Supporting Online Material

Cytokinin oxidase regulates rice grain production

Materials and Methods

Plant materials

An *indica* variety of rice, ‘Habataki’, was crossed with a *japonica* variety, ‘Koshihikari’. The resultant F₁ plants were crossed with ‘Koshihikari’ to produce BC₁F₁ seeds. Ninety-six BC₁F₆ lines (backcross inbred lines, BILs) were developed from the resultant BC₁F₁ plants by the single seed-descent method (1, 2), and used for QTL analysis. NIL- *Gn1* and NIL- *sd1* (BC₄F₂) were developed by repeated backcrossing with ‘Koshihikari’. The *indica* variety 5030, 5150 and 90B2 was kindly provided by the China National Rice Research Institute (Hangzhou, Zhejiang Province).

Molecular marker and Rice databases

Rice genomic tools and information are available on Rice Genome Research Program database; http://rgp.dna.affrc.go.jp/, Oryzabase: Integrated Rice Science Database;


QTL analysis

Grain numbers in main panicles and plant height were determined in 96 BILs grown in a research field at Nagoya University. Linkage maps of the 96 BILs with 200 molecular markers covering the entire rice genome were constructed using MAPMAKER (4). QTL
analysis was performed with phenotypic evaluation and linkage maps using MAPMAKER/QTL (5). Several QTLs associated with grain number have been reported in rice (6-15) and some of them are located near the Gn1 region identified in the present study. It may be possible that this Gn1 locus is responsible for enhancing crop yield in several rice varieties; however, since parental combinations and molecular markers used in previous studies were different, and none of these QTLs have been cloned, it is difficult to conclude whether all QTLs for grain number in this region are identical. Since no QTL that enhances crop yield has definitively characterized, we initiated a project to clone the QTL.

**Molecular cloning of Gn1a**

13,000 BC$_4$F$_2$ plants segregating Gn1a and gn1a were used for positional cloning of Gn1a. Phenotypic evaluation of Gn1a was confirmed using F$_3$ and F$_4$ progenies. DNA fragments of ‘Habataki’ and ‘Koshihikari’ within 6.3 kb of the candidate region were compared.

**RT-Southern blot analysis**

Total RNA was extracted from inflorescence meristems of ‘Habataki’, ‘Koshihikari’, and ‘5150’, and converted into single-strand cDNA. PCR amplification products were generated by using the single-strand cDNA as a template, were separated by agarose gel electrophoresis, and transferred to a Hybond N$^+$ membrane (Amersham Biosciences). The RT-Southern blot analysis was performed with OsCKX2 and Actin cDNA probes.
Transgenic analysis

A full-length genomic DNA of OsCKX2 including the promoter region was inserted into the binary vector pBI-Hm12. An antisense fragment DNA of the 3’-untranslated region of OsCKX2 driven by the native promoter was inserted into the binary vector. These DNA fragments were introduced into a *japonica* variety, ‘TC65’, by *Agrobacterium tumefaciens*-mediated transformation. The empty pBI-Hm12 vector also was transformed into ‘TC65’. Histochemical analysis of GUS activity was performed as previously described by Matsuoka and Sanada (16).

CK analysis

Extraction and determination of CKs from rice tissues (about 100 mg) were performed using a liquid chromatography-tandem mass chromatography system (model 2695/Quattro Ultima Pt, Waters) as described previously (17).

Enzyme assay for CKX

The coding regions of OsCKX2 from ‘Habataki’ and ‘Koshihikari’ were amplified by RT-PCR. The cDNA fragment was cloned into the *Eco*RI site of pBluescript II-KS (Stratagene) and checked by nucleotide sequence analysis. The cDNA was excised by digestion with *Eco*RI and the fragment was cloned into pYES2 (Invitrogen). The resulting plasmids were introduced into the yeast strain INVSc1 (Invitrogen). The empty vector was used as a negative control. Induction of OsCKX2 expression and preparation of total soluble extracts were carried out as described in the supplier’s protocol. The enzyme assay for CKX
was performed essentially as described by Frebolt et al (18), using 0.5 mM 2,6-dichlorophenol indophenol as an electron acceptor and 0.15 mM iP as a substrate.

**Phylogenetical analysis of CKX**

The structural relationships were calculated using CLUSTAL W (19) and the unrooted phylogenetic consensus tree was generated using Treeview (20).
**Fig. S1.** Distribution frequencies of phenotypes for 96 individuals in BILs (A) plant height, (B) number of grains per panicle.

**Fig. S2.** *OsCKX2* expression in the inflorescence meristems of transgenic plants. Lane 1, 2copyCKX2: transgenic TC65 plant carrying two copies of *OsCKX2* derived from ‘Koshihikari’. Lane 2, TC65: control *japonica* line. Lane 3, CKX2/AS: Transgenic rice carrying antisense *OsCKX2* cDNA from ‘Koshihikari’. Lane 4, Koshihikari: control *japonica* line. Actin was used as a control.
References

Figure S1: Analysis of plant height and number of grains per panicle.

A. Plant height (cm) distribution for Koshihikari and Habataki:
- Koshihikari: Average height
- Habataki: Average height

B. Number of grains per panicle distribution for Koshihikari and Habataki:
- Koshihikari: Average number of grains
- Habataki: Average number of grains

Fig. S1 Ashikari et al.
Fig. S2 Ashikari et al.
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4: http://cdna01.dna.affrc.go.jp/cDNA/report/KOME_J023113D01.html
5: http://cdna01.dna.affrc.go.jp/cDNA/report/KOME_J023149003.html
11: http://RiceGAAS.dna.affrc.go.jp/rga-bin/gene.pl?genomeNo=path%3Ddata/chro08/P0690E03.d%26file%3DP0690E03.Predgeneset.genome%26.Predgene12