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Application of rice genomics to plant biology and breeding

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Abbreviations: RFLP, Restriction Fragment Length Polymorphism; GA, Gibberellin; BR, Brassinosteroid; MAS, Marker Assisted Selection; QTL, Quantitative Trait Loci; KD, Kinase Domain.

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Rice Genomics

It has been estimated that 50% of the human population depends on rice (*Oryza sativa* L.) as its main source of nutrition (White, 1994). In particular, it is the most important crop for people living in the monsoonal areas of Asia, where it has a long history of cultivation and is deeply ingrained in the daily lives of Asian people.

For many years, rice has been the subject of numerous breeding studies aimed at developing higher yielding or better tasting cultivars. It has also become a useful plant for studying biology, as a model plant of monocotyledons (monocots). Rice has a small genome (430 Mb) relative to other Gramineae plants, and its genome size is about three times larger than that of *Arabidopsis*, a model plant of dicotyledons (dicots). Last decade, technological innovations in science enabled dramatic advancements in the

field of plant genomics (genome science). Many rice genome projects have been launched, and they are providing very useful information for plant biology and plant breeding.

Between 1966 and 1990 the population of densely populated and low-income countries grew by 1.8 fold while food production more than doubled. Such a rapid increase in the volume of world food production was mainly due to the development of high-yielding varieties of wheat and rice and other technological advancements (Khush, 1999). Now, however, the rate of world population growth has exceeded the rate of growth in food-grain production. It is predicted that the world population will exceed 8 billion people by 2025, with the greatest increase occurring in developing countries. Actually, many people living in Asia and Africa already suffer from malnutrition. To meet these global food demands, grain production will need to increase 50% by 2025 (Khush, 1999). Prompt measures and action should be taken to avoid a large famine and widespread food scarcity.

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The global climate is also changing, and predictions of global warming are of particular concern. Because rice plants can be cultivated under a wide range of environments, from arid highlands to flooded lowlands and in high humidity and high temperature climates as in Asia, rice may be suitable for adapting and propagating under the predicted global warming conditions. Rice genomics is a significant area of research with applications for plant biology and breeding, and its possible role in overcoming problems associated with climate change and food production should be resolved.

The rice genome shows apparent syntenies with many other grasses, such as wheat, barley, and maize (Ahn and Tanksley, 1993; Ahn et al., 1993; Kurara et al., 1994; Dunford et al., 1995; Peterson et al., 1995; Moore et al., 1995; Saghai-Marooof et al., 1996). These syntenies suggest that rice genomics has implications not only for rice breeding but also for other cereal crops.

Over the past 100 years, rice breeders and geneticists have accumulated a large number of rice mutants and a rice classical genetic map using phenotypic markers of these mutants has been developed (Nagao and Takahashi, 1963; Iwata and Omura, 1975; Kinoshita, 1995). The compact nature of the rice genome provides a distinct advantage in gene isolation and genomic sequencing as opposed to other cereal crops, and the results of rice genomics can be directly applied to cereal breeding due to their syntenies, as described above. For these reasons, rice has been selected as a target species for genome research by a number of groups. Developments in DNA technology have enabled the detection of DNA diversity in plants (Mohan et al., 1997; Nagamura et al., 1997). In 1988, a research group at Cornell University first applied the Restriction Fragment Length Polymorphisms (RFLPs) technique to construct a rice linkage map (McCouch et al., 1988), and the group subsequently developed a fine linkage map with 726 DNA markers (Causse et al., 1994). A Japanese group has also constructed a rice RFLP linkage map (Saito et al., 1991). To improve understanding of the rice genome, the Japanese government funded a seven-year (1st phase) Rice Genome Research Program (RGP) between 1991 and 1997. The RGP was initiated as a collaboration between the National Institute of Agrobiological Resources (NIAR) and the Society for Techno-innovation of Agriculture, Forestry and Fisheries (STAFF) under the Japanese Ministry of Agriculture, Forestry and Fisheries (MAFF). This program was composed of four main projects: cDNA analysis, genetic mapping, physical mapping, and bio-informatics (Sasaki, 1998). In the program, a large number of cDNAs were isolated, sequenced (Yamamoto and Sasaki, 1997) and used as DNA markers for constructing a linkage map (Kurata et al., 1994; Harushima et al., 1998) and a physical map of Yeast Artificial Chromosomes (YACs) (Kurata et al., 1997). Then, the rice classical genetic map with morphology markers and the RFLP linkage maps with molecular markers were integrated (Yoshimura et al., 1997). The accumulation of DNA markers, together with the construction of the linkage map and physical map, made it possible to isolate important genes. The *Xa21* gene, which

confers resistance against the bacterial pathogen *Xanthomonas oryzae* pv., was first isolated using a positional cloning strategy (Song et al., 1995). Subsequently, several genes for important agronomic traits have been cloned by the same strategy (Yoshimura et al., 1998; Ashikari et al., 1999; Wang et al., 1999). Rice, having one of the smallest genomes among the food crops, is the focus of basic crop research in many countries and will be the first food crop to be completely sequenced. Progress in understanding rice molecular genetics, established in the first phase of the RGP, and technological innovations in genomics make a reality of the rice genome sequencing projects. Analysis of the human genome promises to help elucidate the characteristics of inherited disease and provide the possibility of overcoming these diseases. It is equally important that staple foods necessary to sustain human life be chosen as targets for genome analysis.

The International Rice Genome Sequencing Project (IRGSP) was launched in 1998 with ten countries participating (Sasaki and Burr, 2000). At the time of writing, August 2001, thirteen research groups in eight countries are involved in the project and share the chromosomes for sequencing: **Japan** (Rice Genome Research Program [RGP], chromosome 1, 2, 6, 7 and 8); **Korea** (Korea Rice Genome Research Program [KRGRP], chromosome 1); **UK** (John Innes Center, chromosome 2); **USA** (Clemson Univ. [CUGI], Cold Spring Harbor Laboratory [CSHL], Washington University School of Medicine-Genome Sequencing Center [GSC], The Institute for Genomic Research [TIGR], Plant Genome Initiative at Rutgers [PGIR], Genome Center of Wisconsin, chromosome 3, 10 and 11); **China** (National Center for Gene Research Chinese Academy of Sciences [NCGR], chromosome 4); **Taiwan** (Academia Sinica Plant Genome Center [ASPGC], chromosome 5); **Thailand** (National Center for Genetic Engineering and Biotechnology [BIOTEC], chromosome 9); and **France** (Genoscope, chromosome 12). The IRGSP immediately releases the rice genomic sequencing data in public databases such as the DNA Data Bank of Japan (DDBJ), GenBank and the European Molecular Biology Laboratory (EMBL). Progress on sequencing is also exhibited in the RGP database (<http://rgp.dna.affrc.go.jp/index.html>). The sequencing data, rice linkage map, and physical map are integrated in the RGP database, the Integrated Rice Genome Explorer (INE: <http://rgp.dna.affrc.go.jp/giot/INE.html>) (Sakata et al., 2000). In 2000, a private company, Monsanto, made 60% of its rice genome sequences public (<http://www.rice-research.org/>). They also released about 7,000 sequence data including microsatellites (http://www.rice-research.org/rice_ssr.html). These data will accelerate the completion of the whole rice genomic sequencing project and make it easier to design DNA markers for specific positions on the rice chromosomes. In 2001, another private company, Syngenta, announced that it had finished sequencing of the rice genome, but the data have not yet been placed in the public domain. The data provided by rice genomics give us very powerful information for rice biology and breeding (Figure 1).

To date, a great number of rice mutants have been reported, and they have also been produced artificially by using mutagens such as irradiation, chemical compounds and DNA insertion, including T-DNA (Jeon et al., 2000), *Ac/Ds* transposon (Greco et al., 2001), and retrotransposon, *Tos17* (Hirochika, 2001). These mutants are very significant materials for functional genomics, enabling us to study and predict the function of genes. Due to progress in the development of genomic tools, molecular markers and genomic libraries, and the accumulation many kinds of sequence data (Figure 1), gene cloning using various mutants has become more and more realistic. Here, we discuss the application of rice genomics to molecular biology, using the analysis of the gibberellin (GAs) biosynthetic and signal transduction pathways in rice as an example.

GAs were first identified in the process of studying rice. A fungal pathogen of rice, *Gibberella fujikroi*, causes an abnormal stem elongation known as Bakanae-disease. Kurosawa discovered that this abnormal stem elongation is due to a water-soluble compound, gibberellin, produced by the fungus (Fosket, 1994). GAs are a large family of tetracyclic diterpenoid plant growth regulators reportedly associated with a number of plant growth and development processes such as seed germination, stem elongation, flowering, fruit development, and regulation of gene expression in the cereal aleurone layer (Reid, 1993; Hooley, 1994; Ross et al., 1997). GA-related mutants in plants show dwarf or elongated phenotypes, and these mutants are crucial for elucidating the regulatory mechanisms governing the GA biosynthetic and signal transduction pathways. Because dwarf characteristics are favored in plant breeding, the study of these characteristics has applications not only for understanding basic plant biology but also for molecular breeding (see below). Many GA-related mutants have been isolated from numerous plant species (Reid, 1993; Hooley, 1994; Ross et al., 1997) and can be roughly classified into two categories: GA-sensitive and GA-insensitive. A GA-sensitive mutant responds to exogenous GA because it cannot produce GA, or it produces insufficient GA due to a deficiency in genes encoding GA catalytic enzymes. On the other hand, a GA-insensitive mutant does not respond to exogenous GA, and genes related to GA-insensitivity may be associated with GA signal transduction. The GA biosynthetic pathway has been

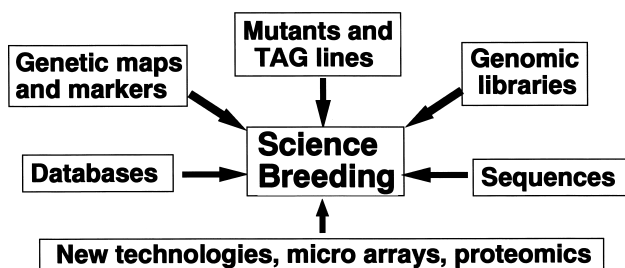


Figure 1. Application of rice genomics to science and breeding.

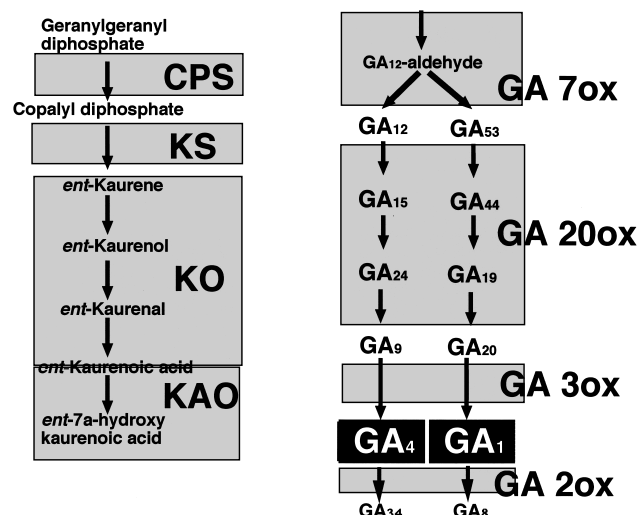


Figure 2. Gibberellin biosynthetic pathway in higher plants. CPS, *ent*-copalyl diphosphate synthase; KS, *ent*-Kaurene synthase; KO, *ent*-Kaurene 19-oxidase; KAO, *ent*-Kaurenoic acid oxidase; GA7ox, GA12-aldehyde 7-oxidase; GA20ox, GA 20-oxidase; GA3ox, GA 3β-hydroxylase; GA2ox, GA 2-oxidase.

well studied in plants, and at least seven enzymes are known to catalyze GA synthesis in plants (Figure 2).

In rice, 61 dwarf mutants have been registered to date. However, only two of these mutants, *d18* and *d35*, are characterized as having defects in their GA biosynthetic pathway (Futsuhara and Kikuchi, 1993; Murakami, 1995). Bio-active GA₁ and GA₄ are catalyzed from geranylgeranyl diphosphate (GGDP) by sequential action of cyclases in the plastids, membrane-associated mono-oxygenases in the endoplasmic reticulum, and soluble 2-oxoglutarate-dependent dioxygenases (2ODD) in the cytosol (Hedden and Phillips, 2000) (Figure 2). In this section, we discuss the isolation of the 3β-hydroxylase (GA3ox) gene, whose product catalyzes the final step in GA biosynthetic pathway, from GA₂₀ to GA₁ and GA₉ to GA₁.

d18 has been characterized as a GA-sensitive dwarf mutant (Murakami, 1972; Kinoshita and Shinbashi, 1982; Kinoshita and Shinbashi, 1982), and four independent alleles have been identified, of which two are strong alleles [*d18-Id18^b* [Hosetsu-waisei dwarf] and *d18-AD* [Akibare-waisei dwarf]], and two are weak alleles [*d18-dy* [Waito-C] and *d18k* [Kotaketamanishiki]]. The strong alleles promote a severe dwarf phenotype, the weak alleles a semi-dwarf phenotype. Physiological and biochemical studies have been mainly carried out using the *d18-dy* mutant. The dwarf phenotype of the *d18-dy* mutant is rescued by the application of GA₁, but not by GA₂₀ (Murakami, 1972). Seedlings of this mutant are deficient in GA₁ and accumulate its immediate precursor, GA₂₀ (Kobayashi et al., 1989). The metabolism of GA₂₀ to GA₁ is much lower in *d18-dy* plants than in wild-type plants (Kobayashi et al., 1994). Based on these observations, it has been predicted that

the *d18-dy* gene encodes a GA 3 β -hydroxylase (Kobayashi et al., 1994). We have cloned two GA 3 β -hydroxylase genes, *OsGA3ox1* and *OsGA3ox2*, from rice by screening a genomic library with a DNA fragment encoding the conserved amino acid sequence of GA 3 β -hydroxylase (Itoh et al., 2001). Both proteins produced by the cDNAs in *E. coli* show 3 β -hydroxylase activity for the steps GA₂₀ to GA₁ and GA₉ to GA₄. Molecular and linkage analyses have mapped the *OsGA3ox1* gene to the distal end of the short arm of chromosome 5, and the *OsGA3ox2* gene to the distal end of the short arm of chromosome 1, which corresponds to the *D18* locus. The association of the *OsGA3ox2* gene with the *d18* locus has been confirmed by sequence and complementation analysis of the *d18* allele. Complementation of the *d18-AD* allele with the *OxGA3ox2* gene results in transgenic plants with a normal phenotype. Northern hybridization has revealed that the genes are expressed in an organ specific manner, with the highest level of *OsGA3ox1* expression occurring in unopened flowers and the highest expression level of *OsGA3ox2* in elongating leaves (Itoh et al., 2001). Therefore, the two 3 β -hydroxylases may be involved in distinctive growth and development events in rice plants.

Recently we have cloned six rice genes catalyzing all steps of the GA biosynthetic pathway except GA7ox, and we have also identified six dwarf mutants which are functionally disrupted in each of the six genes (unpublished results). These results have implications for molecular breeding (see Molecular Breeding below).

GA Signal Transduction Pathway

The mechanisms of signal transduction triggered by GAs are still poorly understood compared to the GA biosynthetic pathway. To elucidate the mechanism of GA signal transduction, the rice GA-insensitive dwarf and slender mutants have been screened. So far, three genes the *d1*, *slr1* and *gid1* genes have been cloned using positional cloning, undoubtedly a very strategic way to isolate a gene. In this section, we present three good examples of the functional analysis of GA signal transduction using rice genomics.

***d1* mutant.** The rice dwarf mutant Daikoku, carrying the *d1* gene, was first isolated as a spontaneous mutant that was not only short, but also had broad, dark green leaves, compact panicles, and short-round grains (Ashikari et al., 1999). These phenotypes are all induced by a recessive allele (*d1*) of the *D1* gene and are thought to reflect aberrant physiological and biochemical pathways in plant growth and development. The rice *d1* mutant was classified as gibberellin-insensitive (Mitsunaga et al., 1994; Ueguchi-Tanaka et al., 2000). The *D1* gene, cloned by positional cloning, encodes the α subunit of a G-protein (Ashikari et al., 1999; Fujisawa et al., 1999). It is well known that G-proteins play an important role in signal transduction in animals and microbes (Near, 1995; Hamm, 1998). The fact that the rice mutant *d1* is GA-insensitive suggests the *D1* gene may be associated with GA signal transduction. The putative function of the α subunit of

the G protein (G α) and the predicted function of the *d1* gene are very consistent. We have investigated GA signaling in *d1* and the role of the G α protein in the GA signaling pathway (Ueguchi-Tanaka et al., 2000), and our findings are discussed here. Compared to the wild-type plants, α -amylase activity in the aleurone cells of *d1* is greatly reduced. The GA₃-treated aleurone layer of *d1* has a lower expression of *RamylA*, which encodes α -amylase, and of *OsGAMYB*, which encodes a GA-inducible transcriptional factor, and no increase in the expression of *Ca²⁺-ATPase* relative to the wild-type. However, in the presence of high GA concentrations, α -amylase induction occurs even in *d1*. The GA sensitivity of second leaf sheath elongation in *d1* is similar to that of the wild-type plants in terms of dose-responsiveness, but the response of internode elongation to GA is much lower in *d1*. Furthermore, *OsGA20ox* expression is up-regulated, and the GA concentration is elevated in the stunted internodes of *d1*. Together, these results suggest that *d1* affects a part of the GA signaling pathway; namely, the induction of α -amylase in the aleurone layer and internode elongation. In addition, a double mutant between *d1* and *slr1* (see below) revealed that *SLR* is epistatic to *D1*, supporting the notion that the G α protein is involved in GA signaling. However, the data also provide evidence for the presence of an alternative GA signaling pathway not involving the G α protein. It is proposed that GA signaling via the G α protein may be more sensitive than that of the alternative pathway, as indicated by the low GA-responsiveness of this G α independent pathway (Ueguchi-Tanaka et al., 2000).

slender (slr) Mutant

The rice *slr* mutant shows a slender phenotype with an elongated stem, leaf sheath, and blade, similar to that of rice plants that have been exogenously treated with GA₃. Consequently, the slender phenotype seems to be the constitutive GA response phenotype, as if it is saturated with GAs. In the *slr* mutant, elongation is unaffected by treatment with Uniconazole, a GA biosynthesis inhibitor. Also, GA-inducible α -amylase is produced in the aleurone cells without GA application, and the endogenous GA content is lower than that of the wild-type. These results indicate that the SLR1 protein is associated with GA signal transduction as a negative regulator.

We have isolated the *SLR1* gene and found that it encodes a putative repressor protein for the GA signaling pathway homologous to *Arabidopsis* GA-Insensitive (*Gai*) / *Repressor of gal-3* (*Rga*), wheat *Reduced height* (*Rht*), and maize *dwarf-8* (*D8*) (Ikeda et al., 2001). Transgenic rice plants that overproduce SLR1 show the dwarf phenotype, supporting the idea that SLR1 functions as a negative regulator of GA signaling (Itoh et al., 2002). Dominant alleles in the *Arabidopsis* *GAI*, wheat *Rht-B1/Rht-D1* and maize *D8* loci confer GA-insensitive mutants with the dwarf phenotype (Koornneef et al., 1985; Harberd and Freeling, 1989; Peng and Harberd, 1993; Winkler and Freeling, 1994; Peng et al., 1997). Molecular cloning of *Arabidopsis* *GAI* has demonstrated that the in-frame dele-

tion of its N-terminal domain occurs in the *gai* mutant (Peng et al., 1997). Similarly, wheat *Rht-B1/Rht-D1* and maize *D8* have mutations in their N-terminal domains as in *GAI* (Peng et al., 2000). According to the dominant phenotype caused by the *gai* mutant protein, it has been suggested that the native *GAI* product represses the action of GA and that its repression can be released by GA (Peng et al., 1997). Peng et al. (1997) also predicted that the internal deletion of the *GAI* protein in the *gai* mutant is resistant to the GA signal. If this were the case, loss-of-function mutants of the *GAI* product should show a constitutive GA response with the slender phenotype regardless of the presence or absence of GA. However, plants with loss-of-function alleles of *gai* only show a slight reduction in GA dependency (Peng et al., 1997). The absence of a clear phenotype in the *GAI*-knockout plants is thought to be due to the presence of redundant genes. Indeed, the *RGA* gene has a highly similar structure to that of *GAI*, and its loss-of-function does not show a typical constitutive GA response phenotype but partially suppresses the dwarf phenotype conferred by the GA-deficiency mutation, *gal-3* (Silverstone et al., 1997; Silverstone et al., 1998). In contrast to these cases in *Arabidopsis*, the rice genome has only one gene encoding an orthologous protein to *GAI/RGA/Rht/d8* (Ikeda et al., 2001), and consequently rice plants with a loss-of-function allele of *SLR1* show a constitutive GA-responsive phenotype (Ikeda et al., 2001). Such non-redundancy in rice should provide an advantage for studying the GA signal transduction pathway. Using transgenic plants that overproduce a fusion protein consisting of SLR1 and green fluorescent protein (SLR1-GFP), we have demonstrated that SLR1 functions in the nucleus to repress GA action and that a GA signal causes SLR1 protein levels in the nucleus to decrease, resulting in the induction of stem elongation (Itoh et al., 2002). Recently, it has also been demonstrated that *Arabidopsis* RGA and GAI proteins occur in the nucleus and that the proteins disappear upon the application of GA₃, as does the rice SLR protein (Silverstone et al., 2001; Fu et al., 2001).

Isolation of New Genes Associated with GA Signaling

We have screened several rice GA-insensitive dwarf mutants from mutagenized M₂ lines in order to elucidate the mechanisms underlying the GA signal transduction pathway. Recently, we selected many mutants showing severe dwarfism, dark green leaves and sterility, which are similar to the severe allele of the rice GA deficient mutant *d18*. We tested the GA sensitivity of these dwarf mutants against three indicators of GA responsiveness, that is, elongation of the second leaf sheath, α -amylase induction in aleurone cells, and expression of *GAC20 oxidase*. Seven of these mutants showed no second sheath elongation or α -amylase induction in response to GA treatment. High-level expression of the *GAC20ox*, which is negatively regulated by active GA in a feedback manner, was observed in the mutants with or without GA treatment. All of these

observations indicate the GA-insensitivity of these dwarf mutants.

gibberellin insensitive dwarf 1 (gid1) Mutant

As a first step towards gaining a better understanding of GA signaling in plants, we selected the *gibberellin insensitive dwarf 1 (gid1)* mutant for detailed characterization. We have identified five *gid1* alleles. In *gid1-1* to *gid1-5*, no elongation of the second leaf sheaths, and no α -amylase induction in aleurone cells, can be observed at any level of GA concentration. The *GAC20ox* is highly expressed in *gid1-1*, and the accumulated level of GA₁ is 100 times higher than that found in wild-type plants. All of these findings demonstrate that *GID1* encodes a positive regulator of GA signal transduction. To elucidate the molecular function of *GID1*, we have cloned *GID1* by positional cloning and identified that *GID1* encodes a novel gene (unpublished results). We are currently investigating the relationship between the *GID1* and *SLR* proteins in GA signaling.

Application to Breeding

In the 1960s, an impending global food crisis was a major concern because it was predicted that demand for food would exceed production. The world population was expanding rapidly due to a significant decline in mortality rates resulting from advancements in modern medicine and human health care, while the availability of land for cultivation had dramatically declined as the result of desertification caused by reckless deforestation and construction. Recognizing the problem, in the 1960s the International Rice Research Institute (IRRI) developed a semi-dwarf variety, IR8, known as “miracle rice.” Widespread adoption of IR8 led to major increases in rice production, and a large famine was averted. The technical revolution and improvements in irrigation and fertilizers also facilitated increases in rice production. All of these advances in the 1960s constitute the so-called rice “green revolution.” As the result, from 1960 to 1990 the global production of grains more than doubled.

Since 1990, growth in the grain harvest has slowed dramatically. However, the population of the world is continuing to increase by 80 million people a year, with 90% of this increase occurring in the developing countries of Asia, Africa, and Latin America. Providing for population growth now requires an expansion in world grain production by 26 million tons per year. In total, world food grain production must increase by 50% (Khush, 1999).

To feed a world population of 8 billion by 2025, we have to act now and develop both political and scientific strategies for meeting the challenge. In scientific terms, there are various strategies for increasing food grain production including: 1) genetic improvement of crop cultivars; 2) technological improvements in irrigation and facilities; and 3) sustainable management of crops and pests, and water, nutrient, and soil resources. In political terms, consistent public policy and fundamental support for research

and basic infrastructure are required. Here, we will focus on the scientific strategies to improve food production. Several biotechnological approaches for increasing crop yield potential are being investigated. These include the introduction of cloned novel genes through transformation and the use of molecular marker technology. In this section, we discuss two strategies for breeding: molecular breeding and Marker Assisted Breeding.

Molecular Breeding

Genetic engineering and biotechnology hold great potential for plant molecular biology and plant breeding as they promise to expedite the time taken to produce crop varieties with desirable characteristics. Because rice breeding by conventional crossing and selections may not catch up with increasing global food demand, other more efficient strategies are required. One such strategy is the molecular breeding approach. Here, we discuss the application of molecular studies of plant hormones to molecular breeding.

Improvement of Plant Height by Controlling GA-Associated Genes

A semi-dwarf variety IR8, produced by the IRRI, increased rice production and brought about a "green revolution" in rice in the 1960s. The *semi-dwarf 1* (*sd1*) gene contributed to this revolution. Around the same time, a wheat semi-dwarf variety produced at CIMMYT (Wheat and Maize Improvement Center) also enabled a green revolution in wheat. In this case, a semi-dwarf gene, *Reduced height* (*Rht*), was important for improving yields in wheat. The *Rht* gene encodes an *Arabidopsis* *GAI* ortholog, a negative regulator of GA signal transduction (Peng et al., 2000). Without these semi-dwarf genes it is unlikely that the green revolutions for rice and wheat would have been possible. In general, nitrogen fertilizer increases grain production, but it also increases culm elongation and the overall height of the crop plant. Wind and rain can readily dislodge tall plants enhanced by fertilizer, causing considerable damage to the crop and yield losses. The semi-dwarf rice variety IR8 responds to fertilizer inputs and produces an increased yield without culm elongation. Particularly in the monsoonal regions of Asia, where typhoons frequently occur during the yielding season, the semi-dwarf characteristic is a very important agronomic trait for crop breeding.

We have attempted to change the height of rice plants by controlling the expression of two GA-related genes, GA 2-oxidase and GA 3 β -hydroxylase gene. To do this, we employed two strategies: overexpression of exogenous GA 2-oxidase in transgenic plants and reducing GA 3 β -hydroxylase gene expression (Figure 2).

In many plant species, bioactive GAs are regulated by metabolism and catabolism of active GAs. The catabolism steps, from active GA₁ to inactive GA₈ and active GA₄ to inactive GA₃₄, are catalyzed by one of the 2-

oxoglutarate-dependent bioxygenases (2ODD), GA 2-oxidase. A rice GA 2-oxidase gene, *OsGA2ox1*, has been cloned (Sakamoto et al., 2001). Because GA 2-oxidase plays an important role in the regulation of plant growth via the reduction of endogenous levels of bioactive GAs, plant height could be controlled by modifying the GA 2-oxidase gene. *OsGA2ox1* has been introduced into wild-type plants to reduce levels of active GAs, and overexpression of the *OsGA2ox1* cDNA in transgenic rice plants inhibits stem elongation (Sakamoto et al., 2001).

In the GA biosynthetic pathway, GA 3 β -hydroxylase catalyzes the reactions from inactive GA₉ to bioactive GA₄ and inactive GA₂₀ to bioactive GA₁. The reduction of GA 3 β -hydroxylase expression in an antisense fashion causes a decrease in production of bioactive GAs. The rice 3 β -hydroxylase gene, *OsGA3ox2*, was isolated by Itoh (Itoh et al., 2001) as described above. Transgenic plants carrying an antisense construct of 3 β -hydroxylase have a semi-dwarf phenotype (Figure 3), and we have successfully produced dominant semi-dwarf rice plants. Because GAs commonly occur in plants and are used in the control of plant growth and development, these data suggest that controlling the height of any crops may be possible via the modification of GA genes.



Figure 3. Molecular breeding. 1, Producing a semi-dwarf rice plant by the controlling a GA biosynthetic gene.

Improvement of Rice Plant Architecture by Expression of the Dominant Negative *OsBRI1*

An erect leaf is a desirable agricultural trait for high yields, because plants with erect leaves can be densely planted in the field and can also perceive sunlight more efficiently under dense planting conditions.

Recently we isolated a novel rice dwarf mutant, *d61*, which shows the dwarf phenotype, erect leaves, and a strange elongation pattern of the internodes. The erect leaves and dwarfism of the mutant are caused by its insensitivity to brassinosteroids (BRs). BRs are a group of plant hormones found at low levels in pollen, seeds, and young vegetative tissues throughout the plant kingdom. BRs share structural similarity with animal steroid hormones, and have been shown to regulate gene expression and stimulate cell division and differentiation (Clouse and Sasse, 1998). *D61* encodes a receptor-like kinase protein, including a putative signal peptide, two conservatively spaced cysteine pairs, a leucine-rich repeat (LRR) domain, a predicted transmembrane domain, and a kinase domain (KD) (Yamamuro et al., 2000). Because the structure of the sub-domains of the *OsBRI1* gene is the same as the *Arabidopsis* BRs receptor, *Bri1*, and also because of its insensitivity to BRs, *OsBRI1* is considered an ortholog of *Bri1* (Li and Chory, 1997). The *OsBRI1* protein may be associated with BRs perception, and the function of *OsBRI1* may well be important in the BRs signaling pathway (Yamamuro et al., 2000). The function of the *Bri1* protein has been studied, and it has been shown that the extracellular domain LRR of *BRI1* is essential for the perception of BRs (He et al., 2000). The intercellular domain is thought to be a KD, and KD domain of *BRI1* has a capacity for auto-phosphorylation (Oh et al., 2000). Recently, Wang et al. (2001) demonstrated that *BRI1* is a receptor kinase that transduces steroid signals across the plasma membrane.

The *d61* mutant shows erect leaves of agronomic usefulness, but it also has severe dwarfism. We have tried to produce erect leaf plants without severe dwarfism by manipulating the expression of *OsBRI1*. At first, we employed an antisense strategy to inhibit the expression of endogenous *OsBRI1*. Transgenic plants with an antisense strand of *OsBRI1* cDNA under control of the rice *Actin1* promoter (Zhang et al., 1991) showed erect leaves and a severe dwarf phenotype (Yamamuro et al., 2000). We thought that the antisense RNA strand had severely reduced the function of the *OsBRI1* protein, and that another strategy was needed to mildly inhibit the *OsBRI1* protein and produce plants with erect leaves but without severe dwarfism. Thus, we employed a dominant negative technique to slightly reduce BR signaling. Because we thought that the recombinant KD might function as a dominant negative factor of endogenous *OsBRI1* and restrain the essential role of *OsBRI1*, we transformed the KD of the *OsBRI1* gene into TC65 and overexpressed it under control of the rice *Actin1* promoter. The transgenic plants showed erect leaves without dwarfism (Figure 4). To study



Figure 4. Molecular breeding. 2, Producing a rice plant with erect leaves by controlling the expression of a brassinosteroid receptor gene.

the phenotype of the dominant negative plants in order to determine their usefulness, we obtained three lines of T3 selfed-progenies of the *OsBRI1* dominant negative plant. To make an accurate quantitative comparison between the transgenic plants and wild-type TC65, the lamina joint angles of the plants were measured. Those of the transgenic plants were 0-5 degrees, and those of TC65 were 15-20 degrees. We also measured and compared several other characteristics, including plant height, culm length, panicle length, internode elongation pattern, tiller number, grain number, weight of 100 seeds, and fertility. However, no differences between the transgenic and wild-type plants were observed. We concluded that the dominant negative plants show erect leaves, but that no other characteristics are affected, including yield.

The transgenic plants with erect leaves yield as well as the wild-type TC65, but they do not require as much space as the wild variety for planting. Computer simulations suggest that, compared to TC65, 1.5 times the number of transgenic plants can be planted in equal sized fields. These figures, however, are based on plants grown under isolated greenhouse conditions. Because many environmental conditions affect rice production, field trials of the transgenic plants are required to confirm these data.

Today, the cloning of genes and production of transgenic plants are common technologies utilized in plant science. Such technologies are very powerful and efficient strategies for producing “ideal” crop plants.

Actually, some transgenic crop plants, commonly referred to as Genetically Modified Organisms (GMOs), with traits such as insect resistance and herbicide tolerance have already been commercialized after passing stringent field trials. Recently, however, concerns about the impact of GMOs on the environment and their safety to humans have received greater attention. Scientists need to be conscious of these concerns as they continue to develop and apply technologies for efficient molecular breeding using gene modification. They will need to be able to prove the safety of GMOs for humans and the environment and consult widely with the general public on matters of concern. Consumers can then make an informed decision about whether to accept or avoid the use of genetically modified products. We believe that in the near future the use of GMOs in everyday life will be commonplace and that this technology can help to resolve the up-coming food crisis.

Marker Assisted Breeding

In the 1800s, Gregor J. Mendel discovered the genetic basis of plant breeding through his experiments using garden peas, and he established Mendel's law. However, for centuries farmers have improved crops and vegetables by crossing and selection based on desired traits such as environmental adaptability, quality, and yield. They planted seeds from individuals bearing the preferred traits and continued further selection to fix those characteristics. A number of plant varieties have been established in this way, and the method used by farmers was based on Mendelian principles. This conventional breeding strategy remains unchanged today, but the use of molecular markers has made it more efficient.

We need to develop rice plants with a range of improved characteristics such as increased yield, resistance to disease, and tolerance to stresses such as drought and salinization. To achieve these plant breeding goals, the identification and isolation of the genetic information held in rice genomic DNA is essential. Here, we discuss the efficient Marker Assisted Breeding strategies, including Quantitative Traits Loci (QTL) approaches.

The use of molecular markers in plant breeding has several advantages over the traditional phenotypic markers previously available to plant breeders. Marker Assisted Selection (MAS) is a very convenient and efficient breeding strategy (Mohan et al., 1997) in which conventional breeding selection is carried out on molecular markers linked to target traits rather than on the traits themselves. Selection is followed by the marker genotype tightly linked to target traits. RFLP markers can be used for MAS, but they cannot efficiently screen genotypes of many plants in a short time because they require intricate and time-consuming experiments. Many kinds of PCR-based markers have replaced RFLP markers. These include RAPDs (Random Amplified Polymorphic DNAs), STS (Sequence Tag Site), SSR (Simple Sequence Repeats, including Minisatellite repeats and Microsatellite repeats), CAPS (Cleaved Amplified Polymorphic Sequences), AFLP

(Amplified Fragment Length Polymorphism), and SNP (Single Nucleotide Polymorphisms). Handling of these PCR-based markers is very simple and efficient, making it possible to hasten the transfer of desirable genes among varieties and to introgress novel genes from related wild species. SSR markers in particular are very convenient for genotyping, mapping, cloning genes, QTL analysis and MAS. Increased recognition of MAS and diffusion of molecular markers to breeders are required for more efficient breeding.

QTL Approach

In contrast to monogenic characteristics, such as disease and insect resistance, many important agronomic traits including yield, heading date, culm length, grain quality and stress tolerance show continuous phenotypic variation. Several genes control these traits. These polygenic characteristics, including QTLs, were previously very difficult to analyze using traditional plant breeding methods, but recent progress in the identification of molecular markers has made it possible to detect the QTL easily. Primary-mapping populations, such as F_2 , Recombinant Inbred Lines (RILs), Backcross Inbred Lines (BILs) and Doubled Haploid Lines (DHLs), are often used in QTL analysis to detect the chromosomal regions controlling target traits. RILs can be developed from F_2 plants by the Single-Seed-Descent (SSD) method to form a population that is useful for the detection of QTLs (Tsunematsu et al., 1996). BILs can be developed from backcross populations, such as BC_1 or BC_2 using SSD (Lin et al., 1998). These populations are very useful for QTL analysis, but SSD can take some time to produce populations. DHLs can be developed via the anther culture of the F_1 hybrid, and this approach is relatively fast because the genotype is fixed by the doubled haploid. These three lines are very useful plant materials for detecting important agronomic traits, but they do have limitations. In QTL analysis, even using a large number of plants and genetic markers and well-developed statistical methods, it is difficult to determine the precise location of individual QTL as a single Mendelian factor in the primary analysis. Consequently, other plant materials are required to allow us to proceed with further analysis, such as fine mapping and characterization of target QTLs.

To achieve high resolution mapping of a QTL, construction of Nearly Isogenic Lines (NILs) harboring the QTL is required. The NILs are constructed by backcrossing with a recurrent parent and selection by MAS. The QTL in NILs can be handled as a single Mendelian factor, and this material can be used for mapping and positional cloning. In rice, a relatively large segment of a particular chromosome from the donor parent is substituted in the recurrent parental background to form Chromosome Segment Substitution Lines (CSSLs) whereas a very small chromosomal segment, containing the target QTLs or genes of a donor line, is substituted (Yano, 2001). Secondary mapping populations, such as CSSLs or NILs, are required to facilitate a more comprehensive analysis of target QTLs.

Several rice primary and secondary mapping populations have been developed (Yano, 2001), and use of these will allow us to perform the molecular cloning of target QTLs. In rice, QTLs for heading days have been well studied. Using a primary population derived from a cross between a Japonica variety, Nipponbare, and an Indica variety, Kasalath, many QTLs for heading days have been detected (Yano et al., 1997), and some have been mapped using a secondary population. So far, two QTLs, *Hd1* and *Hd6*, have been isolated by positional cloning (Yano et al. 2000; Takahashi et al., 2001).

Although artificially induced variations, such as mutants, have mainly been used for genetic and physiological studies in plant species, DNA markers have made it possible to study the naturally-occurring allelic variations underlying complex traits and will become increasingly important for breeding. The use of wild relatives as donor parents would provide a more powerful way to exploit a wide range of allelic variation because these plants are adapted to specific environmental conditions. Because genetic variation gives rise to these different adaptations we might find a wider range of allelic variation in wild relatives than in cultivated species. Inter-specific crosses have already been used for QTL analyses in tomato, and rice (Tanksley and McCouch, 1997). There are many wild relatives in the rice *O. sativa* complex, AA genome, and seven species have been differentiated which have unique geographic distributions with differing environmental conditions. Using these wild species, it should be possible to identify and pull out the genetic information held in the rice genomic DNA for QTL analysis. For more detailed QTL analysis, CSSLs covering whole rice chromosomes have been developed with wild relatives from *O. glaberrima* (Doi et al., 1998) and *O. glumaepatula* (Ikeda et al., 1999). If QTL analysis can be used to locate traits related to adaptability and yield, and reproductive barriers such as hybrid sterility and growth abnormalities, it will give us greater insight into how such crop species have evolved (Yano, 2001). The large degree of variation within the rice species will not only provide us with very important agronomic traits but also with very interesting information on plant biology.

Future Outlook

In 2000, sequencing of the *Arabidopsis* genome sequencing was finished and now functional genomics is being enthusiastically pursued. Many novel genes have been detected and the mechanisms underlying plant growth and development are being revealed by *Arabidopsis* genomics. Now, rice genomics is also progressing, and the rice genomic DNA is being sequenced by IRGSP. Rice genomics has many applications for plant science and the comparison of sequence data from rice, and *Arabidopsis* may improve our understanding of plant evolution and the differences between monocots and dicots. The application of rice genomics will also provide us with very useful and powerful strategies to improve the efficiency of rice breeding. In addition, the rice

genome is very similar to major cereal crops such as maize, barley, and wheat. Therefore, rice genomics has very significant implications not only for rice but also for other cereal breeding programs. We hope that rice genomics will be widely accepted by scientists and breeders and that it makes significant contributions to human welfare.

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