



## Cytokinin Oxidase Regulates Rice Grain Production

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# Cytokinin Oxidase Regulates Rice Grain Production

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Most agriculturally important traits are regulated by genes known as quantitative trait loci (QTLs) derived from natural allelic variations. We here show that a QTL that increases grain productivity in rice, *Gn1a*, is a gene for cytokinin oxidase/dehydrogenase (*OsCKX2*), an enzyme that degrades the phytohormone cytokinin. Reduced expression of *OsCKX2* causes cytokinin accumulation in inflorescence meristems and increases the number of reproductive organs, resulting in enhanced grain yield. QTL pyramiding to combine loci for grain number and plant height in the same genetic background generated lines exhibiting both beneficial traits. These results provide a strategy for tailored crop improvement.

Food shortage is one of the most serious global problems in this century. The United Nations Food and Agricultural Organization (FAO) estimates that 852 million people worldwide were undernourished in 2000 to 2002 (1). The global population, now at 6.4 billion, is still growing rapidly and is projected to reach 8.9 billion people by 2050 (2). Cereals are an important source of calories for humans, both by direct intake and as the main feed for livestock. About 50% of the calories consumed by the world population originate from three cereals: rice (23%), wheat (17%), and maize (10%) (3). To meet the expanding food demands of the rapidly growing world population, crop grain production will need to increase by 50% by 2025 (4).

Many agronomically important traits, including yield, are expressed in continuous phenotypic variation. These complex traits usually are

governed by a number of genes known as quantitative trait loci (QTLs) derived from natural variations (5). QTL analysis has been employed as a powerful approach to discover agronomically useful genes (6–13).

Rice (*Oryza sativa* L.) is a staple food and has been established as a model monocot because it has the smallest genome size (390 Mb) among the major cereals (14), because its genome is syntenic with the genomes of other cereals (15), and because rice can be transformed easily. As a result, many molecular markers for rice have been developed, many mutants have been generated and stocked, and the complete genome of rice has been mapped and sequenced (14, 16–21). These accomplishments have greatly facilitated QTL analysis in rice. Grain number and plant height are important traits that directly contribute to grain productivity. Dwarf rice and wheat varieties were developed by classical plant breeding methods, contributing to the green revolution in the 1960s. Higher yields were obtained from these dwarf crops because their short stature reduced lodging, which is an agronomic term for bending of plants toward the ground after wind or rain storms (22–25). During the past decade, many attempts have been made to characterize QTLs for grain production and plant height; however, the genes involved in

these QTLs have not been identified yet, and their chromosomal positions remain obscure. We aimed to identify genes of QTLs for grain number and plant height, not only to elucidate molecular mechanisms that regulate grain productivity but also to use these genes for breeding.

**QTL analysis.** A choice of parental lines that show wide phenotypic variation in the targeted traits is necessary for QTL analysis, because QTL detection is based on natural allelic differences between parental lines. We chose an *indica* rice variety, Habataki, and a *japonica* variety, Koshihikari, because they not only exhibit large variations in agronomically important traits but also have many molecular markers available (21). On average, Habataki plants are shorter than individuals of Koshihikari but produce more grains in their main panicle (Fig. 1, A to D).

We developed primary-mapping populations of 96 backcross inbred lines (BILs) derived from the cross between Habataki and Koshihikari. Both grain number and plant height seemed to be regulated by QTLs, as these traits were approximately normally distributed in the mapping population (fig. S1). QTL analysis detected five QTLs for increasing grain number (*Gn*) and four QTLs for plant height (*Ph*) (Table 1 and Fig. 1E). The most effective QTL for plant height, *Ph1*, was located close to the *semi-dwarf 1* gene (*sd1*) that encodes gibberellin 20 oxidase (23–25). Comparison of *SD1* between Habataki and Koshihikari revealed that Habataki had a 383-base pair (bp) deletion in the coding region of *gibberellin 20 oxidase*. The resulting loss of function caused the reduced plant height in Habataki. The deletion in the *gibberellin 20 oxidase* is the same as the causal variation found in IR8, a variety that helped lead to the green revolution in rice (23–25).

The most effective QTL for increasing grain number, *Gn1* on chromosome 1, was selected for further analysis. The Habataki *Gn1* allele is expected to produce ~92 more grains per main panicle than the Koshihikari allele; *Gn1* explains 44% of the difference in grain number between Habataki and Koshihikari (Table 1). So far, several QTLs associated with yield have been reported in rice. Some of these QTLs are located near the *Gn1* region on the short arm of

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chromosome 1, suggesting they might be the same QTL (21). Although these QTL genes have not been identified and characterized yet, it is possible that the *Gn1* locus contributes to increased grain productivity in various rice varieties. The importance of *Gn1* for enhancing grain number in rice suggested that this QTL would be a good candidate for cloning.

**QTL cloning.** In QTL cloning, producing nearly isogenic lines (NILs) carrying only one target QTL is necessary to eliminate the effects of other QTLs (5). Consequently, the QTL of interest in the NIL can be considered as a single Mendelian factor (26). We produced the NIL-*Gn1* carrying the *Gn1* region from Habataki in the Koshihikari background and used it for *Gn1* mapping.

We used 96  $F_2$  individuals derived from heterozygote (*Gn1/gn1*) plants of NIL-*Gn1* for coarse mapping of *Gn1*. We found that *Gn1* consisted of two loci, QTL-*Gn1a* and QTL-*Gn1b*. QTL-*Gn1a* was mapped within 2 cM between the molecular markers R3192 and C12072S, whereas QTL-*Gn1b* mapped to the upper region of QTL-*Gn1a* (Fig. 1, F and G). *Gn1a* was chosen as the target for posi-

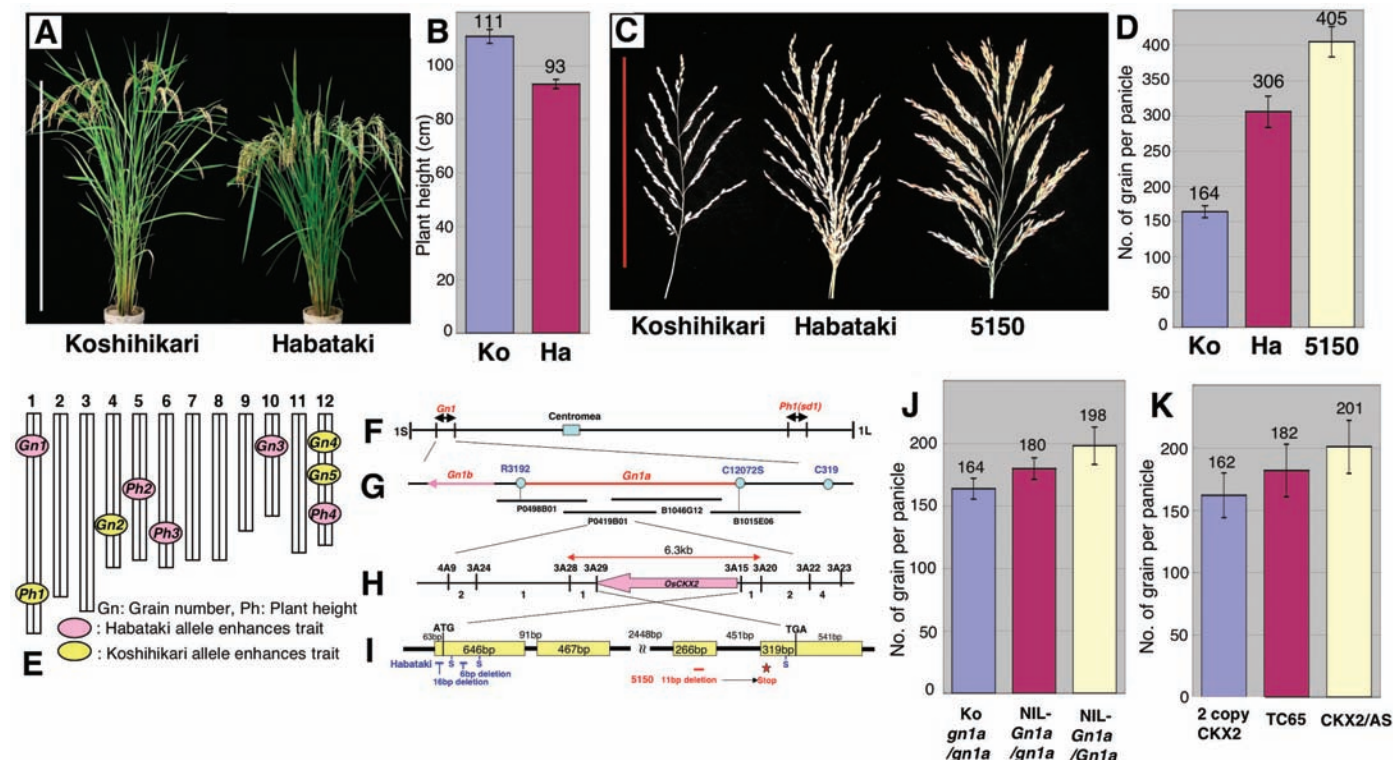
tional cloning, because the effects of Habataki *Gn1a* and *Gn1b* loci were almost identical and because the position of *Gn1a* between the two markers had been unambiguously determined. The *Gn1a* allele of Habataki was semidominant, because the grain number of heterozygote plants (*Gn1a/gn1a*) was intermediate between those of homozygote plants, *gn1a/gn1a* and *Gn1a/Gn1a* (Fig. 1J).

About 13,000  $F_2$  plants derived from heterozygotes (*Gn1a/gn1a*) of NIL-*Gn1a* were used for high resolution mapping of *Gn1a*. The candidate region of *Gn1a* was narrowed down to the 6.3 kb between the markers 3A28 and 3A20 (Fig. 1H). In this region, the Rice Genome Automated Annotation System (27) predicted one reading frame with high similarity to cytokinin oxidase/dehydrogenase (CKX), *OsCKX2* (28) (Fig. 1H). The *OsCKX2* of Koshihikari and Habataki consist of four exons and three introns and encode proteins of 565 or 563 amino acids, respectively. Comparison of the DNA sequences between the cultivars revealed several nucleotide changes, including a 16-bp deletion in the 5'-untranslated region, a 6-bp deletion in the first exon, and

three nucleotide changes resulting in amino acid variation in the first and fourth exons of the Habataki allele (Fig. 1I).

We also analyzed the nucleotide sequences of *OsCKX2* in three alleles of high-yielding rice varieties from China, 5030, 5150, and 90B2. An 11-bp deletion in the coding region was detected in 5150, which produced more than 400 grains in the main panicle in our experimental field (Fig. 1, C and D). This deletion created a premature stop codon, suggesting that 5150 is null for *OsCKX2* (Fig. 1I). The other two varieties had sequences identical to the Habataki allele. The coincidence of the *OsCKX2* null allele and a higher grain number suggested that a reduction or loss of function of *OsCKX2* enhanced grain production.

To confirm that *OsCKX2* corresponds to *Gn1a*, we produced transgenic plants expressing different levels of *OsCKX2* and examined their grain yield. As Koshihikari and Habataki fail to regenerate shoots from the callus, we used the easily regenerable cultivar Taichung 65 (TC65), which possesses the Koshihikari allele of *OsCKX2*. Transgenic plants carrying two copies of the sense strand of *OsCKX2* that



**Fig. 1.** QTL analysis and molecular cloning. (A) Gross morphology of Koshihikari and Habataki at maturity. Scale bar, 1 m. (B) Comparison of plant height at the heading stage in Koshihikari (Ko) and Habataki (Ha). (C) Panicle structure of Koshihikari, Habataki, and 5150. Scale bar, 20 cm. (D) Comparison of grain number in the main panicle of Koshihikari (Ko), Habataki (Ha), and 5150. Values in (B) and (D) are means with SD ( $n = 10$  plants). (E) QTL map of grain number (*Gn*) and plant height (*Ph*) on rice chromosomes. (F) Location of *Gn1* and *Ph1* on chromosome 1. (G) Coarse linkage map and physical map of *Gn1*. (H) High-resolution linkage map of *Gn1a*. The number of recombinants between the

molecular markers is indicated below the high-resolution map. (I) *OsCKX2* structure and mutation sites in Habataki (blue) and 5150 (red). S indicates the site of amino acid substitutions. (J) Comparison of grain number per main panicle in Koshihikari (Ko-*gn1a/gn1a*), NIL-*Gn1a/gn1a*, and NIL-*Gn1a/Gn1a*. (K) Comparison of grain number per main panicle in nontransgenic and transgenic lines. 2 copy CKX2, transgenic TC65 plant carrying two copies of *OsCKX2* derived from Koshihikari; TC65, control *japonica* line; CKX2/AS, transgenic rice carrying antisense *OsCKX2* cDNA from Koshihikari. Values in (J) and (K) are means with SD ( $n = 10$  plants).

was highly expressed showed reduced grain numbers compared to TC65. However, transgenic plants with antisense strands of *OsCKX2* that had reduced levels of expression developed higher grain numbers (Fig. 1K and fig. S2). We conclude that the QTL for increased grain number, *Gn1a*, is *OsCKX2*.

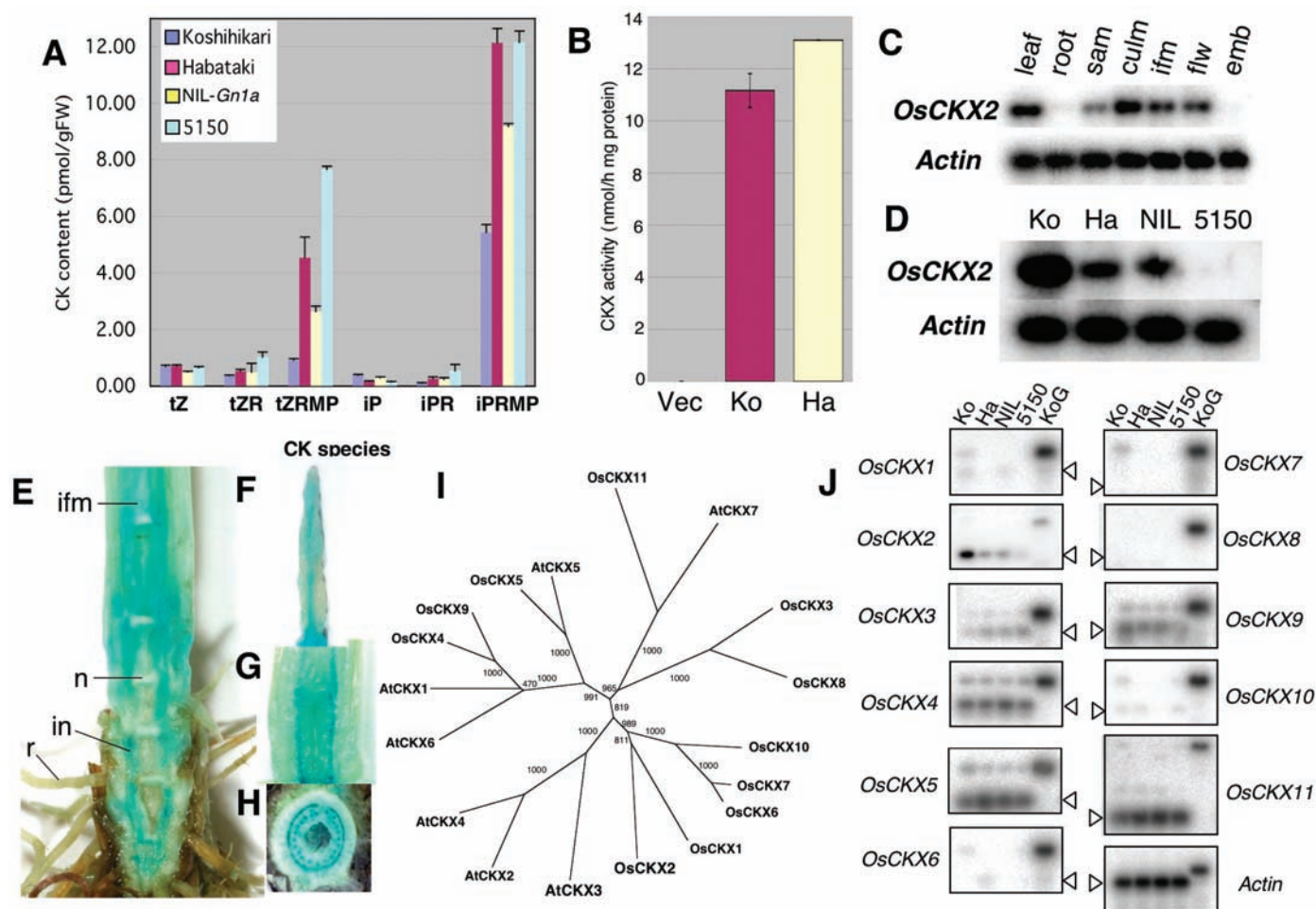
**Molecular analysis of *OsCKX2*.** Cytokinin (CK) was first discovered as a plant hormone that promotes cell division (29). It is now known to influence various aspects of plant growth and development, including seed germination, apical dominance, leaf expansion, reproductive development, and delay of senescence (30). Natural CKs such as *trans*-zeatin (tZ) and isopentenyladenine (iP) are  $N^6$ -substituted adenine derivatives that generally

contain an isoprenoid side chain (31). CKX preferentially and irreversibly degrades nucleobase CKs by cleavage of the unsaturated  $N^6$ -isoprenoid side chains (31). This catabolic enzyme probably plays the principal role in controlling CK levels in plant tissues (32–34).

To examine whether the *OsCKX2* locus affects CK metabolism, we analyzed the levels of CKs in inflorescence meristems of Koshihikari, Habataki, NIL-*Gn1a*, and 5150. Although the contents of active tZ were similar in these lines, CK nucleotides (i.e., tZRMP and iPRMP) were substantially more abundant in Habataki, NIL-*Gn1a*, and 5150 than in Koshihikari (Fig. 2A). Because the CK metabolism modifying the adenine moiety is partially shared with the purine salvage pathway,

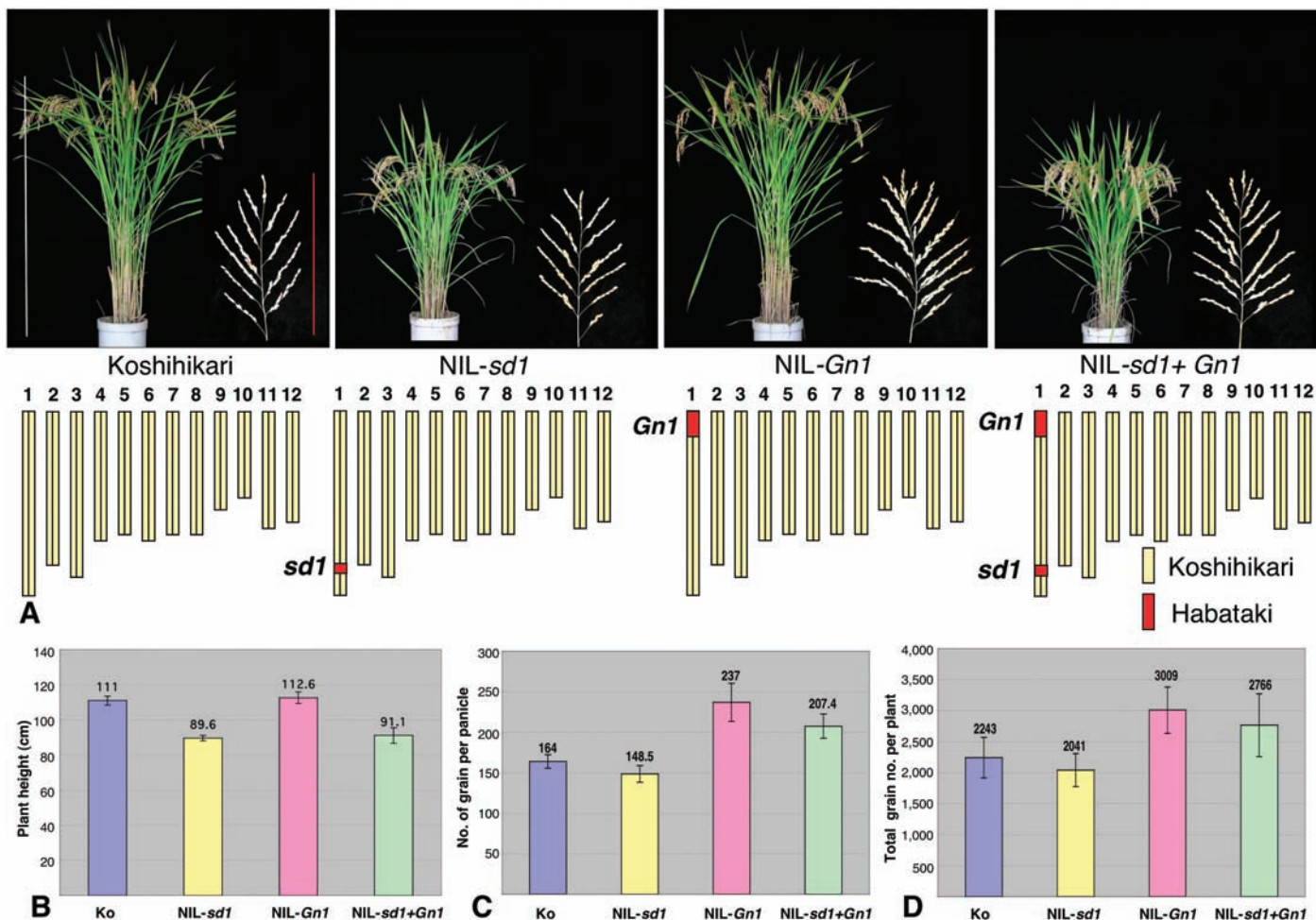
nucleobase CKs are readily converted to the corresponding nucleotides and nucleosides (31). This metabolic flow plays an important role in the homeostasis of active CKs (31). In this context, the accumulation of the nucleotide- and nucleoside-species is explainable by the reduction in CKX activity in Habataki, NIL-*Gn1a*, and 5150, and the increased production of CK conjugates to reduce the overall CK activity (see below).

To test whether *OsCKX2* encodes an active enzyme in Koshihikari and Habataki, we isolated the cDNAs and expressed the proteins in budding yeast, *Saccharomyces cerevisiae*. Although several amino acids varied between the two *OsCKX2* proteins, they catalyzed the cleavage of iP side chains with similar specific ac-



**Fig. 2.** Molecular characterization of *OsCKX2*. (A) Comparison of CK levels in the inflorescence meristem of Koshihikari, Habataki, NIL-*Gn1a*, and 5150. tZ, *trans*-zeatin; tZR, tZ riboside; tZRMP, tZR 5'-monophosphate; iP, isopentenyladenine; iPR, iP riboside; iPRMP, iPR 5'-monophosphate; gFW, grams fresh weight. Values are means with SD ( $n = 3$  measurements). (B) Enzymatic CKX activity in yeast cells transformed with empty vector (Vec), the *OsCKX2* allele from Koshihikari (Ko), and that from Habataki (Ha). (C) Expression analysis by RT-Southern blot of *OsCKX2* in various organs [leaf, root, shoot apex meristem (sam), culm, inflorescent meristem (ifm), flower (flw), and embryo (emb)] of rice. (D) *OsCKX2* expression in the inflorescence meristem of Koshihikari (Ko), Habataki (Ha), NIL-*Gn1a* (NIL), and 5150. Actin was used as a control in (C) and (D). (E to H) GUS expression under the control of the

*OsCKX2* promoter: (E) longitudinal section of a culm (ifm, inflorescent meristem; n, node; in, internode; r, root); (F) young flower; (G) longitudinal section of node and internode; and (H) transverse section of internode. (I) Phylogenetic relationship of CKX proteins in rice and *Arabidopsis*. *OsCKXs*, *O. sativa* CKXs (table S1); *AtCKXs*, *Arabidopsis* CKXs (28). (J) Expression analysis of *OsCKX1* to *OsCKX11* in the inflorescence meristem of Koshihikari (Ko), Habataki (Ha), NIL-*Gn1a* (NIL), and 5150 by RT-Southern blot. The Koshihikari genomic DNA was used as a template for the positive control (KoG) in the polymerase chain reaction (PCR) with primers designed for each *OsCKX*. The PCR produced bands at higher molecular weights than those generated from cDNA because they contained intron sequences. The expected signal sizes of the cDNAs are indicated by arrowheads.



**Fig. 3.** Phenotypic characterization of NIL-QTLs. (A) Plant morphologies and chromosome maps of Koshihikari, NIL-*sd1*, NIL-*Gn1*, and NIL-*sd1+Gn1*. White and red scale bars indicate 1 m and 20 cm, respectively. (B) Comparison of plant height, (C) grain number in the main panicle, and (D) grain number in whole plants for Koshihikari, NIL-*sd1*, NIL-*Gn1*, and NIL-*sd1+Gn1*. Values in (B) to (D) are means with SD ( $n = 10$  plants).

tivities (Fig. 2B). This result shows that both alleles of *OsCKX2* encode functional enzymes.

We next studied the expression profiles of *OsCKX2* in various organs by reverse transcription (RT)-Southern blotting, because RNA gel blot analysis did not detect any signals because of the low expression levels. The RT-Southern blot showed that *OsCKX2* was preferentially expressed in leaves, culms, inflorescence meristems, and flowers (Fig. 2C). The highest levels of *OsCKX2* expression in inflorescence meristems were found in Koshihikari. Transcript accumulation was less abundant in Habataki and NIL-*Gn1a* and extremely low in 5150 (Fig. 2D). As these differences indicated a correlation between *OsCKX2* expression levels and grain number, they suggested that the phenotypic differences observed might have been caused by differential transcription of *OsCKX2*.

We next examined the tissue specificity of *OsCKX2* expression in transgenic rice harboring an *OsCKX2 promoter:: $\beta$ -glucuronidase* (GUS) construct. GUS expression was observed mainly in the vascular tissue in developing culms,

inflorescence meristems, and young flowers in the T2 generation of transgenic plants (Fig. 2, E to H). The expression of *OsCKX2* in inflorescence meristems might regulate the CK level to control flower number. CK is known to be translocated acropetally via the xylem and systemically via the phloem (35). The high levels of expression in these tissues suggest that *OsCKX2* plays a role in regulating CK levels in the vascular system of developing culms, where CK is transported to the inflorescence meristems.

At least 11 putative *CKX* genes (*OsCKX1* to *OsCKX11*) are present in the rice genome (Fig. 2I and table S1). This redundancy suggests that the *OsCKXs* could be functionally differentiated by their temporal and spatial expression patterns. In tobacco and *Arabidopsis*, overexpression of *CKX* results in reduced levels of endogenous CK and lower meristem activity (33, 34). In transgenic *Arabidopsis*, overexpression of *AtCKX3*, the allele showing the highest similarity to *OsCKX2* of the seven *AtCKXs* (Fig. 2I), reduced flower number because of a decreased rate of primordia formation in the

flower meristem (34). Our findings are in agreement with these results from *Arabidopsis*.

We examined the expression of all *OsCKX* genes in the inflorescence meristems of the four lines, Koshihikari, Habataki, NIL-*Gn1a*, and 5150, to elucidate why their phenotypes were different despite the apparent high redundancy of *OsCKX* genes in the rice genome. *OsCKX2* was the dominant *OsCKX* expressed in Koshihikari inflorescence meristems (Fig. 2J), underlining its role in crop productivity. In contrast to *OsCKX2*, the expression patterns of all other *OsCKX* genes did not differ among these cultivars (Fig. 2J), indicating that *OsCKX2* functions in inflorescence development.

**QTL pyramiding.** In this experiment, *Gn1a* was identified as *OsCKX2*, a gene that increased grain number by ~21% (Fig. 1J). *Gn1b*, another QTL, has not been identified yet and will be the next target for characterization. The Habataki alleles of *Gn1a* and *Gn1b* additively increase grain number; both components of *Gn1* (*Gn1a* and *Gn1b*) are ideally suited for application to a practical breeding program. The NIL-*Gn1* carrying the *Gn1* locus

**Table 1.** Putative QTLs for grain number (*Gn*) and plant height (*Ph*). QTL names are designated with the abbreviation of the trait name. NML, nearest marker locus of putative QTLs; PVE, phenotypic variation explained by each QTL; LOD, logarithm of odds.

QTL name	Chromosome number	NML	Position of NML (cM)	Change in effect*	PVE	LOD
<i>Grain number</i>						
<i>Gn1</i>	1	BB-85	22.6	H92	44%	9.863
<i>Gn2</i>	4	AE-19	102.1	K41	10%	1.922
<i>Gn3</i>	10	AJ-65	30.2	H39	7%	1.38
<i>Gn4</i>	12	BI-20	30	K35	9%	1.701
<i>Gn5</i>	12	BB-23	47	K35	11%	2.147
<i>Plant height</i>						
<i>Ph1</i>	1	BC-55	146.4	K24	30%	6.531
<i>Ph2</i>	5	BF-37	66.5	H10	7%	1.389
<i>Ph3</i>	6	CC-84	107.3	H11	9%	1.629
<i>Ph4</i>	12	BB-48	91.4	H9	8%	1.565

\*H indicates a Habataki-enhanced trait; K, a Koshihikari-enhanced trait. In grain numbers or cm of plant height.

(*Gn1a* and *Gn1b*) of Habataki has a heavier panicle weight and is more susceptible to lodging.

To resolve the problem, we employed a QTL pyramiding breeding strategy. In this approach, desirable QTLs are combined through crossing of NIL-QTLs into a common genetic background. First, we developed an NIL carrying the Habataki *sd1* allele in the Koshihikari background (Fig. 3A). This NIL-*sd1* was ~20% shorter than Koshihikari, as expected because of the effect of the *sd1* allele (Fig. 3, A and B). Simultaneously, an NIL-*Gn1* carrying the Habataki *Gn1a*+*Gn1b* chromosome fragment that produced ~45% more grain than Koshihikari (Fig. 3, A and C) was also selected. The degrees of increase in grain number (45%) and reduction of plant height (20%) in the NILs corresponded to the phenotypic variation effects of *Gn1* (44%) or *Ph1* (30%), respectively, as predicted by the QTL analysis (Table 1). NIL-*sd1*+*Gn1* was generated by crossing NIL-*Gn1* and NIL-*sd1* (Fig. 3A). The grain number in the main panicle was 26% higher, and plants were 18% shorter in this line than in Koshihikari (Fig. 3, A to C). The reduction in grain number for NIL-*sd1*+*Gn1* (207 grains), as compared to NIL-*Gn1* (237 grains), seemed due to pleiotropic effects of the *sd1* allele. The same degree of grain number reduction was also found in NIL-*sd1*, relative to Koshihikari (Fig. 3C). The positive effect of *Gn1*, however, outweighed the negative effect of *sd1*, in NIL-*sd1*+*Gn1* (Fig. 3C). Because total grain number per plant is the most important factor for increasing grain yield under field production conditions, grain numbers per plant were compared rather than grain numbers per main panicle. When the comparison was based on grain numbers per plant, 34% and 23% increases were found in NIL-*Gn1* and NIL-*sd1*+*Gn1*, respectively (Fig. 3D). No difference in grain size was observed for these two NILs. Thus, *Gn1* is a useful locus for increasing grain productivity.

### Toward the application of QTLs for a new green revolution.

We succeeded in cloning a QTL (*Gn1a*) that increased grain number in rice. *Gn1a* encodes OsCKX2, an enzyme that degrades bioactive CK. A null allele of *OsCKX2* had been selected for increasing crop yield in a conventional breeding program in China. Genome synteny allows breeders to integrate traits and genes among cereals (15). For example, rice chromosome 1 shows regions of sequence similarity with chromosomes 3, 6, and 8 in maize (36), where some QTLs for grain yield traits have been mapped (37–40). *Gn1a* in rice might correspond to these maize QTLs, and orthologous CKX genes in other species might regulate yield in other cereal crops as in rice.

In molecular studies of rice and wheat varieties, the phytohormone gibberellin has been identified as a key player in controlling crop plant architecture (22–25). We demonstrate here that CK metabolism also contributes to crop productivity. Because CK controls cell division and lateral meristem activity (30, 31), CK accumulation in the inflorescence meristem can explain the significantly higher grain numbers.

Identification of agronomically important QTLs and pyramiding of such QTLs presents a useful strategy for efficient crop breeding. Interspecific crosses between *O. sativa* and wild relatives could lead to the discovery of useful QTLs from a range of allelic variations much wider than those present in cultivated lines. Furthermore, wild rice species are likely to provide access to QTLs not only for yield but also for disease resistance, stress tolerance, and other desirable traits (6–8, 21), because these plants have adapted to unique geographic and environmental conditions. Discovering useful genes, improving agricultural traits hidden in the plant genome, and applying these findings to crop breeding will pave the way for a new green revolution.

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### Supporting Online Material

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References and Notes

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