mapping 223 AFLP markers in the common buckwheat genome and showed that it is possible to prepare linkage markers of useful genes using AFLP methods. However, AFLP method contains time- and money-consuming steps, such as restriction enzyme reaction, ligation reaction and electrophoresis using acrylamide gels. So, AFLP markers to screen hundreds of plants must be converted into SCAR markers. Indeed, AFLP-derived SCAR markers linked to a self-incompatibility gene (Yasui *et al.* 2008) and a shattering gene (Matsui *et al.* 2004) have been developed. Yasui *et al.* (2008) constructed a Bacterial Artificial Chromosome (BAC) buckwheat library; the availability of genome-wide AFLP markers and the construction of a BAC library finally allowed buckwheat genes to be identified by map-based cloning. Moreover, since AFLP analysis can be used to detect many DNA fragments, AFLP banding patterns have been used for phylogenetic analysis. Konishi *et al.* (2005) compared 396 AFLP dominant markers (i.e., AFLP bands) obtained from wild and cultivated common buckwheat from Yunnan Province, Sichuan Province, and the Tibet Autonomous Region of China. Cluster analysis revealed that cultivated and wild species in the Sanjiang area could be grouped into one highly reliable phylogenetic group (bootstrap value = 100), coinciding with the results of the abovementioned allozyme analysis by Ohnishi (2009). The AFLP method has greatly facilitated studies of the genetics and breeding of buckwheat, making it possible to genotype large numbers of markers with high accuracy.

PCR-based DNA markers II: simple sequence repeat (SSR) and expressed sequence tag (EST) markers

AFLP and RAPD markers must be converted to SCAR markers for increased convenience and reliability. Therefore, SSR and EST markers, which do not require SCAR conversion, have also been used as DNA markers. SSR markers contain hyper-variable repeat polymorphisms, such as simple di- or tri-nucleotide repeats. Using acrylamide gel-electrophoresis, SSRs are detected as co-dominant length polymorphism markers. The first SSR marker system in common buckwheat was described by Iwata *et al.* (2005). Using five SSR markers, the authors compared the daylength responses of three agro-ecotypes (summer, intermediate, and late-summer types) of Japanese common buckwheat and revealed lower diversity of the summer type than the other types. Konishi *et al.* (2006) developed 54 marker sets, and a common buckwheat linkage map consisting of SSR and AFLP markers were constructed (Konishi and Ohnishi 2006). SSR markers have also been used to demonstrate that there is a low level of gene flow between wild and cultivated common buckwheat (Konishi and Ohnishi 2007). Furthermore, Ma *et al.* (2009) developed 136 SSR markers, and this system was mainly used to investigate genetic diversity in Korean common buckwheat varieties (Ma *et al.* 2009, Song *et al.* 2011).

Studies using allozyme (Ohnishi 1993b) and SSR (Iwata *et al.* 2005) markers have revealed a high level of intra-species diversity in common buckwheat. Therefore, DNA polymorphisms are likely present even in ‘conserved’ EST regions in common buckwheat, allowing markers to be developed for this crop. Hara *et al.* (2011) designed 170 PCR primer sets based on EST sequences, and by

examining length polymorphisms and restriction enzyme site polymorphisms of the PCR products, successfully elucidated the linkage relationships among 63 co-dominant markers. The authors also performed QTL analysis of photoperiod sensitivity, representing a milestone for QTL analysis in common buckwheat. Thus, advances in population genetics and breeding studies of common buckwheat have been greatly facilitated by the development of SSR and EST markers.

NGS-based markers: microarray analysis and GBS

Since 2010, NGS technology has been used in genetic and breeding studies of common buckwheat. Microarray probes (50–75 bp long) were designed based on a partial genome sequence of this crop obtained by NGS (Yabe *et al.* 2014). In this study, the pseudo-testcross strategy (Grattapaglia and Sederoff 1994) was carried out for linkage analysis. Genome-wide linkage array-based markers (8,884 markers spanning 756 loci) were mapped onto eight linkage groups (the number of chromosomes in common buckwheat), and a QTL controlling main stem length was successfully identified. This microarray technique was also successfully used for genomic selection to increase buckwheat yields (Yabe *et al.* 2018).

In 2016, a draft genome sequence for buckwheat was published and the Buckwheat Genome DataBase (BGDB) was established (Yasui *et al.* 2016). Local BLAST (Altschul *et al.* 1990) and keyword searches for gene names and their annotations can now be performed using the BGDB. Several agronomically useful genes have already been identified from the BGDB (Yasui *et al.* 2016). Furthermore, GBS analysis using this draft genome as a reference was carried out to detect the genomic region specific to common buckwheat plants with short-styled flowers, namely *S*-allele linked region (Yasui *et al.* 2016). Using genome-wide genotype data obtained by GBS analysis, Mizuno and Yasui (2019) detected 255,517 SNP sites (one SNP per 28 bp) from 46 cultivated common buckwheat plants and revealed their high genetic diversity. The nucleotide diversity of common buckwheat (0.0065) is comparable to that of other outcrossing plants, such as *Zea mays* (0.0064; Wright *et al.* 2005) and *Helianthus annuus* (0.0056; Liu and Burke 2006) and higher than that of selfing plants such as *Oryza sativa* (0.0024, Huang *et al.* 2012) and *Glycine max* (0.0019, Lam *et al.* 2010). These studies helped confirm the validity of the draft genome of common buckwheat for use in genomics-assisted breeding and population genetics research.

Mizuno and Yasui (2019) demonstrated that cultivated common buckwheat comprises two genetically distinct groups, i.e., the Asian and European types, and that the degree of differentiation between these groups is low, as shown in previous studies (Ohnishi 1993a, 1993b). In addition, based on phylogenetic analysis using *S*-allele linked region, where recombination is suppressed, they showed that common buckwheat has largely differentiated into three groups. This finding suggests that gene flow likely occurred from wild to cultivated buckwheat and that cultivated common buckwheat may have had multiple origins. The authors concluded that the higher nucleotide diversity of cultivated common buckwheat is likely due to buckwheat's outcrossing mating system and/or gene flow from wild buckwheat.

Genome-wide genotyping can easily be performed via genome re-sequencing and/or RNA-seq. High-density marker information can be obtained at low cost and with

Table 1. Summary of the milestone studies associated with the development of genetic marker systems for common buckwheat

Year	Genetic marker type	Major findings	References
1987	Morphological and allozyme	World's first linkage map of common buckwheat was constructed, with six allozyme and 15 morphological markers.	Ohnishi and Ohta 1987
1993	Allozyme	Results obtained from allozyme analyses using 160 worldwide populations (32,000 samples) with 19 loci were summarized, revealing high diversity and low population differentiation.	Ohnishi 1993b
1998	RAPD and SCAR	DNA markers for an agronomically important self-compatible gene (*Ho*) were developed.	Aii *et al.* 1998
2004	AFLP	Chromosome maps of common buckwheat using genome-wide markers were developed for the first time.	Yasui *et al.* 2004
2006	SSR	Microsatellite markers (54 loci) for common buckwheat were developed.	Konishi *et al.* 2006
2011	EST	QTL analysis using 50 EST markers uncovered photoperiod-sensitivity genes.	Hara *et al.* 2011
2014	NGS-based DNA array	Using DNA microarrays, a high-density linkage map (756 loci and 8,884 markers) was constructed.	Yabe *et al.* 2014
2016	NGS	Draft genome sequences were decoded, the genome database (BGDB) was established, and several agronomically useful genes were identified from the BGDB.	Yasui *et al.* 2016
2018	NGS-based DNA array	Genomic selection using 14,598–50,000 markers resulted in a 20.9% increase in the selection index compared to the initial population.	Yabe *et al.* 2018
2019	GBS	GBS detected 255,517 SNP sites from 46 cultivated common buckwheat plants, pointing to the likelihood of gene flow from wild to cultivated buckwheat.	Mizuno and Yasui 2019

low labor input. Therefore, NGS technologies will greatly advance plant breeding and genetics studies.

Perspectives

In summarizing the genetic marker systems for common buckwheat, it became clear that Japanese scientists have been global leaders in constructing genetic marker systems for this crop (**Table 1**). Genome re-sequencing and/or RNA-seq using NGS will no doubt play a leading role in this field. The common buckwheat genome will need to be fully decoded to produce reference sequences for short read mapping. Indeed, genome assembly mapping of common buckwheat using DenovoMAGIC™ (NRGene, Israel) is currently underway (https://www.nrgene.com/nrgene-assembles-buckwheat-genome/), and the complete genome assembly will soon be released. In this new era, we will be able to focus on identifying agronomically important phenotypic traits; the most important goals are to create mating populations and to establish/manage genetic resources for common buckwheat, including landraces and wild species. In addition, a genome-editing system should be constructed to facilitate the development of robust new common buckwheat varieties. The complete decoding of the common buckwheat genome will also be useful for designing genome-editing vectors.

Author Contribution Statement

YY wrote the main text and prepared the table.

Acknowledgments

This study was supported by JSPS KAKENHI Grant Numbers 18H02177 and 18KK0172.

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