Development of chromosome segment substitution lines harboring *Oryza nivara* genomic segments in Koshihikari and evaluation of yield-related traits

Tomoyuki Furuta1), Kanako Uehara1), Rosalyn B. Angeles-Shim1), Junghyun Shim1), Keisuke Nagai1), Motoyuki Ashikari1) and Tomonori Takashi*2)

Chromosome segment substitution lines (CSSLs) are rich genetic resources that can be mined for novel, agriculturally useful loci or that can be used directly as materials for breeding. To date, a number of rice CSSLs have been developed by crossing rice cultivars with its wild relatives as a means to tap into the potential of wild alleles in rice improvement. *Oryza nivara* is a wild relative of rice that is thought to be a progenitor of *O. sativa* spp. *indica*. In the present study, 26 CSSLs that covers the entire genome of *O. nivara* as contiguous, overlapping segments in the genomic background of a *japonica* cultivar, *O. sativa* cv. Koshihikari were developed. Evaluation of the CSSLs for several agriculturally important traits identified candidate chromosome segments that harbors QTLs associated with yield and yield-related traits. The results of the study revealed the potential of *O. nivara* as a source of novel alleles that can be used to improve the existing *japonica* cultivar.

**Key Words:** *Oryza nivara*, chromosome segment substitution lines (CSSLs).

**Introduction**

Wild species are morphologically and genetically more diverse compared with the domesticated rice varieties. As such, they tend to possess several biotic and abiotic stress resistances that allow them to survive naturally harsh environments. Wild rices have also been reported to have genes that can potentially improve the yields of existing rice cultivars (Hajjar and Hodgkin 2007, Vaughan 1994). Recently, many breeding programs have started introducing useful traits from wild rice species into cultivated varieties (Khush 1997, Sanchez et al. 2013). Of these 5 wild relatives, *O. rufipogon* is the wild progenitor of *O. sativa* spp. *japonica* which was originally domesticated in the middle area of the Pearl River in southern China (Huang et al. 2012). On the other hand, *O. nivara* is thought to be a progenitor of *O. sativa* spp. *indica*, although *indica* has a more complicated domestication history in which *indica* underwent crossing between multiple wild species and primitive *japonica* cultivar (Huang et al. 2012, Vaughan et al. 2003). In the previous genetic study of *O. nivara*, loci improving yield and resistence for bacterial blight and green leafhopper have been reported (Cheema et al. 2008, Fujita et al. 2004, Kaur et al. 2005, Mahmoud et al. 2008, Natarajkumar et al. 2009, Swamy et al. 2011). Genetic survey of *O. nivara* can potentially lead to the discovery of novel genes and alleles that can be used to breed new varieties of rice (Juneja et al. 2006).

Here, we report the development and evaluation of CSSLs harboring *O. nivara* genomic segments in the genetic background of *O. sativa* cv. Koshihikari. Chromosomal segments from the entire genome of *O. nivara* were successfully introduced into the genomic background of Koshihikari as contiguous, overlapping segments without any gaps. The CSSLs exhibited significantly different phenotypes for number of days to heading, culm length, panicle length, number of primary branches, number of grains per panicle, grain length, grain width and 100-grain weight.
Materials and Methods

Development of the CSSLs

The donor parent, *O. nivara* Acc. W0054 and the recurrent parent *O. sativa* cv. Koshihikari were provided by the National Institute of Genetics (NIG), Japan. Even though *O. nivara* is categorized as an annual type of *O. rufipogon* in NIG, we prefer referring to this species as *O. nivara*. The CSSLs, hereinafter referred to as NSLs, were developed by first crossing *O. nivara* with cv. Koshihikari to produce the F1 hybrid. The resulting F1 was then backcrossed to Koshihikari to produce 57 BC1F1 plants. Successive backcrossing with the recurrent parent produced 75 BC2F1, 59 BC3F1, 107 BC4F1 and 143 BC5F1 plants. Genotyping using 149 single nucleotide polymorphisms via the AcycloPrime-FP Detection System and Fluorescence Polarization Analyzer (Perkin Elmer Life Science, Boston, MA, USA) was carried out for 59 BC2F1, 107 BC3F1 and 143 BC4F1 to identify backcross lines having 2–4 long, contiguous chromosome segments at a few target chromosomes. The SNP markers, which were developed using the Build 2 Pseudomolecules of cv. Nipponbare, were evenly distributed across the 12 rice chromosomes at an average marker interval of 2.4 Mb. The selected backcross lines were then subjected to additional backcrossing with Koshihikari and subsequent self-pollination to fix the genotypes to homozygotes. All of the F1 hybrid and backcrossed lines that were derived from the successive backcrossing of the F1 with Koshihikari were cultivated at the experimental field of Honda Research Institute (HRI) in Kisarazu, Chiba, Japan. The breeding scheme used to develop the NSLs is presented in Fig. 1.

![Fig. 1](image)

Fig. 1. Flowchart of the breeding process for the development of NSLs. Numbers in parenthesis indicate the number of lines produced for each backcross generation whereas boxed numbers show the number of candidate lines for NSLs selected by MAS. Numbers in brackets present the number of NSLs finally selected from the resulting backcross inbred lines.

Agronomic evaluation of the NSLs

The NSLs were evaluated relative to Koshihikari for several agriculturally important traits in the experimental field of Nagoya University in Togo, Aichi, Japan in 2015. Seedlings of all plant materials were first grown in the greenhouse and then transplanted in the field 30 days after sowing. Ten plants of each NSL and Koshihikari were planted in single-row plots at a spacing of 30 cm between rows and 15 cm between hills. Five plants at the 3rd–7th hills of each row were measured for culm length, panicle length and number of primary branches per panicle. Data on the number of grains per panicle, grain length, grain width and 100-grain weight were also obtained from seeds that were air-dried for 2 months in the glasshouse. The number of tillers was counted at the maturity stage. Days-to-heading was recorded as the number of days from sowing to flowering of 50% of the plants in each line. Grain length and grain width were measured using a scanned image analyzing software, SmartGrain (Tanabata et al. 2012). Dunnett’s multiple comparison test at 95% confidence interval (*P* < 0.05) was used to evaluate significance of difference in the NSLs.

Results

Characteristics of the NSLs

To evaluate the potential of *O. nivara* as a genetic resource to improve existing rice varieties, we developed a series of chromosome segment substitution lines harboring *O. nivara* genomic segments in the genetic background of *O. sativa*, which we named as NSLs. A total of 26 NSLs carrying homozygous chromosome segments from *O. nivara* were developed. The whole genome of *O. nivara* was represented as contiguous or overlapping chromosome segments in the NSL set, although a fragment of *O. nivara* chromosome 3 (defined by SP112-SP1299) remains to be fixed in NSL-7 (Fig. 2). Multiple chromosome substitutions were observed in some of the NSLs. In particular, most of the NSLs except for NSL-2, 6, 7, 8 and 25 have substituted segments on chromosome 6.

Putative QTLs controlling important agronomic traits in the NSLs

Data on the agronomic performance of the NSLs under experimental field conditions are presented in Table 1. A total of 98 significant changes in the tested traits were identified in the NSLs, 22 of which had positive and 76 had negative changes on the phenotypic values.

Days-to-heading

A total of 19 lines showed significantly different days-to-heading compared with Koshihikari. NSL-1, 2, 3, 6, 7, 9, 12, 22 and 25 exhibited delayed flowering. NSL-7 recorded the most delayed flowering (95.8 days), which was 6.8 days later than Koshihikari. On the other hand, 10 lines including NSL-4, 5, 10, 13, 14, 15, 16, 19, 23 and 26 flowered earlier than Koshihikari. NSL-23 recorded the earliest heading date (79.4 days), which was 9.6 days earlier than Koshihikari.
This result indicates that *O. nivara* has many genes affecting flowering time which can be used to fine-tune the flowering time of Koshihikari.

**Culm and panicle length**

Significant changes in culm length were detected in 12 NSLs that gave significantly higher or lower values for the trait compared to Koshihikari. A 15.5–22.91% increase in culm length were recorded in NSL-3, 6, 7, 10, 11, 12, 22 and 25, whereas a 10.34–15.02% decrease in the value of the trait was recorded in NSL-14, 16, 17 and 23 compared to Koshihikari.

In contrast to culm length, only a negative change in panicle length was noted in the NSLs. NSL-13 had the shortest panicle of 14.2 cm, which is 31.7% shorter than the average panicle length of Koshihikari. NSL-14, 16, 17 and 23 exhibited reductions in both culm and panicle length, whereas NSL-12 showed longer culms but shorter panicles relative to those of Koshihikari. A moderate positive correlation between culm length and panicle length was observed (correlation coefficient = 0.562).

**Number of tillers, primary branches and grains per panicle**

Significant differences in the number of tillers were observed in NSL-16, 17 and 19, relative to Koshihikari. Both NSL-16 and 17 recorded more than twice the number of tillers of Koshihikari, whereas NSL-19 showed a significant decrease of 4.6 tillers compared to Koshihikari in average. NSL-13, 16 and 17 exhibited significantly fewer primary branches per panicle compared with Koshihikari. A significant increase in the number of primary branches per panicle was not observed in any line. In addition, a trade-off between the number of tillers and primary branches per panicle was observed in NSL-16 and 17.

Number of grains per panicle is one of the most important components of yield and it has been one of the main targets for improvement in the history of rice breeding. No significant increase in grain number was observed in any of the NSLs. However, 13 lines showed a 20–50% reduction in the numbers of grains per panicle relative to Koshihikari, with NSL-17 recording the lowest value for the trait (75.3 grains per panicle). Even though many of the NSLs did not show significant differences in the number of primary branches, a strong positive correlation between the number of primary branches and grains per panicle was detected (correlation coefficient = 0.858). These results indicate that *O. nivara* Acc. W0054 has several inferior alleles controlling the numbers of primary branches per panicle which leads to reduction in the number of grains per panicle.

**100-grain weight, grain length and width**

Two lines (NSL-15 and NSL-22) showed significantly higher 100-grain weight compared to Koshihikari which recorded an average of 2.39 g. These NSLs carry a fragment of chromosome 6 and 10 of the donor genome and increased 100-grain weight by 7.95–11.72%. Reduction in 100-grain weight was observed in NSL-4, 7, 11, 12, 13, 17, 18, 19, 20.
and 26. All of these NSLs also recorded decreases in grain length and/or grain width. A significant increase in grain length was observed only in NSL-23, which recorded a 4.35% increase compared to Koshihikari. No significant increase in grain width was observed in any of the NSLs. In contrast, several NSLs produced significantly smaller grains than Koshihikari. NSL-3, 8, 12 and 18 showed significant decreases only in grain width, whereas NSL-5, 14, 16, 19 and 20 showed significant decreases only in grain length. Reductions in both grain width and length were observed in NSL-4, 6, 7, 11, 13, 17 and 26. Surprisingly, NSL-6 recorded an almost similar grain weight to that of Koshihikari even though this line had significantly smaller grains in terms of both width and length compared to Koshihikari.

**Discussion**

In the past couple of decades, not a few sets of CSSLs, introgression lines and several kinds of inbred lines have been developed not only in rice but also in other crops. These genetic resources have contributed significantly not only in advancing science but also agriculture (Eshed and Zamir 1995, Korff et al. 2004, Lippman et al. 2007, Warschefsky et al. 2014). Lines having one or few introgressed chromosomal segments in a uniform genetic background such as CSSLs, provides the advantage of identifying loci controlling complex, quantitatively inherited traits (Doi et al. 1997, Furuta et al. 2014, Kubo et al. 2002, Shim et al. 2010). In an ideal set of CSSLs, each line has one introgressed chromosome segment from a donor parent in the genetic background of a recipient parent. This simple genomic structure enables us to use a simple statistical method to detect correlations between the phenotypes and the introgressed chromosome segments (Ali et al. 2010). A significant difference between CSSLs and the recipient parent in the value of any trait examined directly points to a causal gene located on the substituted chromosome segment from the donor parent. Any CSSL showing a significant difference in the value of the trait of interest can be used for further genetic analysis (i.e. fine mapping of a causal gene) or used directly to breed for new varieties.

In this paper, we developed 26 NSLs harboring *O. nivara* genomic segments in the background of the *japonica* cultivar, Koshihikari. Most of the NSLs unexpectedly have a substituted segment in chromosome 6. This may be due to the well-known reproductive isolation-related locus, *S6*, found in this region (Koide et al. 2008, 2012). Apart from this, the whole genome of *O. nivara* Acc. W0054 was successfully introgressed in the set of NSLs as 26 chromosomal
segments individually substituted in the Koshihikari genomic background without any gaps between the segments. 

A lot of significant differences in the traits examined were detected between the NSLs and Koshihikari (Table 1). Nineteen lines out of 26 NSLs, for example, showed significantly different numbers of days to heading compared to Koshihikari. The earliest flowering was 9.4 days earlier than Koshihikari, while the latest was 6.8 days later compared to the average flowering time of Koshihikari. The earlier flowering was a preferable property for the cropping in the area having a relatively short period of an ideal climate for rice cultivation (Izawa 2007). NSL-23, which showed the earliest flowering in the NSLs, has O. nivara derived chromosomal segments in chromosome 6 and 11 (Fig. 2). Based on the rice QTL database “Q-TARO”, the previously reported QTLs in the substituted chromosomal regions were searched (Yonemaru et al. 2010). No flowering related QTL was found in chromosome 11 but Hd1 is located in the substituted segment in chromosome 6 of NSL-23 (Yano et al. 2000). However, the other NSLs, except for NSL-2, 3, 6, 7, 8, 10, 11, 12, 22, 24, and 25, also have O. nivara derived chromosome segments in the Hd1 location and showed different phenotype from that of NSL-23 in the days to heading (Table 1). Due to the multiple substituted segments in the NSLs which might have genetic interactions to control the traits, it is difficult to speculate the positions of candidate QTLs. For the improvement of yield, the number of grains per panicle and 100-grain weight are the traits having a direct effect on yield. We could find 2 lines, NSL-15 and NSL-22, that showed significant increases in 100-grain weight, whereas no line showed a significant increase in the number of grains per panicle. Unfortunately, NSL-15 and NSL-22 have reductions in the number of grains per panicle, although the differences were not significant. Furthermore, these two lines exhibited no significant changes in the grain length or width. These results suggest that the increase in 100-grain weight might be due to increased starch filling in the grains of these two NSLs. NSL-22 has a substituted segment at the end of the long arm in chromosome 10. A QTL for 1000-grain weight (qTGWT-10) has been reported in this region (Zhuang et al. 2002). The number of tillers and primary branches per panicle are also important traits for improvement of yield. Only 3 NSLs showing significant differences were found for each of the traits. These results might be caused by the property of the data that are relatively smaller and discrete variables compared to the other traits. Increasing the number of replicates per NSL may improve the statistical detection power to detect loci that controls the number of tillers and primary branches per panicle in O. nivara. Remarkable increase in the number of tillers was observed in NIL-16 and 17. However, these lines also exhibited decreases in culm length, the number of grains and primary branches per panicle, and grain length. The increase in the number of tillers can increase yield, whereas the decrease in the number of grains per panicle cause the reduction of yield. In the previous study in recombinant inbred lines derived from the cross between O. sativa and O. rufipogon, a QTL cluster controls several traits for plant architecture has been reported at the end of the short arm in chromosome 7 where NIL-16 and NIL-17 commonly have substituted segments (Onishi et al. 2007). Further detailed genetic studies are required to know whether the trade-off effect is caused by pleiotropic effects of one gene or closely linked genes. Most of the NILs did not show outstanding effect on the yield in this study. However, several lines could improve yield of Koshihikari, even though Koshihikari is one of the well-bred elite cultivar. For example, NIL15 showed a significant increase in 100-grain weight with shortening days-to-heading. Overall, these results point to the potential of wild rice relatives as donors of alleles that can be used to fine-tune the agricultural traits of current elite cultivars.

In the present study, NSLs were mainly evaluated for yield and yield components. However, O. nivara has also been reported to harbor genes for disease and insect resistance (Cheema et al. 2008, Fujita et al. 2004, Vaughan 1994). Evaluation of the performance of the 26 NSLs under different biotic stresses would facilitate the identification of genes controlling resistance to diseases and pests. In addition, O. nivara is known as annual plant while O. sativa and its ancestor O. rufipogon are perennial plant. This feature enables NSLs to be applicable to a study of annuality. Thus, NSLs could be the useful genetic resource not only for improvement of agricultural traits, but also for understanding physiological and ecological differences between O. nivara and O. sativa.

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