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Short Communication

A visible assay for meiotic homologous recombination in pollens of rice

Yoshinori Shingu[1,*], Yasuko Kanno[2], Takeshi Tokai[2], Takehiko Shibata[1], Kyo Wakasa[2,a]

[1] Cellular & Molecular Biology Laboratory, RIKEN Institute, Wako, Saitama 351-0198, Japan; [2] Faculty of Agriculture, Tokyo University of Agriculture, Atsugi, Kanagawa 243-0034, Japan
* E-mail: shingu@riken.jp Tel: +81-48-467-9796 Fax: +81-48-462-1227

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Abstract Meiotic homologous recombination generates genetic diversity for adaptations to environmental changes, and is the underlying principle of breeding by genetic crossing. Meiotic recombination in plants is solely manifested by the phenotypes of the progeny after a series of genetic crosses, and thus its assessment is laborious and time-consuming. A facile assay for meiotic recombination in higher plants, especially in crops, will innovate molecular breeding technology. In this study, we developed a fluorescence-based quantitative assay for meiotic gene conversion in rice pollens, using a pair of homologous genes: an active gene encoding cyan-fluorescent protein (CFP) as the recipient gene, and a yellow-fluorescent protein (YFP) gene without a promoter as the donor gene for the conversion. Pollens emitting YFP fluorescence, representing meiotic gene conversion products, were detected at frequencies of 2.7×10^{-6} to 4.9×10^{-5} in five transgenic lines, whereas the mitotic gene conversion rate between the two genes was less than 10^{-6} in calli derived from one of these lines. Southern hybridization analyses of the transgenic lines revealed that the CFP and YFP genes were integrated at various independent loci in each line. This method provides a rapid technique to assess meiotic gene conversion.

Key words: Fluorescent protein, meiotic recombination, transgenic rice.

Homologous recombination plays an essential role in the repair of DNA double-stranded breaks (DSBs), to maintain genetic integrity in all living organisms. In eukaryotes, meiotic homologous recombination is essential in the disjunction of homologous chromosomes and in genetic diversification for adaptation to environmental changes. Genetic diversification by homologous recombination is an important underlying principle of breeding by genetic crosses. If the manipulation of homologous recombination becomes possible as a breeding tool, such as for gene targeting, then it will shorten the breeding periods of crops. While mitotic recombination is a stochastic event, meiotic recombination is regulated by a genetic program, which activates Spo11 at an early stage of meiosis to induce DSBs at specified recombination hotspots in eukaryotes, from yeasts to flies, plants and animals (Bergerat et al. 1997; Keeney et al. 1997; Szostak et al. 1983). In yeasts, the frequency of meiotic recombination is 100 to 1,000-fold higher than that of mitotic recombination. In a successful gene targeting trial, the attachment of the DNA binding domain of Gal4 (Gal4BD) to Spo11 increased targeted meiotic recombination events more than 10-fold, at Gal4BD target sequences that were located at naturally inert loci for recombination in *Saccharomyces cerevisiae* (Pecina et al. 2002). In rice (*Oryza sativa* L.), we identified a new Spo11 homologue, OsSPO11D, which possesses DSB forming activity, although its *Arabidopsis* counterpart lacks this activity (Shingu et al. 2012). A novel gene targeting technique could be developed by using Spo11 proteins in rice, as in the case of yeast. Despite the essential roles of meiotic recombination, the monitoring of recombination is laborious and time-consuming, especially in higher plants. Recently, high-throughput recombination assays using a fluorescent marker in seed-based and pollen tetrad-based analyses have been developed in *Arabidopsis* (Francis et al. 2007; Melamed-Bessudo et al. 2005), in which pollen tetrad analysis was shown to be a robust method for analyzing recombination events. *Arabidopsis* has a suitable flowering system for harvesting pollens, and flowers are produced successively throughout development. In the case of rice, the period of flower development is very restricted, because the

Abbreviations: CFP, cyan-fluorescent protein; YFP, yellow-fluorescent protein; BSD, blasticidin S deaminase; OASA1D, rice anthranilate synthase α-subunit gene; 5MT, 5-methyltryptophan.

[a] Present address: Department of Bio-Science, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan.

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flowering time is usually only once per year, and meiosis progresses within only a week. However, the pollens are still suitable for assaying recombination in rice, because they can be harvested in large quantities at one time. Two advantages of using pollens for recombination analyses are: firstly, recombination can be directly detected by the phenotypes in haploid gametes, and secondly, high-throughput analyses by microscopy, as well as pollen tetrad analyses, are available.

Homologous recombination is classified as either crossing-over (reciprocal recombination) or gene-conversion (non-reciprocal recombination). Crossing-over is the exchange of chromosomal arms at a homologous sequence, and it can cause chromosomal aberrations if it occurs between nonallelic loci. Gene conversion is the overwriting of part of a recipient gene by the homologous part of a donor gene, without any change of the donor, and thus does not cause chromosomal aberrations. This characteristic makes gene conversion suitable for targeted gene manipulation, a proposed innovative breeding technology.

For the quantitative analysis of gene conversion, we constructed two types of vectors and introduced them into rice calli (cv. Nipponbare). We thus generated transgenic rice lines that have a pair of homologous genes for a quantitative assay: an active gene encoding cyan-fluorescent protein (CFP) as the recipient gene, to assess the population of cells with the gene to be recombined, and a yellow-fluorescent protein (YFP) gene without a promoter, as the donor gene for gene conversion. Both genes share 98% identity in their coding DNA sequences (Supplementary Figure 1). Pollens producing YFP fluorescence indicate the products of meiotic gene conversion, in which a CFP-specific sequence was replaced by a YFP-specific sequence. The fluorescence signals of CFP and YFP can be discriminated with optical filters.

The recipient DNA construct (pREC, Figure 1) was assembled by using a fused gene encoding CFP and blasticidin S deaminase (BSD), as a resistance marker to select CFP transformants. Both genes were controlled by the rice actin promoter, as described (Ochiai-Fukuda et al. 2006). The donor DNA construct (pDON, Figure 1) have a promoter-less gene encoding YFP fused with the BSD-E56D (BSD56) gene, in which the BSD was inactivated (Kimura et al. 2000) and an altered rice anthranilate synthase α-subunit gene (*OASA1D*), as a selectable marker controlled by the 35S promoter of cauliflower mosaic virus (Yamada et al. 2004), conferring 5-methyltryptophan (5MT) resistance to select the YFP transformants. The inactivated BSD gene was used for elongating homologous sequence between recipient and donor DNA constructs.

We delivered both DNA constructs to rice seed calli by two rounds of particle bombardment, using a PDS-1000/He system particle gun (Bio-Rad Laboratories, Hercules, CA). In each bombardment, we used a mixture of 2 μg each of pREC DNA and pDON DNA. Rice seed calli were induced from scutella, as described previously (Tozawa et al. 2001). After one day, the bombarded calli were transferred to 2N6 medium (Urushibara et al. 2001) containing 20 mg l^{-1} blasticidin S. After three weeks, the blasticidin S-resistant calli were examined for CFP fluorescence by a FUJIFILM Luminescent Image analyzer LAS-3000 (FUJI PHOTO FILM, Tokyo), using the Lite mode of 460 nm EPI and the 510DF10 filter. The selected calli that showed blasticidin S-resistance and CFP fluorescence were transferred to MSRE plant regeneration medium (Rashid et al. 1996) containing 150 μM 5MT (Sigma-Aldrich), and were cultured for one to two weeks. They were then transferred to MS medium (Murashige and Skoog 1962) containing 150 μM 5MT. The regenerated plants were transferred to pots containing podosol soil (Sumitomo Chemical, Osaka), and were grown at 28°C and 60% humidity under natural light conditions in a containment greenhouse. Genomic DNA was extracted from the leaves of the regenerated plants, and the presence of the CFP- and YFP-transgenes was confirmed by a PCR amplification test. Five independent transgenic lines were used in the following experiments.

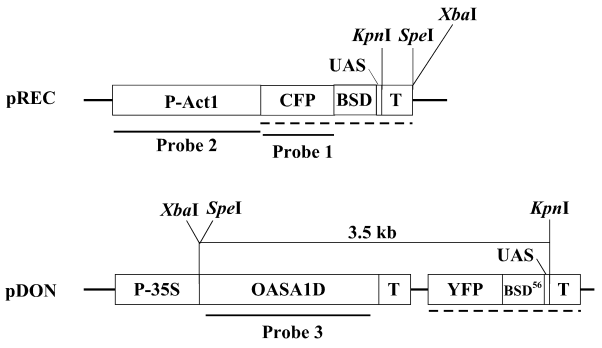

Figure 1. Recipient (pREC) and donor (pDON) vectors. The pREC vector carries a CFP-BSD fused gene driven by the rice *ACT1* promoter. The pDON vector carries *OASA1D* driven by the *CaMV35S* promoter, and a gene encoding YFP fused with blasticidin S deaminase containing the E56D replacement (BSD^{56}, a defective mutation) without a promoter. The UAS region was used to replace the yeast Gal4BD binding site, and was inserted between BSD or BSD^{56} and a terminator (T). Underlines indicate regions probed by Southern blot analyses. The dotted lines indicate homologous regions of DNA.

To detect pollens with YFP fluorescence, as the products of meiotic gene conversion, anthers were collected from the five rice lines at the time of flowering, and the mature pollens within them were examined by fluorescence microscopy. The total numbers of pollen examined were calculated, to combine the number of pollens per anther. The average pollen number per anther was determined by counting the number of pollens among ten randomly selected anthers, using a Burker–Turk counting chamber. The recombination rate was

calculated, based on the numbers of pollens emitting CFP fluorescence. We observed 4.58×10^6 pollens in total, with a range of 0.7×10^6 to 1.09×10^6 pollens from each transgenic line. Almost half of the pollens showed CFP fluorescence in each transgenic line (Table 1), suggesting that the CFP gene had integrated mostly in a single chromosome. Under our experimental conditions, the YFP pollen was clearly detected (Figures 2C and F), and the number of YFP fluorescent pollens in each line is shown in Table 1. Among the five transgenic rice lines, line 17 produced the greatest number of YFP pollens, which were as high as 25 among 1.09×10^6 pollens. Using the definition of the recombination rate as the frequency of YFP fluorescent pollens appearing per CFP fluorescent pollen, the rate varied from 7.0×10^{-6} to 4.92×10^{-5} (Table 1). To test whether the recombination observed here was meiotic recombination, we examined the YFP fluorescent signals in mitotic cells of line 12. Protoplasts were isolated from line 12 calli that was maintained by subcultures after transduction, by treating with Cellulase Onozuka and Macerozyme R-10 (Yakult Pharmaceutical Co. Ltd., Japan), according to the previously described protocol (Wakasa et al. 1984). Because physiological condition of cells influences emission and a part of cells sometimes loses fluorescence or light, a recombination rate was calculated based on a influencing cells. Among 1.05×10^6 protoplast cells that showed CFP fluorescence, YFP fluorescence was not detected, although this line exhibited a recombination rate of 1.58×10^{-5} in meiosis (Table 1). This result suggests that the recombination observed in pollen depends on meiotic recombination.

The observed variation in the recombination rates would be caused by the differences in the integration sites of the CFP gene and the variations in the copy numbers of CFP and YFP in the transgenic plants. In addition, it is noted that in line 17, which showed the highest recombination rate, the YFP fluorescent pollen (Figure 2F) lacked CFP fluorescence under a CFP filter (Figure 2E, arrows). This suggested that the line 17 cells have a single copy of the CFP gene, which was converted to the YFP gene. Therefore, we performed Southern hybridization analyses. The genomic DNA was isolated from the leaf cells of each transgenic line, and was digested with the *Kpn*I and *Xba*I or *Spe*I and *Xho*I restriction enzymes. After the electrophoresis of the digested DNA samples, the DNA fragments were hybridized with a DIG (Roche Applied Science) labeled CFP-YFP probe (probe 1), the *ACT1* promoter probe

Table 1. Meiotic recombination rates of transgenic lines.

Line	Number of anthers examined	Total Pollen ($\times10^6$)	Rate of CFP pollen	Total CFP pollen ($\times10^5$)	YFP pollen	Recombination rate ($\times10^{-5}$)*
12	864	1.08	52.4%	5.66	9	1.58
17	857	1.09	45.1%	5.08	25	4.92
22	864	0.85	44.8%	3.81	10	2.62
23	648	0.71	52.3%	3.70	1	0.27
53	756	0.85	51.5%	4.38	10	2.28

* Recombination rate is defined as detection of YFP pollen per CFP fluorescent pollen.

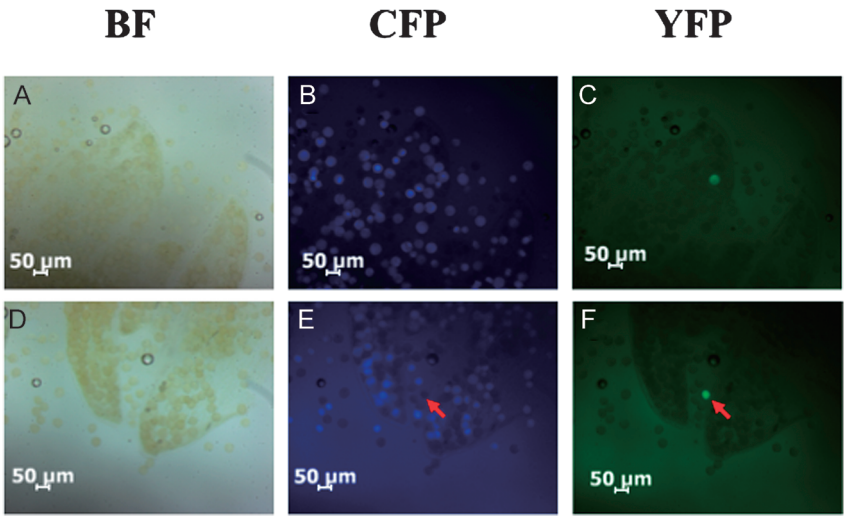

Figure 2. Detection of fluorescent pollens. Anthers from the five transgenic rice lines shown in Table 1 were collected at the time of flowering, and the mature pollens within them were examined with a fluorescence microscope, model MZFL3 (Leica Microscopy systems, Heerbrugg, Switzerland), equipped with an AxioCam digital camera (Zeiss, Gottingen, Germany). Images were collected and analyzed, using the Axio vision software, ver. 4.1. A to C, line 12; D to F, line 17. A and D, Bright field (BF) images; B and E, CFP images; C and F, YFP images. It is notable that the pollens emitting YFP fluorescence in F were not detected in E (marked by arrows).

(probe 2), and the *OASA1D* probe (probe 3; Figure 1, underlined). As shown in Figure 3A, we detected a number of signals corresponding to either the CFP or YFP gene by probe 1 in the transgenic rice genomes. To distinguish between the CFP and YFP genes, we used probes 2 and 3 for another hybridization. The signals that hybridized with probe 2 were identified as the CFP gene (blue arrows) and those hybridizing with probe 3 were identified as the YFP gene (red arrows) (Figures 3, A to C). It should be noted that since probes 2 and 3 contain rice genes, the authentic *Act1* promoter and anthranilate synthase α-subunit genes, respectively, the probes also hybridized with these genes (Figures 3, B and C). If the transgene of pDON illustrated Figure 1 was inserted into the rice genomic DNA correctly, as shown for lines 17, 22, 23 and 53, then the 3.5-kb fragments should be detected in lanes I in Figure 3C. However, although both marker products of OASA1D and BSA were functional, the 3.5-kb fragment was not detected in line 12, suggesting that part of the transgene was lost during integration.

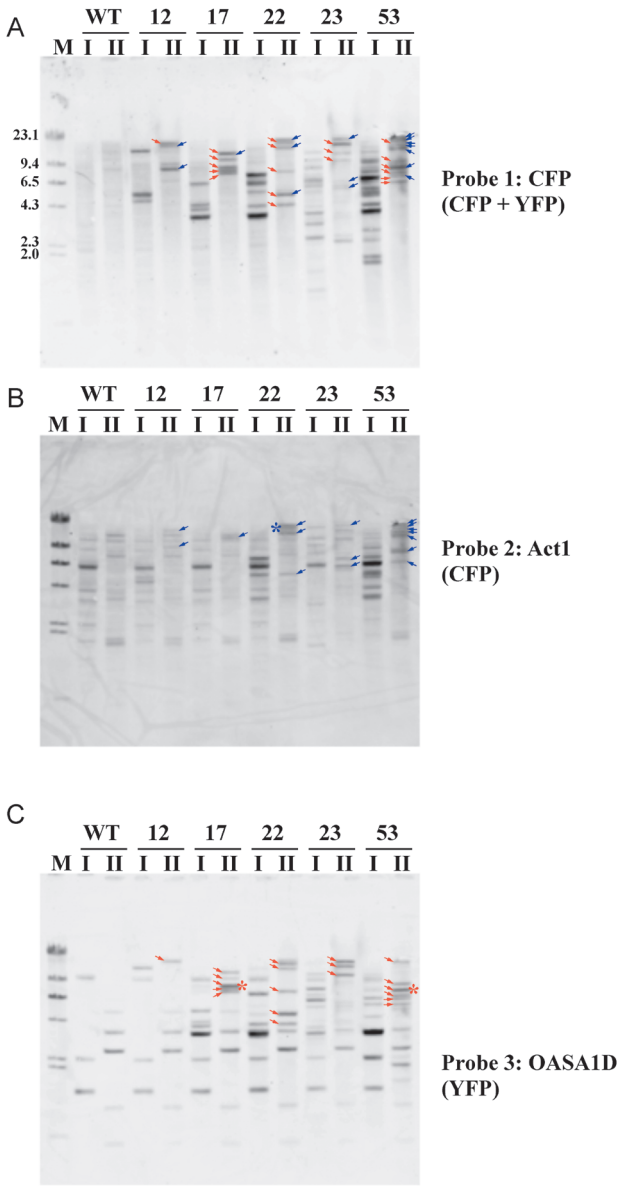

Figure 3. Number of integrated loci of CFP and YFP genes, as detected by Southern hybridization. The genomic DNA samples were isolated from leaf cells of the wild type (WT) and transgenic lines 12, 17, 22, 23 and 53, and were digested with the *Kpn*I and *Xba*I (lanes I) or *Spe*I and *Xho*I (lanes II) restriction enzymes. After electrophoresis of the digested DNA samples, the DNA fragments were hybridized with a DIG (Roche Applied Science) labeled CFP-YFP probe (probe 1, in A), the *Act1* promoter probe (probe 2, in B), and the *OASA1D* probe (probe 3, in C). These probes are illustrated in Figure 1. The expected band size of endogenous genes of *Act1* promoter and *OASA1D* were 6.4 kb in lane I and 7.7 kb in lane II (B), 10.3, 2.3, 1.4 and 1.4 kb in lane I and 3.7, 2.7, 1.0 and 0.5 kb in lane II (C), respectively. In Figure 3B, extra bands were detected. The identified CFP and YFP genes are indicated by blue and red arrows, respectively, in lanes II. Lane M, DNA Molecular Weight Marker II, DIG-labeled (Roche). Asterisks represent bands possibly consisting of several signals (line 22 in B and line17 and 53 in C).

The numbers of loci at which the CFP or YFP gene were integrated in the transgenic rice genomes were estimated by the numbers of signals in lanes II (blue and red arrows) in Figures 3B and 3C, and the results are listed in Table 2. The variation in the signal strength is likely to represent the copy number of the proved gene at each locus. Line 17 has only a single locus of the integrated CFP gene in the genome (Figure 3, see Table 2). Considering the loss of CFP fluorescence in the YFP fluorescent pollen in line 17 (Figures 2 E and F), line 17 has a single copy of the CFP gene at the locus. The recombination rate is strongly influenced by the transgene integration locus in yeasts, but we were unable to determine whether this also occurs in the transgenic rice analyzed here.

The recombination frequencies detected by the YFP fluorescence in the rice analyzed here were about 10-fold lower than those obtained by the GUS gene reporter assay in *A. thaliana*, which reached a frequency of approximately 10^{-4} (Sun et al. 2008). This difference is probably due to the different relative locations of the homologous sequences to be recombined. In the Sun et al. system, the genes to be recombined were tandemly placed only 1.5 kbp apart on a continuous DNA region and detected intrachromosomal recombination. Our results do not exclude the possibility that the CFP and YFP genes were both integrated tandemly at the same

Table 2. The number of loci with an integrated CFP or YFP gene in the transgenic genome.

Line	Number of CFP transgene loci	Number of YFP transgene loci
12	2	1
17	1	5 to 6
22	3 to 4	5
23	3	3
53	7	6 to 7

Each number corresponds to the arrows and asterisks in Figures 3B or C.

locus. However, this is unlikely, since in our system, both members of a pair of homologous genes, the active recipient CFP gene and the inactive donor YFP gene, were integrated independently at random sites in the rice genomic DNA. This is supported by our Southern blot analyses, which showed that the sizes of the CFP (blue arrows) and YFP (red arrows) signals mostly varied by more than two (Figure 3A, lanes II). These results have shown that our assay system mimics meiotic homologous recombination and is useful to estimate the rate of meiotic gene conversion, which occurs between pairs of homologous sequences on different chromosomes (i.e., homologous chromosomes). This type of recombination would occur less frequently than that within a short (a few kbp) region.

Our system has additional advantages over single gene reporter systems such as firefly luciferase and GUS; i.e., our system enables us to calculate the homologous recombination rates between homologous sequences among cells that are emitting both/either CFP (recipient gene) and/or YFP (recombinant gene) fluorescence. This provides more accurate estimation of recombination rate, since single gene reporter systems estimate recombination rates based on large population of cells. Our assay system is useful for any plant organs or tissues; however, the use of pollens permits the detection of a recombination event at an early generation. This sensitive and effective assay system provides a useful tool to evaluate various genetic and environmental factors that may influence meiotic homologous recombination.

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References

Bergerat A, deMassy B, Gadelle D, Varoutas PC, Nicolas A, Forterre P (1997) An atypical topoisomerase II from archaea with implications for meiotic recombination. *Nature* 386: 414–417

Francis KE, Lam SY, Harrison BD, Bey AL, Berchowitz LE, Copenhaver GP (2007) Pollen tetrad-based visual assay for meiotic recombination in *Arabidopsis*. *Proc Natl Acad Sci USA* 104: 3913–3918

Keeney S, Giroux CN, Kleckner N (1997) Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell* 88: 375–384

Kimura M, Sekido S, Isogai Y, Yamaguchi I (2000) Expression, purification, and characterization of blasticidin S deaminase (BSD) from *Aspergillus terreus*: The role of catalytic zinc in enzyme structure. *J Biochem* 127: 955–963

Melamed-Bessudo C, Yehuda E, Stuitje AR, Levy AA (2005) A new seed-based assay for meiotic recombination in *Arabidopsis thaliana*. *Plant J* 43: 458–466

Murashige T, Skoog E (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol Plant* 15: 473–497

Ochiai-Fukuda T, Takahashi-Ando N, Ohsato S, Igawa T, Kadokura K, Hamamoto H, Nakasako M, Kudo T, Shibata T, Yamaguchi I, et al. (2006) A fluorescent antibiotic resistance marker for rapid production of transgenic rice plants. *J Biotechnol* 122: 521–527

Pecina A, Smith KN, Mezard C, Murakami H, Ohta K, Nicolas A (2002) Targeted stimulation of meiotic recombination. *Cell* 111: 173–184

Rashid H, Yokoi S, Toriyama K, Hinata K (1996) Transgenic plant production mediated by Agrobacterium in Indica rice. *Plant Cell Rep* 15: 727–730

Shingu Y, Tokai T, Agawa Y, Toyota K, Ahmed S, Kawagishi-Kobayashi M, Komatsu A, Mikawa T, Yamamoto M, Wakasa K, et al. (2012) The double-stranded break-forming activity of plant SPO11s and a novel rice SPO11 revealed by a Drosophila bioassay. *BMC Mol Biol* 13: 1

Sun X, Zhang Y, Yang S, Chen JQ, Hohn B, Tian D (2008) Insertion DNA promotes ectopic recombination during meiosis in Arabidopsis. *Mol Biol Evol* 25: 2079–2083

Szostak JW, Orr-Weaver TL, Rothstein RJ, Stahl FW (1983) The double-strand-break repair model for recombination. *Cell* 33: 25–35

Tozawa Y, Hasegawa H, Terakawa T, Wakasa K (2001) Characterization of rice anthranilate synthase α-subunit genes *OASA1* and *OASA2*. Tryptophan accumulation in transgenic rice expressing a feedback-insensitive mutant of OASA1. *Plant Physiol* 126: 1493–1506

Urushibara S, Tozawa Y, Kawagishi-Kobayashi M, Wakasa K (2001) Efficient transformation of rice suspension-cultured cells by *Agrobacterium tumefaciens*. *Breed Sci* 51: 33–38

Wakasa K, Kobayashi M, Kamada H (1984) Colony formation from protoplasts of nitrate reductase-deficient rice cell lines. *J Plant Physiol* 117: 223–231

Yamada T, Tozawa Y, Hasegawa H, Terakawa T, Ohkawa Y, Wakasa K (2004) Use of a feedback-insensitive α subunit of anthranilate synthase as a selectable marker for transformation of rice and potato. *Mol Breed* 14: 363–373

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    1
CFP ATGGTGAGCA AGGGCGAGGA GCTGTTCACC GGGGTGGTGC CCATCCTGGT CGAGCTGGAC GGCGACGTAA ACGGCCACAA GTTCAGCGTG TCCGGCGAGG
YFP ATGGTGAGCA AGGGCGAGGA GCTGTTCACC GGGGTGGTGC CCATCCTGGT CGAGCTGGAC GGCGACGTAA ACGGCCACAA GTTCAGCGTG TCCGGCGAGG
    101
CFP GCGAGGGCGA TGCCACCTAC GGCAAGCTGA CCCTGAAGTT CATCTGCACC ACCGGCAAGC TGCCCGTGCC CTGGCCCACC CTCGTGACCA CCCTGACCTG
YFP GCGAGGGCGA TGCCACCTAC GGCAAGCTGA CCCTGAAGTT CATCTGCACC ACCGGCAAGC TGCCCGTGCC CTGGCCCACC CTCGTGACCA CCTTCGGCTA
    201
CFP GGGCGTGCAG TGCTTCAGCC GCTACCCCGA CCACATGAAG CAGCACGACT TCTTCAAGTC CGCCATGCCC GAAGGCTACG TCCAGGAGCG CACCATCTTC
YFP CGGCCTGCAG TGCTTCGCCC GCTACCCCGA CCACATGAAG CAGCACGACT TCTTCAAGTC CGCCATGCCC GAAGGCTACG TCCAGGAGCG CACCATCTTC
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    701                720
CFP TGGACGAGCT GTACAAGTAA
YFP TGGACGAGCT GTACAAGTAA
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Supplementary Figure 1

The coding DNA sequence alignment of CFP and YFP.