Chapter 10
Genomics-Assisted Allele Mining and its Integration Into Rice Breeding

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Abstract Understanding the association between nucleotide changes and phenotypic changes is necessary for germplasm enhancement but has been a significant challenge in the molecular genetics and breeding of rice. In this article, we summarize our efforts to develop plant materials such as chromosome segment substitution lines to enhance the genetic analysis of traits of interest. The power of genetic dissection of phenotypic traits by use of novel populations is illustrated by our genetic analysis of heading date. We also present examples of the discovery of useful alleles involved in disease resistance and drought avoidance. Finally, we describe the discovery of genome-wide single-nucleotide polymorphism, which facilitate genetic analysis. This new type of genetic marker has allowed us to uncover the genome architecture of modern cultivars in Japan. These areas of progress will gradually change the landscape of selection in rice breeding.

10.1 Introduction

It has been more than 8 years since the whole genome sequence of rice was published (IRGSP 2005). This information has contributed to improved selection strategies for rice breeding (marker-assisted selection: MAS) as well as to the functional analysis of rice genes. Elucidation of the associations between nucleotide changes and phenotypic changes is necessary to most effectively use MAS but has been a significant challenge in the molecular genetics and breeding of rice. Over the last decade, many studies have been performed to clarify the relationships between sequence variation and phenotypic changes (Yamamoto et al. 2009; Yonemaru et al. 2010). To enhance our ability to genetically dissect complex phenotypes, appropriate plant materials are needed. Discovery of genes with economic value can be achieved most effectively through the use of plant materials specifically designed for use in molecular analysis (Fukuoka et al. 2010a). These efforts have already resulted in the discovery and use of several new genes in modern rice breeding programs

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(Fukuoka et al. 2009; Uga et al. 2011). To facilitate allele mining using the novel plant materials that have been developed, several studies have also been undertaken for genome-wide discovery of single-nucleotide polymorphisms (SNPs) (McNally et al. 2009; Huang et al. 2010; Ebana et al. 2010; Nagasaki et al. 2010; Yamamoto et al. 2010; Arai-Kichise et al. 2011; Zhao et al. 2011). These SNPs facilitate cost- and labor-effective analysis of a large number of individuals in genetic studies and breeding programs (Meuwissen et al. 2001; Bernardo and Yu 2007; Nordborg and Weigel 2008; Huang et al. 2010; Zhao et al. 2011). These advances in plant materials and molecular markers are acting as a force to change the landscape of selection in rice breeding. In this article, we summarize our recent activity in developing resources such as plant materials and genetic markers, and we discuss the application of these tools to rice breeding. In the last decade, new technologies and enabling platforms have revolutionised our ability to dissect genomes and decipher gene function not only in rice, but also other crop species (Yano and Tuberosa 2009). Therefore, we believe that the strategy described in this paper can also be integrated with other novel approaches enhancing allele mining and its application to improve rice and other crops.

10.2 Discovery of Natural Variation for Use in Rice Breeding

10.2.1 Developing Advanced Plant Materials from Diverse Rice Accessions

Genetic dissection of the wide range of naturally occurring variation in rice has progressed substantially through the use of quantitative trait locus (QTL) analysis, resulting in molecular cloning of genes and loci of biological and agronomic interest. The success of these analyses depends strongly on the plant materials used. Several types of plant materials, such as recombinant inbred lines (RILs), backcrossed inbred lines (BILs), introgression lines (ILs), and near isogenic lines (NILs), can be used in molecular genetic analysis (Fig. 10.1). Each type of population has its own advantages and disadvantages for use in genetic analysis. For example, F_2 and BC_1F_1 populations are most frequently used in genetic analysis because of the relatively short time needed for their development, but they also have several disadvantages in terms of the reproducibility and reliability of phenotyping. In such populations, the phenotype must be measured in individual plants, without replication, which sometimes compromises the reliability of genetic analyses such as the detection of QTLs. RILs can also be used for QTL mapping: these lines allow replication, thereby providing more statistical power for the genetic dissection of complex traits. Although RILs can be used for reliable phenotyping of many traits because they can be used in replicated trials, one major problem with RILs as well as other primary mapping populations is the variation in heading date. In general, progeny derived from a cross between diverse accessions often exhibit wide variation in heading date, and transgressive segregation is often observed. Several morphological and physiological

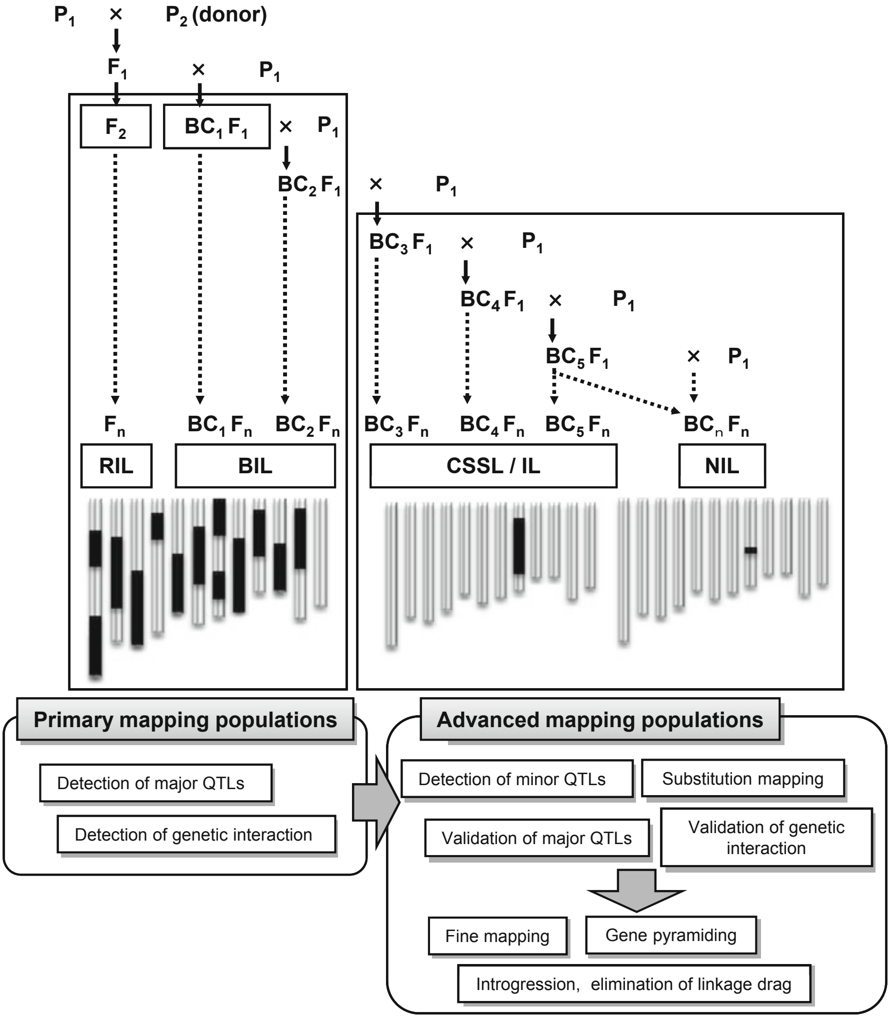

Fig. 10.1 Plant materials used for the genetic dissection of naturally occurring variation. *RIL* recombinant inbred line; *BIL* backcross inbred line; *CSSL* chromosomal segment substitution line; *IL* introgression line; *NIL* near-isogenic line

traits of agronomic interest, such as yield potential, eating quality, culm length, cold tolerance at the booting stage, and source (photosynthetic) ability, can be affected by heading date (reviewed by Fukuoka et al. 2010a). Therefore, it may be difficult to precisely evaluate such traits among segregants with large variation in heading date.

To more effectively detect relatively small allelic variations of agronomic value, it will be necessary to use mapping populations with a small range of variation in heading date and a more uniform genetic background. Eshed and Zamir (1995) proposed the novel concept of designing plant materials for genetic dissection based on their experience with a series of tomato introgression lines (ILs), each with one

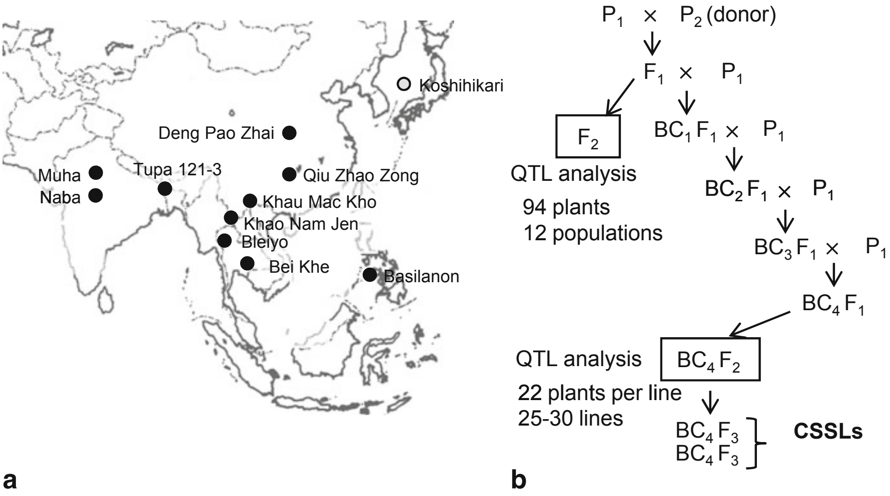

Fig. 10.2 (**a**) Geographical origin of Asian rice accessions. Koshihikari (*gray circle*) was used as a common parental strain for crosses with the 10 other accessions (*black circles*). (**b**) Pedigree of populations used in the analysis of heading date

or a few segment substitutions. So far, similar types of mapping populations have been developed as ILs and chromosome segment substitution lines (CSSLs) or single segment substitution lines (SSSLs) in rice (reviewed by Fukuoka et al. 2010a).

To develop CSSLs from multiple crosses, we selected 10 accessions from a rice core collection to comprehensively characterize the diversity of Asian cultivated rice (Kojima et al. 2005) (Fig. 10.2a). We selected these accessions based on the presence of sequence variation detected by means of restriction fragment length polymorphisms (RFLPs) and on their geographical origin (Kojima et al. 2005). The accessions originated from different regions of Asia and belong to three different cultivar groups: *japonica*, *aus*, and *indica* (Fukuoka et al. 2010a). Most of the plant materials are now at the stage of advanced backcross generations such as BC_4F_3 or BC_4F_4, and a set of CSSLs from these crosses will soon be available for public use. Once we have developed a series of CSSLs, a large-scale phenotyping experiment will be designed. By using CSSLs, the effects of variation in heading date should be minimized. Precise and reliable phenotyping of these lines will provide us more comprehensive understanding of rice morphological and physiological traits, allowing for a more effective mining of different types of alleles in diverse germplasms.

10.2.2 Genetic Architecture of Heading Date Revealed by Analysis of Novel Plant Materials

As noted above, a wide range of variation in heading date has been observed among rice cultivars. Although information on the genetic control of rice heading has accumulated rapidly (Izawa 2007; Tsuji et al. 2008), the genetic architecture underlying heading date variation still remains to be clarified because of the limited number of cross combinations used in the analyses. It has also been very difficult to precisely compare QTLs detected in different studies because of the different level of mapping resolution in each study (Yonemaru et al. 2010). In general, because QTLs are detected based on the allelic differences between parental lines, it is difficult to determine whether a particular QTL is shared among different cross combinations. Therefore, to understand the genetic factors controlling heading date in diverse germplasms, a common parental line should be used for the development of mapping populations.

To understand the genetic control of heading date, we previously performed a QTL analysis in 12 populations derived from crosses of the *japonica* cultivar Koshihikari, as a common parental line, with diverse cultivars that originate from various regions in Asia; 10 of these accessions have been used as donor cultivars for CSSL development (Fig. 10.2a). QTL analyses using multiple crosses revealed a comprehensive series of loci involved in natural variation in flowering time. Interestingly, the chromosomal locations of those QTLs corresponded well with the locations of QTLs detected in other studies, such as *Hd1, Hd3a, Hd6, RFT1*, *Ghd7*, and *DTH8* (*Ghd8*) (Xue et al. 2008; Wei et al. 2010; Ebana et al. 2011; Yan et al. 2011). Candidate genes for several of these QTLs have been cloned and sequenced, confirming the involvement of these genes in phenotypic variation. Taking together the allelic differences found in the QTL analyses and the sequence variation within candidate genes for heading date, Ebana et al. (2011) concluded that a large portion of the wide range of phenotypic variation in heading date and daylength response could be generated by combinations of different alleles, possibly representing both loss and gain of function, of the QTLs *Hd1*, *Hd2*, *RFT1*, *Ghd7*, *DTH8* (*Ghd8*), and *Hd6*/*Hd16* (Ebana et al. 2011; Shibaya et al. 2011). Although we successfully detected several major QTLs by using F_2 populations, it is very likely that some additional QTLs with minor effects might be also involved in the phenotypic variation in these F_2 populations. To detect QTLs with minor effects, we have been performing genetic analysis using advanced backcross progeny (BC_4F_2) (Fig. 10.2b). Preliminary results have clearly demonstrated that a limited number of additional QTLs may be involved in natural variation for heading date (unpublished data). Once we complete the development of the CSSLs, we will attempt to verify the results obtained in the analysis of the BC_4F_2 populations. Furthermore, we might discover additional QTLs with minor or epistatic effects under diverse environmental conditions using these CSSLs, leading to more comprehensive understanding of the genetic factors that control natural variation in the heading date of Asian cultivated rice.

As mentioned above in our discussion of heading date, CSSLs derived from different donor cultivars will be valuable resources for the genetic dissection of naturally occurring phenotypic variations in diverse germplasms. In addition, Koshihikari, the recurrent parent in the CSSLs, is a current leading cultivar in Japan. Therefore, if we identify traits of agricultural and economical value in a particular CSSL, the line itself can be used as new breeding material.

10.3 Genetic Dissection of Agriculturally Important Traits

10.3.1 Characterization of Durable Resistance Genes for Rice Blast

Rice blast caused by the fungal pathogen *Magnaporthe oryzae* is the most serious disease in rice. During the last decade, many studies have been performed to detect and clone major genes involved in race-specific resistance to rice blast, and a series of race-specific resistance genes have been isolated by map-based cloning. However, breeding efforts using race-specific resistance have not usually led to the development of varieties with durable resistance because of the rapid breakdown of resistance caused by the emergence of new blast fungus races (Fukuoka et al. 2009). Therefore, many geneticists and breeders have been interested in using genes for partial resistance to rice blast, which might be more durable in the long term.

To understand the genes and biological mechanisms involved in durable resistance, two genes for rice blast resistance, *pi21* and *Pb1*, have been cloned by map-based cloning (Fukuoka et al. 2009; Hayashi et al. 2010). *Pb1* is considered to be a durable resistance gene, and recently it has been clarified to encode a typical plant disease resistance gene of the NBS-LRR type. Introduction of *Pb1* from *indica* cultivars into *japonica* cultivars has been performed, and resistance has not yet broken down after 30 years. Japanese upland rice cultivars exhibit a high level of resistance to rice blast, and this resistance is considered to be durable. Although many efforts have been made to introduce the genes involved in such resistance into lowland rice cultivars, no success in developing new cultivars has been achieved. The main reason for the failure of these efforts has been linkage of blast resistance and low eating quality. The blast resistance found in upland rice cultivars has been genetically dissected (Fukuoka and Okuno 2001), and one of the QTLs, *pi21*, has been cloned (Fukuoka et al. 2009). *Pi21* encodes a putative heavy metal binding protein with proline-rich sequences. A combination of two deletions, each within a putative proline-rich motif thought to be important for protein–protein interaction, causes a loss-of-function mutation in the *Pi21* gene, which normally suppresses defense response. It should be noted that the rapid onset but slow development of the *pi21*-mediated defense response may be beneficial both for the optimization of disease control and for reducing detrimental effects on agricultural traits.

Although the molecular mechanism of durable resistance generated by *pi21* needs to be clarified further, the resistant allele of *pi21* found in a limited group of *japonica* rice cultivars is a potential source for improving the blast resistance of rice worldwide. These efforts have opened up the possibility of introducing durable resistance into Japanese lowland cultivars without simultaneously introducing low eating quality. The durable blast-resistance gene *pi21* was found to be closely linked (within a 40-kb distance) with one or more genes associated with inferior eating quality (Fukuoka et al. 2009). In that study, desirable recombinants between *pi21* and the genes conferring inferior eating quality were successfully selected from a large breeding population by using DNA markers for the region around *pi21*.

The availability of DNA markers closely linked to genes of interest has enabled a breakthrough in the development of durably resistant cultivars that had not been achieved during 80 years of conventional breeding in Japan. This is a clear example of the power of MAS in breeding that could not have been achieved without the determination of the precise location of the gene of interest.

10.3.2 Genetic Control of Rice Root Morphology

Rice roots play a key role in the absorption and translocation of water and nutrients, but breeding for improvement in root traits poses unique challenges. Quantification of root traits in fields, especially paddy fields, is laborious and time consuming because the root spreads in a complicated pattern underground. Nevertheless, many researchers have applied the best available evaluation methods under several environmental conditions to detect QTLs for root morphological and anatomical traits such as maximum length, thickness, volume, and distribution. Environments used in these studies have included field plots (Li et al. 2005; Yue et al. 2005; Uga et al. 2008), cylinders or pots (Champoux et al. 1995; Yadav et al. 1997; Ali et al. 2000; Price et al. 2000; Zheng et al. 2000, 2003; Zhang et al. 2001; Kamoshita et al. 2002a, b; Price et al. 2002; Venuprasad et al. 2002; Courtois et al. 2003; Yue et al. 2006), and hydroponic culture (Price and Tomos 1997). As a result, a large number of QTLs have been identified as candidates underlying the natural variation of root traits. Courtois et al. (2009) summarized 675 QTLs for root traits that were reported in previous studies.

Why have researchers focused so intently on the exploration of root QTLs? The main reason may be that root traits appear to enable the plant to avoid drought stress by absorbing water deposited in deep soil layers (Yoshida and Hasegawa 1982). The wide extent of natural variation of root traits in rice has been revealed in previous studies (O'Toole and Bland 1987; Lafitte et al. 2001; Uga et al. 2009). For example, upland rice typically shows thicker and deeper rooting than lowland rice (O'Toole and Bland 1987). This diverse natural variation in rice could be a useful resource for improving drought avoidance in the field (Yoshida and Hasegawa 1982; Fukai and Cooper 1995). In fact, some root QTLs have been used for MAS in drought-resistance breeding (Shen et al. 2001; Steele et al. 2006). Steele et al. (2006) performed MAS to introduce the Azucena (upland *japonica*) allele at several QTLs for root traits into Kalinga III (upland *indica*), which had not been used in previous QTL analyses. Some advanced progenies having the selected QTL alleles showed significantly improved root traits, although other lines containing these alleles did not show positive effect for root phenotype. Therefore, QTLs affecting root traits across multiple genetic backgrounds have yet to be identified.

Recently, several QTLs for root traits have been fine-mapped as single loci by using advanced progeny: *qRL6.1*, a QTL for root length, on chromosome 6 (Obara et al. 2010); *Sta1*, a QTL for stele transversal area, on chromosome 9 (Uga et al. 2010); *Dro1*, a QTL for deep rooting, on chromosome 9 (Uga et al. 2011); *qFSR4*, a QTL for root volume per tiller, on chromosome 4 (Ding et al. 2011); and *qSOR1*, a QTL associated with soil-surface rooting in paddy fields, on chromosome 7 (Uga et al. 2012). These root QTLs may be useful gene resources for improving rice production. For example, *DRO1* is expected to contribute to drought avoidance. *DRO1* was detected in RILs derived from a cross between the lowland cultivar IR64, with shallow rooting, and the upland cultivar Kinandang Patong (KP), with deep rooting (Uga et al. 2011; Uga et al. 2013). A near-isogenic line homozygous for the KP allele of *DRO1* (Dro1-NIL) in the IR64 genetic background showed significantly deeper roots than that of IR64 under upland field conditions. Moreover, the Dro1-NIL plants had a significantly larger panicle weight than that of IR64 under upland, drought-stressed conditions, suggesting that *DRO1* is involved in drought avoidance. Thus, QTL cloning and development of advanced progeny containing target QTLs within a defined genetic background can elucidate the relationships between root QTLs and abiotic stresses such as drought. In the future, it is expected that these QTLs will be isolated as single genes and their biological functions analyzed. Gene isolation is also beneficial from the point of view of breeding programs. If haplotype data of many rice accessions at a target QTL are obtained by DNA sequencing, we can detect the presence of favorable root QTL alleles in the different varieties. Such information will become a powerful tool for the breeding of root traits, which are difficult to investigate in the field.

10.3.3 Isolation and Pyramiding of Yield-Related Genes

Yield is the most important agronomic trait and the most common objective of breeding programs, but its genetic basis is so complex that, with only few exceptions, allele mining of yield-related traits is far behind that of other traits. Yield is the final mass of harvested organs, such as grain or straw, and is the product of numerous morphological and physiological traits. Moreover, each component trait is controlled by the effects of numerous loci and their interactions with environmental conditions. However, with the aid of recent progress of rice genomics research, some important genes involved in high yield have been revealed.

In crop physiological studies, yield is divided into three major conceptual components: sink size, i.e. the size and number of harvested organs; source strength, the ability to produce sucrose via photosynthesis; and translocation, the delivery of source products to sink organs. Among these three, the most QTLs have been identified for sink size because of the relative ease of sink measurement. In particular, some QTLs involved in culm length (Sasaki et al 2002), panicle architecture (Huang et al. 2009a; Miura et al. 2010), seed number (Ashikari et al. 2005), and grain size (Song et al. 2007; Shomura et al. 2008) have been isolated by map-based cloning, and their biological mechanisms have been clarified. Most of these genes are discovered independently in current high-yielding rice varieties, so enhancing the current level of yield potential will require combining these alleles by MAS. However, rice breeders and geneticists understand that there are trade-offs among yield-related traits that prevent the expected increases from being fully attained. For example, Ohsumi et al. (2010) reported that gene pyramiding of two major sink-size genes, *Gn1a* (Ashikari et al. 2005) and *APO1/SCM2* (Ookawa et al. 2010), did not increase total grain yield because the increase in grain number was offset by changes in the ripening ratio, panicle number, and single-grain weight. The authors (Ohsumi et al. 2010) also reported that enlargement of sink size might enhance translocation of carbohydrate stored in the stem. This finding suggests that identification of major genes for source and translocation followed by combination with sink-size genes would be a promising way to improve rice yield.

Compared to the recent developments on sink traits, there has been substantially less progress toward isolation of genes involved in source and translocation traits except for reports of QTLs involved in grain filling (Wang et al. 2008), non-photochemical quenching, which regulates energy conversion in photosystem II (Kasajima et al. 2011) and leaf photosynthesis rate (Takai et al. 2013). Unlike the relatively straightforward methods that can be used to assess sink-size traits, the crop physiological evaluation systems for source and translocation traits that have been used for many years as the authoritative standards are difficult to apply to genetic analysis. For example, the most precise open gas-exchange system (LI-6400, Li-Cor Inc., Lincoln, NE, USA) for measuring leaf photosynthesis ability takes more than 10 min per sample for stabilizing the chamber environment. Given that a QTL analysis requires about 100 individuals derived from each cross, the length required for each measurement and the need for constant environmental conditions during the measurements makes it impractical to assess a population of this size in the field. Nevertheless, some QTLs related to source traits have been reported (Takai et al. 2009, 2010a; Xu et al. 2009; Adachi et al. 2010, 2011a, b; Gu et al. 2011), and One of such QTLs has recently been identified (Takai et al. 2013). Technological innovations are making field-scale measurements easier. For example, Takai et al. (2010b) revealed that leaf temperature measured by an infrared thermograph provides a promising indirect evaluation of stomatal conductance of the expanded leaf at the maximum tillering stage. We are currently applying this technique to detect QTLs involved in canopy photosynthesis.

For the future, it is important to pursue identification and functional analysis of QTLs for source and translocation. At this point, we still do not know whether genes that promote final yield (biomass) without affecting other traits actually exist. If such candidates are identified, it will be interesting to see whether these QTLs effectively increase source strength without having any pleiotropic effects on other growth-related traits.

10.4 Application of Genome-Wide SNP Analysis

The main problem in current MAS has been the shortage of available DNA markers showing high frequency of polymorphism among breeding varieties. Because of the time and labor required for genotyping experiments, it is unrealistic to develop a custom-made marker set for each breeding population. As the second best

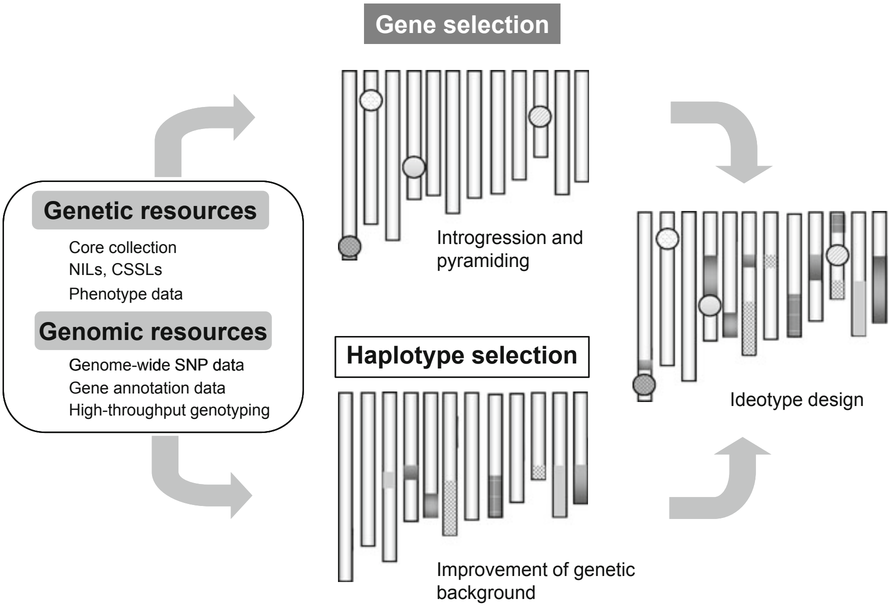

Fig. 10.3 Genomics-assisted breeding: integration of gene selection and haplotype selection

way, researchers have usually developed "semi-universal" marker sets selected from large numbers of SSRs or InDel sites distributed in the rice genome, which show an intermediate level of polymorphism among rice ecotypes (Orjuela et al. 2010). However, recent progress in DNA sequencing and genotyping has been reducing this limitation in a large number of crops (Feltus et al. 2004; Feuillet et al. 2010). Also, genome-wide resequencing of any of rice accession of interest to the researcher or breeder is now possible (Huang et al. 2009b). With concurrent innovations in genotyping technology (Gupta et al. 2008), it is possible to monitor the genomic constitution of breeding materials even with relatively similar genetic backgrounds (McNally et al. 2009; Yamamoto et al. 2010; Arai-Kichise et al. 2011).

The initial phase of MAS in rice was direct or indirect selection of useful gene loci from a donor variety and transfer into modern elite varieties. But the current lineup of such useful genes is limited to QTLs with major effects and mutated alleles whose phenotypic effects are easy to recognize. Assuming that slight modification of phenotype may be possible by introducing QTLs with minor effects or epistatic interactions, both of which are difficult to handle by MAS, we should be able to establish the relationship between each haplotype and specific phenotype. Genomics-assisted breeding will make it possible not only to select a useful allele at a specific locus but also to improve the total genetic background through haplotype selection (reviewed by Fukuoka et al. 2010b; Fig. 10.3). Plant breeders are always conscious of the extent of linkage blocks (haplotypes) being transferred during the breeding process. A comprehensive survey of modern rice varieties by genome-wide SNP analysis clarified the combinations of haplotype blocks within varieties that had been derived from intercrossing of particular founder varieties (Yamamoto et al. 2010, Yonemaru et al. 2012). When we can annotate a haplotype in the pedigree record with a specific phenotypic trait, it will be possible to select the best cross combination for a certain breeding objective and to predict the level of improvement.

10.5 Conclusions and Future Prospects

Rice breeding can be described as a three-step process: (1) identify a favorable phenotype from among diverse genetic resources, (2) produce new gene combinations by crossing, and (3) select favorable segregants from the progeny of these crosses and make them genetically uniform. Allele mining is part of the first step of the above process and one of the major determinants for successful crop improvement. We have demonstrated the feasibility of genomics-assisted allele mining by developing CSSLs covering the natural variation of rice and using them to improve traits such as heading date. The technique can also be applied to agriculturally important traits, such as blast resistance, root morphology, or yield-related traits. We have also emphasized the importance of haplotype selection by genome-wide SNPs as a complementary approach to major-gene selection by MAS.

In addition to our approach described above, recent progress in genomics might further improve current existing strategy for allele mining. The accuracy of genome-wide association might be improved by the availability of new high-density genome-wide SNPs (Huang et al. 2010; Jahn et al. 2011), but these statistically predicted results need to be confirmed by observations of actual phenotypic differences. With respect to experimental materials, the utility of nested association mapping populations (McMullen et al. 2009) and multi-parent advanced-generation intercross populations (Cavanagh et al. 2008) has been validated in maize and *Arabidopsis*, respectively. These approaches combine diverse donors into one experimental population in return for keeping genetic uniformity, which is a major advantage of the CSSLs. These materials are expected to complement each other, and both types need to be developed in rice. Also, genomic selection (Hayes et al. 2009; Heffner et al. 2009) is now being applied in some crops (Zhong et al. 2009). To increase the number of new allele combinations, recurrent selection, which is commonly adopted in allogamous crops, should be considered in rice. Genome-wide SNPs could be helpful in verifying the effectiveness of recurrent selection by enabling surveys of changes in genome composition after each recurrent selection step.

With the aid of technological innovations resulting in automated and low-cost procedures, genotyping is no longer a limiting factor for MAS. On the other hand, the genetic basis of many economically important traits, most of which are complex traits, still remains to be elucidated. Therefore, both basic researchers and plant breeders should focus their efforts on improving methods for accurately evaluating small phenotypic differences to enable effective allele mining. The combination of improved evaluation tools and well-designed experimental materials such as CSSLs will enhance genomics-assisted breeding.

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