Methodology to identify dwarfing gene d60 that complements gamete lethal gene gal by Next-generation DNA sequencing analysis

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Abstract

The author previously discovered dwarfing gene, d60, and it complements the gametic lethal gene, gal, to cause gametic lethality in rice. Namely, in the F1 hybrid (genotype D60d60Galgal) of Koshihikari (D60D60galgal) × Hokuriku 100 (d60d60GalGal), male and female gametes having both gal and d60 become gametic lethal and the pollen and seed fertility decrease to 75%. Therefore, F2 progeny shows a unique mode of inheritance that is segregated into a ratio of 6 fertile long-culm (4D60D60: 2D60d60GalGal) : 2 partially fertile long-culm (D60d60Galgal = F1 type) : 1 dwarf (d60d60GalGal).Prior to Next-Generation Sequencing analysis targeting d60, it is required to develop isogenic genome of reference except for target d60 gene. When the F1 (D60d60Galgal) progenies of 'Koshihikari' × 'Hokuriku 100' were backcrossed to 'Koshihikari' (D60D60galgal), BC1 F1 individuals segregated in a ratio of 1 tall and 25% sterile (D60d60galGal) : 2 tall (1 D60D60Galgal : 1 D60D60galgal). Here, D60d60Galgal-heterozygous plants can be recognized by pollen sterility prior to anthesis. Then Tall and 25% sterile BC1F1 plants (D60d60galGal) were selected by 25% pollen sterility and backcrossed with 'Koshihikari' as female parent to produce BC2F1 seeds. Hereafter, D60d60Galgal-heterozygous plants were selected in the first generation of each backcross (BCnF1) and immediately backcrossed repeatedly with Koshihikari to obtain isogenic genome useful for next-generation sequencing. That is to say, the target d60 DNA region narrowed down with each back cross generation, enabling identification of the target DNA mutation point by whole genomic sequencing.

1. Introduction

The breeding program that has made the greatest contribution in the history of mankind is the 'Green Revolution,' in which the production of rice and wheat grain was dramatically increased in the 1960s with the development of dwarf varieties (Khush 1999). Dwarfing prevents plants from lodging at their full-ripe stage, makes them lodging-resistant to wind and rain, has enhanced their adaptability for heavy manuring and has dramatically improved the productivity up to double in rice and quadruple yields in wheat. In recent years, dwarf genes, which have been used as breeding materials to improve lodging-resistance, have been isolated. *sd1*, which is a dwarf gene that contributed to the 'Green Revolution' of rice, is a defective C20-oxidase gene in a late step in the gibberellin (GA) synthesis pathway (defective GA20-oxidase gene) (Sasaki et al. 2002). Surprisingly, rice semidwarf varieties developed independently by using different native varieties or artificially induced mutant lines as mother plants happen to be controlled by a same single dwarfing gene, *sd1* (Sasaki et al. 2002). In addition, both *d35* from Tanginbouzu, which became the best rice cultivar in Japan between 1955 and 1964, and *d18* from Kotake-tamanishiki were also kaurenoic acid oxidase- or 3-beta hydroxylase-defective in the same GA synthesis pathway (Itoh et al. 2001). On the other hand, *Rht*, which contributed to the 'Green Revolution' of wheat, is missing a gene involved in GA's signaling (Peng et al. 2000; Hedden 2003), and the Daikoku-type dwarf gene *d1* in rice is defective in the alpha subunit of the heterotrimeric G protein, affecting GA signal transduction (Ueguchi-Tanaka et al. 2000). Above all, available dwarf genes are limited to those associated with gibberellin (GA) for both rice and wheat.

Although dwarf varieties of rice have contributed to the dramatic improvement and stabilization of yields all over the world, *sd1* is the world’s only short-culm gene usable in actual rice breeding so far. Namely, options are limited. In consideration of the purpose to maintain/expand genetic diversity of plant

cultivars, we should not rely on GA synthesis-defective gene *sd1* and should discover more new dwarfing genes and promote their use in lodging-resistant breeding. Therefore, it is necessary to acquire a wide range of novel dwarfing genes in order to cope with climate changes.

2. A unique genetic mode of novel semidwarfing gene *d60*

In order to find a novel dwarf gene that can take the place of the green revolution gene sd1, the author conducted gene analyses focusing on Hokuriku 100, which is a radiation-induced mutant line with culms approx. 15 cm shorter than those of variety Koshihikari (Samoto & Kanai 1975), however, despite its promising phenotype, had been left aside because of unclear genetic mode. As a result, the author discovered a novel dwarf gene, *d60*, which brings about a good plant type with erect leaves by shortening culms by about 20% (Tomita 1996). Furthermore, the author discovered that this dwarfing gene, *d60*, and that it complements the gametic lethal gene, *gal*, to cause gametic lethality (Tomita et al 2003). Therefore, in the F_1 hybrid (genotype *D60d60Galgal*) of Koshihikari (*D60D60galgal*) × Hokuriku 100 (*d60d60GalGal*), male and female gametes having both *gal* and *d60* become gametic lethal and the pollen and seed fertility decrease to 75% (Fig.1). Namely, F_2 progeny shows a unique mode of inheritance that is segregated into a ratio of 6 fertile long-culm (4*D60D60*: 2*D60d60GalGal*) : 2 partially fertile long-culm (*D60d60Galgal* = F_1 type) : 1 dwarf (*d60d60GalGal*). In other words, as the gametes with *gal* and *d60* result in death, *d60* is not transmitted to progeny without *Gal*. Furthermore, isogenic lines that were developed by introducing both *d60* and *sd1* genes into Koshihikari by backcrossing, namely the *d60sd1* line, became additively extreme-dwarf (Tomita 2012), indicating demonstrating that *d60* is functionally independent from *sd1* and not involved in the GA1 synthesis pathway.

3. Genome analysis in Koshihikari genetic background

The threat of strong typhoons is

increasing, probably as a result of global warming. This is a serious problem in rice production, because strong winds cause stem lodging and consequent yield losses and deterioration in crop quality. Koshihikari is the major rice cultivar and has approximately 40% of crop acreage in Japan. However, Koshihikari has shown a reduction in yield and loss of quality due to lodging and immature chalky grains, which have been caused by recent climate change due to global warming. Therefore, there is an urgent need to develop and disseminate a semidwarf Koshihikari variety that is resistant to lodging. Hence there is a pressing need to develop new short-culm rice cultivars resistant to strong winds. The genome wide association analysis was conducted in the rice genome by using target gene-substituted isogenic Koshihikari.

Today's advantage of genomics is developing Next-generation sequencer that is able to decode giga level of DNA sequences. Development of Next-Generation DNA sequencer is advanced under Obama care that aims to realize a societal implementation of medical genomics (Bahassi & Stambrook 2014; Levy & Myers 2016). NGS can read 15 million DNA fragments at one run (that are equivalent to 30 times of the rice genome which can be sequenced) and yielding high throughput, high-quality data. Prior to Next-Generation Sequencing, it is required to develop isogenic genome of reference except for target *d60* gene. Then the author crossed Koshihikari with cultivars with *d60* and then the offspring carrying the target gene *d60* was selected in the offspring and repeatedly backcrossed with Koshihikari. Except for *d60* gene region, the genome returned to Koshihikari as reference genome, every time backcross was repeated. When the F_1 *(D60d60Galgal*) progenies of 'Koshihikari' × 'Hokuriku 100' were backcrossed to 'Koshihikari' (*D60D60galgal*), BC_1 F_1 individuals segregated in a ratio of 1 tall and 25% sterile (*D60d60galGal)* : 2 tall (1 *D60D60Galgal* : 1 *D60D60galgal*) (Fig. 1). Here, *D60d60Galgal*-heterozygous plants, can be recognized by pollen sterility prior to anthesis. Then Tall and 25% sterile BC_1F_1 plants (*D60d60galGal*) were selected by 25% pollen sterility and backcrossed with

'Koshihikari' as female parent to produce BC_2F_1 seeds. Namely, *D60d60Galgal*-heterozygous plants were selected in the first generation of each backcross (BC_nF_1) and immediately backcrossed repeatedly with Koshihikari to obtain isogenic genome useful for next-generation sequencing (Fig. 1). That is to say, the target *d60* DNA region narrowed down with each back cross generation, enabling identification of the target DNA mutation point by whole genomic sequencing (Fig. 2). This process is called genome-wide association analysis. Using the high-throughput, long-read next-generation sequencer, a library of 15-20 million reads (insert length 500 bp) was set up from 2 Gp, covering the rice genome size of over 400 Mb x 30. Clusters then were formed on flow cells and pair-end sequencing of 250-bp-long read was performed. The read sequences were mapped on the reference genome of Koshihikari. Thus, introgression of target genes and that the rest of the sequences are the same as the original becomes obvious at a glance by sequencing the entire genome via next-generation sequencer. Our process of genome wide association analysis would be expected to lead to the rapid completion of a cultivar that has the same genome of Koshihikari except for the integrated useful target gene, at the same time.

4. References

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Figures:

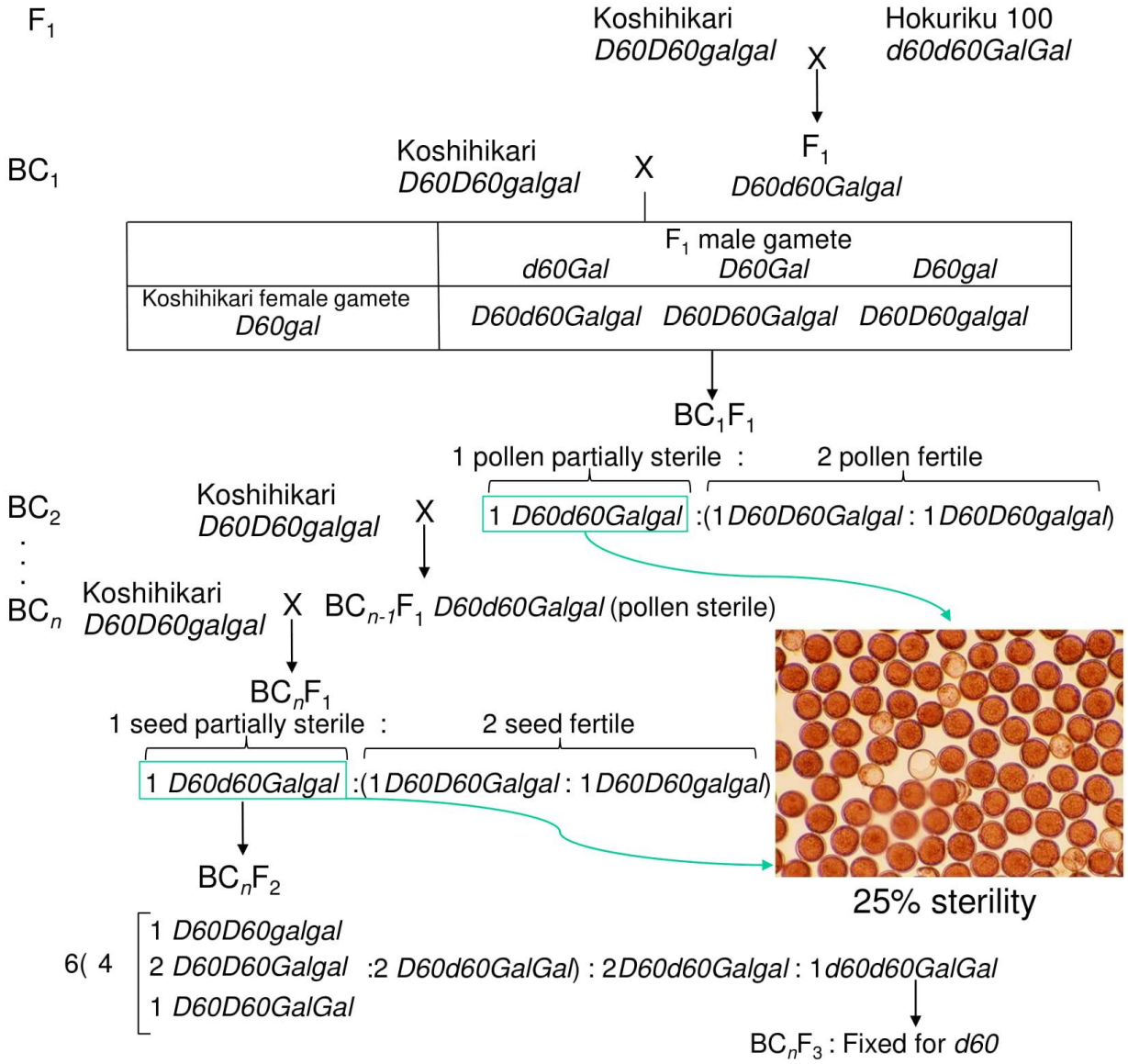

Fig. 1. Back-cross introgression of semidwarfing gene *d60* by using *D60d60Galgal*-heterozygous BC_nF_1 plants as male parent to obtain isogenic genome, which is devised from Tomita (2012), for whole genomic Next-generation sequencing

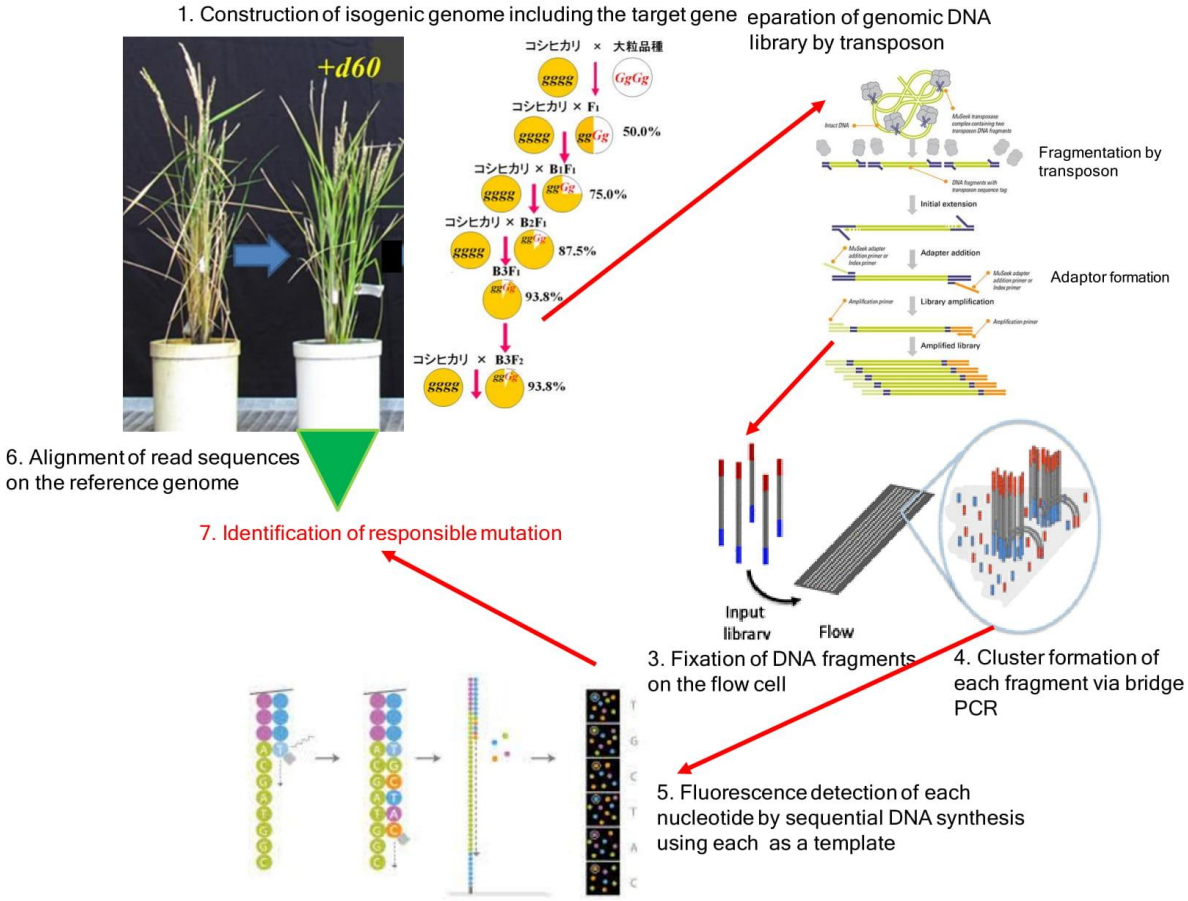

Fig. 2. Next-generation sequencing analysis of isogenic genome to identify responsible gene by using Illumina system (2016)

