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Preface

Increase in agricultural production and productivity is a recent primary issue to secure food for growing population worldwide while harmonizing it with sustainability in agro- and natural-ecosystems. However, fruitful outputs of plant breeding have often led to genetic erosion or loss of crop genetic diversity. Integration of plant genomics and biotechnology with plant breeding is expected to generate innovative enhancement of agricultural production and productivity in sustainable manner. Bio-resources produced by plant genomic research and biotechnology tools give a great impact on breeding science and technology. On the other hand, genetically modified organisms (GMO) have faced to a campaign against GMO and may pose ethical problems and require substantial debate among policy makers, researchers and the public at large. Particularly in cultivation and use of GMO, the debate has become quite polarized and there is therefore an increasing need for high quality research data related to these issues.

With this background, the main theme of the 10th International Congress of SABRAO in Tsukuba, 2005 is, "How to utilize crop diversity for productivity and sustainability: Breeding science and technology for the new era". The congress comprised plenary sessions, seven symposium sessions and poster sessions. Symposium sessions were organized into seven primary themes as follows:

- Rice Genome: Challenges and Opportunities
- Perspectives of Utilization and Conservation of Plant Genetic Resources
- Germplasm Improvement with DNA Markers in Rice.
- Genetic Improvement of Abiotic Stress Tolerance in Crop Plants
- Genetic Diversity of Soybean and its Use for High Benefit Production
- Sharing the Experience on Transgenic Research and Crop Development in Asia
- Information Sciences for Plant Breeding

National Institute of Agrobiological Sciences (NIAS) sponsored the 13th NIAS International Workshop on Genetic Resources as parts of symposium sessions during the 10th International Congress of SABRAO (The Society for the Advancement of Breeding Researches in Asia and Oceania) which was held 22-23 August, 2005 in Tsukuba. In this proceedings, two research highlights on "Rice Genome: Challenges and Opportunities" and "Perspectives of Utilization and Conservation of Plant Genetic Resources" are discussed. I expect all these research outputs recorded in the proceedings will contribute to further advance in breeding science and strategies today and tomorrow.

March 2006

Kazutoshi Okuno
Director of Genebank, NIAS
Tsukuba, Japan

Topic 1

Rice Genome: Challenges and Opportunities

COMPLETE RICE GENOME SEQUENCE AND ITS APPLICATION TO GENOME BIOLOGY

Takashi Matsumoto, Jianzhong Wu, Yuichi Katayose, Hiroshi Mizuno, Baltazar Antonio, Takuji Sasaki
National Institute of Agrobiological Sciences
2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602 Japan
mat@nias.affrc.go.jp
Tel: +81-298-7441
Fax: +81-298-7468

Hiroyuki Kanamori, Masaki Fujisawa, Nobukazu Namiki
STAFF Institute
446-1 Kamiyokoba, Tsukuba, Ibaraki 305-0854 Japan

Abstract

The International Rice Genome Sequencing Project (IRGSP), which was officially initiated in 1998, completed the sequencing of the *japonica* rice cultivar Nipponbare in December 2004. The total nucleotide sequence of ca. 370 Mbp corresponding to 95% of the entire genome has been elucidated and submitted in the public databases. Initial analysis predicted 37,544 protein-coding genes, about one third of which are clustered in tandem. Similarity analysis among all the protein sequences revealed clear segmental duplications within the genome. The nearly completed genome sequence also shed light on regions previously classified as "dark side" due to the absence of protein coding genes. Analysis of the centromere and telomere regions revealed the presence of many repetitive sequences which maybe crucial in chromosome maintenance. Some transcriptionaly active genes were also identified in these heterochromatic regions. The high-quality Nipponbare genome sequence could be used as a common framework for comparative analysis of closely related genomes. The end-sequences from BAC clones of an *indica* variety, Kasalath have been succesfully mapped using the Nipponbare sequence as reference. This could lead to the detailed sequence comparison between *japonica* and *indica* subspecies of rice cutivars, both of which are important to modern rice breeding, and could provide many SNPs and Indels which could become fine genetic markers. Combining the fine scale genetic maps, genome sequence and gene annotation could accelerate gene discovery and identification of regions responsible for various biological phenomena. This could pave the way for a genome-based breeding strategy to generate new rice varieties with highly desirable agronomic traits. The Nipponbare sequence is therefore a gold standard vital to the understanding and utilization of the genomic variation in the genus *Oryza* and other Graminae species sharing syntenic relationships with rice.

Rice is one of the most important crops in the world. It is the staple for 34 countries around the world and it provides 27% of dietary energy supply. Global rice annual production has steadily increasing and reaches to 545 Million tons in 2004. But the predicted demand for rice production at 2025 is 700 Mt. While the late "Green Revolution" from 1966 with IR8 greatly increased food supply, land and water resources that support our food supply are diminishing. Breeders from all over the world are trying to produce novel varieties which is characterized as even higher yield than modern varieties, resistant to severe diseases which are caused by various pathogens, global environmental changes cause abiotic stress which might it impossible for the crop plants to survive. One of the goals of the current breeding is to produce crops which is

tolerant to these stresses such as drought,flood, global warning and salinity.Current dietetics requires for food the balanced nutrients, not just calories in order to live healthy life. Supplementation of the nutrients whose lack will cause severe disease is new target for modern breeding.These phenotypic changes as targets for breeding are governed by the genes and genetic systems, and genome sequence, which is the blueprint of the whole activities, will greatly accelerate the gene-targeted molecular breeding by rational design. The International Rice Genome Sequencing Project, or IRGSP, completed sequencing of japonica rice variety, Nipponbare last year (IRGSP, 2005). Established in 1998, the IRGSP has set up materials for analysis, constructed a detailed physical maps by PAC,BAC, and fosmid clones, and sequenced the map-based clones by the hierarchal shotgun strategy. In the course, in 2002, a whole genome sequence with a 10x high-quality shotgun was revealed, since many rice researchers need the relatively acuurate rice sequences(IRGSP, 2002).And after two more years, it could publish the completed rice genome sequence which would be invaluable for crop biology and biotechnology. The IRGSP assigned the twelve chromosomes of rice to each sequencing participants, and 10 countries, devoted to sequence several hundred PAC/BAC clones until it meets Bermuda standard whose accuracy is 99.99%. 3,453 clones generated 370 million nucleotides in total and the sequence has already been open to the public. They are submitted in the public databases such as DDBJ, EMBL, and GenBank in the appropriate format. Moreover reserachers can obtain the sequences from the IRGSP website(http://rgp.dna.affrc.go.jp/IRGSP/Build3/build3.html) by ftp. These data are called pseudomolecules as they mimic each chromosome structure except the unsequenced regions.

Adding the sequenced 370Mb length, 36 of the contig gaps, Centromere gaps, rDNA regions, and telomere gaps, the total size is calculated as 388.8Mb. Sizes of rice genome was calclated as 415-430Mb by flow cytometry, 400Mb by the physical map estimation of genomic clones. The current size is the most precise calculation concerning japonica rice genome, and it is also confirmed we have 95% of them as the sequences.

Next we have predicted gene domains within the sequenced region. First the regions of various repeats such as transposons, retrotransposons, centromere repeats, and other repeats were detected and removed. The remaining regions were subjected into the gene prediction by using FGENESH software trained by monocot dataset. In total, 37,544 genes are predicted. This number is almost double the number of genes in Arabidopsis as 25,000 in the original annotation (AGI, 2000), The gene density or more accurately, gene interval is one gene per 10kb on average, about twice larger than Arabidopsis. In fig.1 the statistics on the predicted genes are briefly summarized. 61% has some rice transcript support, 52% has a protein match, 71% has a Arabidopsis counterpart , and 62% has at least one motif or domain which is annotated. 17% of the total predicted genes have neither transcript support, nor protein or domain database hit. Functional assignment of these gene models should be done by experiments. One of the striking feature of the rice genome is the abundance of the tandemly arrayed genes. About 30 % of rice genes exists as clusters which consitutes tandem gene arrays. In terms of protein function, many of these large arrays contain protein kinase genes. Another analysis of protein domain detection shows that the top 5 domains are all protein kinase or kinase-like domains showing rice genome is protein kinase-rich. Another finding about rice genome evolution is the segmental duplication. As we have and also Andy Paterson indicated in 2004 (Paterson et al., 2004), all against all similarity searches of the rice predicted proteins clearly show the conserved giant gene clusters. Plotting only the most conserved region pairs indicated the the large scale segmental duplication or polyploidization which is estimated at about 70MYA.

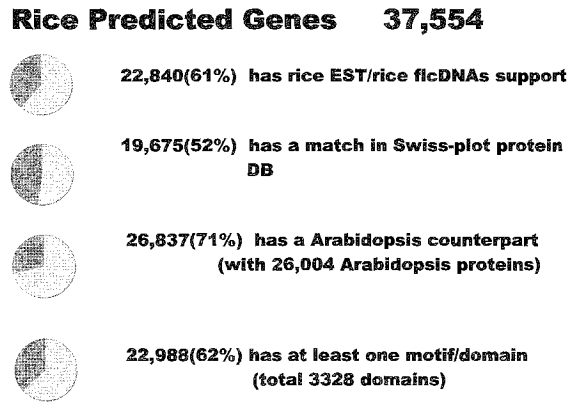

figure 1 Statistics on rice predicted genes

It might be interesting to investigate whether these genes are also diverged in other Grass genomes, because these duplications were occurred after divergence of the Poaceae. It might also be intriguing that the duplicated genes have same function or have subjected to subfractionation within the evolution and selection process, because all these genes are predicted as intact ORFs. Sequences and gene structures from maize and sorghum, and barley, which will be revealed in the near future will tell the exact correlation among these taxons.

Analysis of the complete rice genome by several investigators, inside and outside IRGSP found that rice genome has some remarkable sequences besides protein coding regions. A large chroloplast and mitochondria insertion were found within nuclear genome. More than 400 cp inserts and 1000 mt inserts were found in all chromosomes, which covers up to 0.4% of rice genome. 1/3 of the rice genome was occupied by various kinds of transposable elements. So far we do not exactly know the correlation of transposon and genome function. Moreover, more than 450 non-coding RNAs excluding rDNA, tRNAs are found. These ncRNA are charcterizend as micro RNA, small nucleolar RNA, and spliceosomal RNAs. Moreover many candidates for DNA markers for breeding, such as SSR and SNPs are found between the two major subspecies of Oryza sativa, japonica and indica. These non-protein-coding region are current research topics, because these non-canonical type of gene regulation might important for the plant systems. Here the completed genome of Nipponbare could make it possible to assign sequence of other genome to the japonica sequences accurately.

Figure 2 shows the sequenced region within rice genome by IRGSP. The green bar indicates sequence contigs. Although most of the region is covered by green bars, there are still gaps and dotted regions. Other than the contig gaps shown as the disconnected bars, sequences from special part of the genome has not been fully obtained and recognized as "dark side". As the heterochromatin region which might be transcriptionally silent, are centromeres as indicated by red triangles, NORs as dotted green lines,and telomeres at the chromosomal ends. Most of these sequences were left unseqenced, but since they are important for the chromosomal maintenance or chromosomal recognition and other important function, we have been continuing sequencing of these regions to complete the rice genome overall sequencing.

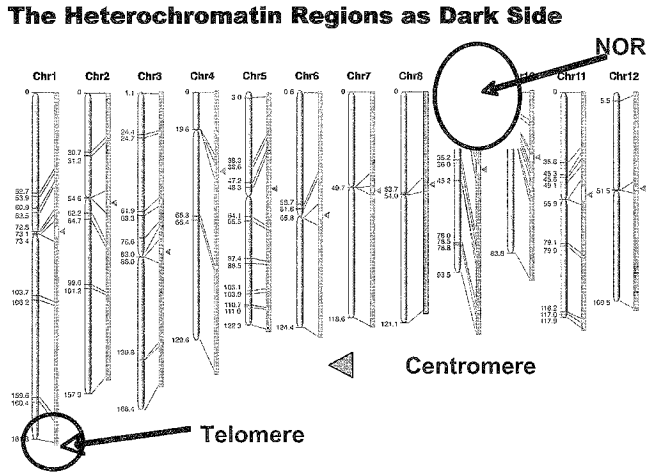

Figure 2 The sequenced and gapped region of rice genome.

We have already sequenced and published the overall structure of the 2Mb region of centromere and pericentromere in chromosome 8 in 2004. Furthermore we have been analyzing some regions within NOR (nucleolar organizing region) where a major rDNA cluster exsits, and telomere regions to obtain full-scale image of rice genome.

Since rice is the most studied crop, the sustainable food supply is the one of the most important objectives for crop breeding. How rice sequence would accelerate this goal? There might be two approaches for that. First functional analysis of the total rice gene set will lead to understand how gene works solely or as part of a network. These analysis will go to the knowledge how to regulate specific gene function without disturbing cell's other functions. These technique will enable the targeted gene improvement by human design by either crossing or gene manipulation. Other approarch to utilize the genome sequence is comparative genomics studies. There are two information flow in this area. One is to utilize rice sequence and other information to elucidate the orthologus gene identification of Grasses by synteny. The other application is to look into Oryza itself. Utilization of vast Oryza genetic resources worldwide would cause the second green revolution to rice breeding to establish a novel varieties.

The Genus Oryza consists of two cultivars and 21 wild species. But human utilized only two cultivars, O.sativa and O. glaberrima for agricluture. For the time being, studies on rice molecular biology have been concentrated on these two subspecies of O.sativa, japonica and indica. Within the AA species, in other word, Oryza complex,O. sativa is believed to be evolved from O.rufipogon, while O.glaberrima from O.barthii. Moreover breeders and researchers of genetic resources understand there might be vast gene resources to improve modern variety in wild rice . For example, in 1998, Tanksley's group found yield QTL in the O.rufipogon species, and in the recent history of rice breeding, some novel disease resistance genes from O.rufipogon, O.longstaminata, O.officinalis, O.ridely O.minuta, have been introgressed into rice cultivar by unknown mechanism. Therefore our objectives for rice comparative genomics will be symbolized as these two questions. How rice cultivar generated and diverged from the same ancester? What is the wild rice? How it is useful to agriculture? In 2004, we have constructed BAC library of Kasalath, an indica cultivar, and a Kasalath BAC *in silico* physical maps which covers 70% of Nipponbare genome are constructed (Katagiri et al., 2004). We are in the process of the comparative work of japonica and indica sequences in the chromosomal wide scale. On the other hand, the RGP constructed a rice transcript map for the basis of BAC physical maps in 2002.

There 6,591 ESTs from the unique regions of 3'UTR of rice cDNAs were mapped by PCR screening(Wu et al., 2002). We applied these Nipponbare EST markers to the wild rice accessions. By the courtesy of Dr. Vaughaun and Dr. kadowaki at NIAS, DNAs from 45 accession s from 21 species from AA to HHJJ genomes were subjected to PCR amplification by using the 943 ESTs throughout the genome as the source of PCR primer. There is a clear difference for amplification degree for AA species, BB to EE species, and others. This means there is a discrete difference of sequence similarities of 3'UTR among wild rice complexes.

References

AGI Nature 408: 796-815 (2000)
IRGSP http://rgp.dna.affrc.go.jp/rgp/Dec18_NEWS.html (2002)
IRGSP Nature 436: 793-800 (2005)
Katagiri et al. Breed. Sci. 54: 273-279 (2004)
Paterson et al. PNAS 101:9903-9908 (2004)
Wu et al. The Plant Cell 14: 525-535 (2002)

GENOME COMPARATIVE ANALYSIS OF DOMESTICATED RICE *ORYZA SATIVA INDICA* AND *JAPONICA* SUBSPECIES

Bin Han
National Center for Gene Research, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 500 Caobao Road, Shanghai, 200233 China, bhan@ncgr.ac.cn

Abstract

As part of international efforts to completely sequence the entire rice genome, the National Center for Gene Research (NCGR) of the Chinese Academy of Sciences has finished the sequence of rice *Oryza sativa japonica* chromosome 4 through a map-based strategy. The finished sequence spans 35.5Mb and represents 98.2% of the chromosome. To characterize the blueprint of intra-specific DNA-sequence variations between *Oryza sativa indica* and *japonica* subspecies, NCGR has conducted an *indica-japonica* chromosome 4 comparison at the nucleotide sequence level. A 22.1-Mb of the *indica* Guangluai 4 (GLA4) chromosome 4 has been sequenced, the largest map-based high-quality *indica* sequence, and compared this sequence with 23.2 Mb of the *japonica* orthologous chromosome 4. This comparison provided a fine scale *indica-japonica* comparison. A sequence comparison of the collinear regions from *indica* and *japonic*a chromosome 4 has revealed their extensive micro-colinearity in gene order and content, but also the intraspecific sequence polymorphisms in both coding and non-coding regions. Comparative genome analysis between cultivated rice subspecies shows that there is an overall syntenic relationship between the chromosomes and divergence at the level of single-nucleotide polymorphisms and insertions and deletions.

1. Chromosome-wide comparison of *indica* and *japonica* subspecies

Rice is the principal food for over half of the population of the world. With a compact genome spanning ca. 430 megabase (Mb) pairs, an extensive genetic map (Harushima et al. 1998), and established synteny with other cereal crops (Gale & Doves 1998), the cultivated rice species Oryza sativa represents a model for cereals and as well as other monocot plants (Shimamoto & Kyozuka 2002). The completion of the genome sequence of rice opens a new and exciting chapter in our quest to functionally characterize all of the approximately 37,500 annotated genes in rice (Feng et al. 2002; Sasaki et al. 2002; Rice Chromosome 10 Sequencing Consortium 2003; International Rice Genome Sequencing Project, 2005). A systematic approach to characterizing these genes will allow us to dissect and understand the regulatory networks and evolutionary selection controlling such complex traits as yield, grain quality, biotic and abiotic stresses, reproductive barriers, epigenetics and flowering time. The next essential steps towards deciphering the sequenced genome are to develop complete and accurate maps of actively transcribed regions during rice development, and to carry out genome comparative analysis of taxonomically closely related rice subspecies. These will facilitate the identification of all the genes and proteins encoded in the DNA sequence. Such information will allow further analysis of their function, regulation, and how they cooperate in complex biological processes in a systems manner.

Domesticated Asian rice Oryza sativa, which consists of two major subspecies *indica* and *japonica* partially isolated in terms of sexual reproduction, has become the most important rice crop in the world (Linares et al. 2002; Goff et al. 2002). *Indica-japonica* genome comparison has been chosen as a model system for understanding of origin, speciation, domestication and

genome evolution of rice. *Indica* and *japonica* cultivars can be classified based on their agronomic traits (Oka & Morishima 1997) and the use of molecular markers (Glaszmann 1987; Cheng et al. 2003). Rapid progresses in both *indica* and *japonica* genome sequencing have been made (IRGSP, 2005; Goff, et al., 2002; Yu et al. 2002; Yu et al. 2005). The International Rice Genome Sequencing Project (IRGSP) has adopted the clone-by-clone approach for obtaining a finished rice genome sequence, because it is modular, allows efficient gap filling, avoids problems arising from distant repetitive sequences and results in the early completion of larger contiguous segments of a genome (Sasaki & Ben 2000). As part of international efforts to completely sequence the entire rice genome, the National Center for Gene Research (NCGR) of the Chinese Academy of Sciences has finished the sequence of rice *japonica* chromosome 4 through a map-based strategy (Zhao et al. 2002; Feng et al. 2002). This was one of the first two rice chromosomes to have been completely sequenced. The finished sequence spans 35.5Mb and represents 98.2% of the chromosome. To characterize the blueprint of intra-specific DNA-sequence variations between *indica* and *japonica* subspecies, NCGR has conducted an *indica-japonica* chromosome 4 comparison at the nucleotide sequence level. A 22.1-Mb of the *indica* Guangluai 4 (GLA4) chromosome 4 has been sequenced, the largest map-based high-quality *indica* sequence, and compared this sequence with 23.2 Mb of the *japonica* orthologous chromosome 4 (Han et al. unpublished data). This comparison provided a fine scale *indica-japonica* comparison. A sequence comparison of the collinear regions from *indica* and *japonica* chromosome 4 has revealed their extensive micro-colinearity in gene order and content, but also the intraspecific sequence polymorphisms in both coding and non-coding regions (Feng et al. 2002; Han & Xue 2003; Li et al. 2004). Comparative genome analysis between cultivated rice subspecies shows that there is an overall syntenic relationship between the chromosomes and divergence at the level of single-nucleotide polymorphisms and insertions and deletions. The rice genome has been well mapped both genetically and physically and has a syntenic relationship with other cereals. Identification of the sequence polymorphisms of the two subspecies has produced the most density of polymorphic sequence maps of the *indica* and *japonica* chromosome 4. Importantly, this comparison revealed the dynamic changes that may reflect major evolutionary events occurring throughout the domestication and natural selection of rice.

A complete sequence of a chromosome centromere is necessary for fully understanding centromere function. The rice chromosome 4 centromere sequence has been completely determined and its structures have been analyzed. Complete sequencing of the 124-kb rice chromosome 4 centromere revealed that it consisted of 18 tracts of 379 tandemly arrayed repeats known as CentO and a total of 19 centromeric retroelements (CRs) but no unique sequences were detected. The preferential insert of the CRs among CentO repeats indicated that the centromere-specific retroelements may contribute to centromere expansion during evolution. The presence of three intact retrotransposons in the centromere suggests that they may be responsible for functional centromere initiation through a transcription-mediated mechanism (Zhang et al. 2004).

2. A rice chromosome 4 tilling array chip: chromosomal level regulation

As a first attempt to decipher the rice genome, computational annotation has been successful, although improvements are needed (Yuan et al. 2003). Recent efforts to verify experimentally the gene model structure by sequencing cDNA and expressed sequence tags (ESTs) have provided valuable information toward our understanding of gene structure and genome coding capacity (Wu et al. 2002; Rice Full-Length cDNA Consortium 2003; Rensink & Buell 2004). An essential and necessary step in this effort is the determination of the coding information and expression

patterns of each sequenced chromosome. However, until now only about half of the predicted genome coding capacity had any cDNA or EST expression support. Clearly, experimental approaches complementary to computation-based genome annotation are essential for an understanding of genome structures. Because of the presence of large amounts of unfinished sequence data, unusual compositional gradients in genes, and the large size of the rice genome (Rensink & Buell 2004), there is even greater need for experimental approaches in rice genome annotation.

A chromosome-scale transcriptional analysis will expand our knowledge of possible chromosomal level transcriptional regulation. One prominent feature of eukaryotic chromosomes is their organization into heterochromatic and euchromatic regions. Heterochromatin was first distinguished from euchromatin cytologically as more intensely staining nuclear material throughout the cell cycle in Bryophyta. In fact, heterochromatin has emerged as a key regulator in the epigenetic control of gene expression, chromosome behavior, and evolution. Using tiling path microarray analysis as a tool, it is now possible to perform high-throughput profiling of the transcriptional activities along an entire sequenced chromosome to examine potential connections between transcription and cytologically-defined chromatin organization. Xing-Wang Deng's and Bin Han's laboratories have developed a tiling path DNA microarray consisting of overlapping PCR amplified genomic fragments covering over 33 Mb (95.5%) of *japonica* rice chromosome 4 (Figure 1). Using this array, they analyzed the transcriptional activity of chromosome 4 in six representative Rice Chromosome 4 Transcriptome organs or tissues. Chromosomal-scale transcription patterns were analyzed in comparison with cytologically observed chromatin organization and the distribution of transposon-related and various other gene model groups (Jiao et al. 2005). Six representative rice organ types were examined using this microarray to catalogue the transcribed regions of rice chromosome 4 and to reveal organ- and developmental stage-specific transcription patterns. This analysis provided expression support for 82% of the gene models in the chromosome. Transcriptional activities in 1,643 non-annotated regions were also detected. Comparison to cytologically defined chromatin features indicated that in juvenile stage rice euchromatic region is more actively transcribed than transposon-rich heterochromatic portion of the chromosome. Interestingly, elevated transcription of transposon-related gene models in certain heterochromatic regions was observed in mature stage rice organs and in suspension cultured cells. These results suggest a close correlation between transcriptional activity and chromosome organization and developmental regulation of transcription activity at the chromosomal level (Jiao et al. 2005; Li et al. 2005).

Recent completion of both *indica* and *japonica* genome sequence analysis now allowed an unbiased interrogation of transcriptional activity of the whole genome and experimental verification of computational genome annotation. A comparative tiling analysis of the entire chromosome 4 of both *indica* and *japonica* genomes has also been analyzed. This analysis detected expression of about 80% of the unsupported gene models without previous experimental evidence in both subspecies. This tiling analysis also identified 141 new models for the *japonica* chromosome, representing a 3.6% increase in the annotated protein-coding capacity. This analysis also revealed that a chromosomal level transcriptional regulation largely coincides with the heterochromatin and euchromatin domains.

References

Cheng, C., Motohashi, R., Tsuchimoto, S., Fukuta, Y., Ohtsubo, H., & Ohtsubo, E. 2003 Polyphyletic origin of cultivated rice: based on the interspersion pattern of SINEs. *Mol. Biol.Evol.* **20,** 67-75.

Feng, Q. *et al.* 2002 Sequence and analysis of rice chromosome 4. *Nature* **420,** 316-320.

Gale, M.D. & Devos, K.M. 1998 Comparative genetics in the grasses. *Proc. Natl.Acad. Sci. USA* **95,** 1971-1974.

Glaszmann, J.C. 1987 Isozymes and classification of Asian rice varieties. *Theor. Appl. Genet.* **74,** 21-30.

Goff, S.A. *et al.* 2002 A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). *Science* **296,** 92–100.

Han, B. & Xue, Y. 2003 Genome-wide intraspecific DNA-sequence variations in rice. *Current Opinion in Plant Biology* **6,** 134-138.

Harushima, Y. *et al.* 1998 A high-density rice genetic linkage map with 2275 markers using a single F2 population. *Genetics* **148,** 479-494.

International Rice Genome Sequencing Project. 2005 The map-based sequence of the rice genome. *Nature.* **436:** 793-800.

Jiao, Y. *et al.* 2005 A Tiling Microarray Expression Analysis of Rice Chromosome 4 Suggests a Chromosomal Level Regulation of Transcription. *Plant Cell,* 17(6):1641-57.

Li, C., Zhang, Y., Ying, K., Liang, X., & Han, B. 2004. Sequence variations of simple sequence repeats on chromosome-4 in two subspecies of the Asian cultivated rice. *Theor. Appl. Genet.* **108,** 392-400.

Li , L., Wang, X., Xia, M., Stolc, V., Su, N., Peng, Z., Li, S., Wang, J., Wang, X. & Deng, X. W. 2005 Tiling microarray analysis of rice chromosome 10 to identify the transcriptome and relate its expression to chromosomal architecture. *Genome Biol.* In press.

Linares, O. F. 2002. African rice (*Oryza glaberrima*): History and future potential. *Proc. Natl.Acad. Sci.* **99,** 16360-16365.

Oka, H., & Morishima, H. 1997. Wild and cultivated rice. In Science of the rice plant (vol 3: enetics, eds Matsuo, T., Futsuhara, Y., Kikushi, F., & Yamaguchi, H.).pp. 88-111.

Rensink, W. A., & Buell, C. R. 2004. Arabidopsis to rice. Applying knowledge from a weed to enhance our understanding of a crop species. *Plant Physiol.* **135,** 622-629.

Rice Chromosome 10 Sequencing Consortium. 2003 In-depth view of structure, activity, and evolution of rice chromosome 10. *Science* **300,** 1566-1669.

Rice Full-Length cDNA Consortium. 2003 Collection, mapping, and annotation of over 28,000 cDNA clones from *japonica* rice. *Science* **301,** 376-379.

Sasaki, T. *et al.* 2002 The genome sequence and structure of rice chromosome 1. *Nature* **420,** 312–316.

Sasaki, T. & Burr, B. 2000 International rice genome sequencing project: the effort to completely sequence the rice genome. *Curr. Opin. Plant Biol.* **3,** 138–141.

Shimamoto, K. and Kyozuka., J. (2002). Rice as a model for comparative genomics of plants. nnu. Rev. Plant Biol. 53, 399-419.

Wu, J. *et al.* 2002 A comprehensive rice transcript map containing 6591 expressed sequence tag sites. *Plant Cell* **14,** 525-535.

Yu, J. *et al.* 2002 A draft sequence of the rice genome (*Oryza sativa* ssp. *indica*). *Science* **296,** 79–91.

Yu, J. *et al.* 2005 The genomes of *Oryza sativa:* A history of duplications. *PLoS Biol* **3(2)**, e38.

Yuan, Q., Ouyang, S., Liu, J., Suh, B., Cheung, F., Sultana, R., Lee, D., Quackenbush, J., & Buell, C.R. 2003 The TIGR rice genome annotation resource: annotating the rice genome and creating resources for plant biologists. *Nucleic Acids Res.* **31,** 229-233.

Zhang, Y. *et al.* 2004 Structural features of the rice chromosome 4 centromere. *Nucleic Acids Res.* **32 (6),** 2023-2030.

Zhao, Q. *et al.* 2002 A Fine Physical Map of the Rice Chromosome 4. *Genome Research* **12,** 817-823.

THE RICE GENOME RESOURCE CENTER AS THE CORE FOR CEREAL GENOMICS

Baltazar A. Antonio, Akio Miyao, Yoshiaki Nagamura, Takuji Sasaki
National Institute of Agrobiological Sciences
2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan
*Corresponding author: antonio@nias.affrc.go.jp (Tel: +81 29 838 7456)

Abstract

The ultimate goal for rice genomics is to elucidate the biology of the rice at the cellular, organismal and evolutionary level. With the complete sequence of the rice genome in the public domain, researchers can now have access to the sequence of about 40,000 genes that defines a rice plant. This must be supplemented with an expanded genetic toolkit that includes biological materials which could be used for experimental verification of gene function, viable strategies to execute large-scale characterization of the genome and a robust informatics infrastracture to facilitate integration of all knowledge about the rice plant. Efforts in providing the research community with genome resources including full-length cDNA clones, mutant lines and biological materials for genetic analysis will be described with emphasis on how these resources will shape research in cereal genomics in the future. The next trend in rice genomics will focus in implementing a whole-systems approach to the identification of gene function which will require knowledge of overall gene expression profiles at the cellular, subcellular and organ level under a variety of defined conditions. Achieving this goal will also require databases and bioinformatics tools to integrate all information that can be queried from any perspective and provide a platform for comparative genomics that will lead to better understanding of other cereal genomes.

Introduction

Rice is considered a model cereal crop because of its small genome size and syntenic relationships with other major crop species. The completion of the rice genome sequence will therefore herald a new era for cereal genomics with rice providing a reference sequence for elucidating the biology of maize, wheat, barley and other cereal genomes (International Rice Genome Sequencing Project 2005). However, in order to completely understand the biology of this important cereal crop and effectively use it as an experimental model system for understanding other major cereal crops, the following challenges in rice genomics should be addressed immediately: (1) elucidating the actual function of about 37,000 genes predicted in the genome; (2) establishing the protein profile in rice plant; (3) understanding the metabolic pathways and their interactions; (4) clarifying the cellular mechanics that occur throughout the life cycle; and (5) deciphering how rice genome evolves and how it diversifies. These areas of research basically correspond to the field of functional genomics, which includes understanding the function from the basic structural sequence of the DNA, the RNA, proteins and accompanying interactions.

Since 1991, the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan has been continuously funding comprehensive large-scale projects for rice genome research. Foremost among these projects is the Rice Genome Research Program (RGP), which led to the establishment of the high-density genetic map, physical map, transcript map and eventually the complete rice genome sequence. During the same period, other genome related projects have been organized to facilitate efficient utilization of genome information derived from mapping and

sequencing. These include projects on DNA marker breeding, gene cloning / functional analysis, proteome, microarray, full-length cDNA analysis etc., which further generated biological materials and genetic resources which could be used for functional characterization of the genome. As part of an effort to ensure that the large investment in these projects will benefit the scientific community at large and encourage more innovative researches in functional and applied genomics, the Rice Genome Resource Center (RGRC, http://www.rgrc.dna.affrc.go.jp/) was established in 2003. It also aims to facilitate efficient management and distribution of genome resources and to provide access to high-throughput technologies in functional genomics. The three major genome resources, namely, rice full-length cDNA, *Tos17* mutant lines and genetic analysis materials (Table 1) will be described with emphasis on their utilization in functional characterization of the genome.

Rice Full-length cDNA

The full-length cDNA sequences are important resources to facilitate the characterization of gene functions. Such information is important for the confirmation of genes identified by genome annotation using various prediction programs and for identification of alternative splicing sites for RNAs. The Rice Full-length cDNA Project has generated sequence data on 175,642 rice full-length cDNAs clustered into 28,469 nonredundant clones (The Rice Full-length cDNA Consortium 2003). The starting materials for library construction represent various tissues such as root, shoot and panicle as well as calli subjected to various treatment and growth conditions and therefore represent the complete array of genes expressed in rice. A total of 21,596 clones have been assigned with tentative protein functions through homology searches of publicly available sequence data. In addition, more than 94% of the clones could be mapped to subspecies japonica and indica genomic sequences indicating that the nucleotide sequences of the gene-coding regions are very similar in these two subspecies. All sequence data are available through the database KOME (Knowledge-based Oryza Molecular Biological Encyclopedia, http://cdna01.dna.affrc.go.jp/cDNA/). The database provides the nucleotide sequence and encoded amino acid sequence information, results of the homology search with the public databases, mapping information, pattern of alternative splicing, protein domain information, transmembrane structure, cellular localization and gene function by Gene Ontology. Access to specific information for each full-length cDNA clone can be made by BLAST search, accession number of the clone, specific domain name and general key word search.

The availability of rice full-length cDNA clones will greatly enhance the rate of gene discovery, patterns of splicing and the understanding of gene function and protein interactions in rice. Since the annotation of the sequence simply classifies various regions of the genome into genic regions, non-transcribed region, transposable elements etc., homology with full-length cDNA sequence will address whether the predicted genes are ever expressed or not to facilitate more informative characterization of the gene.

***Tos17* mutant lines**

An alternative to the nucleic acid characterization through full-length cDNA homology is by direct demonstration that the gene has a function. This can be done by disrupting the gene function via inserting either a T-DNA or a transposon sequence. The insertional mutagenesis approach is expected to play an important role in determining the function of the genes predicted in rice. A transposon tagging strategy, which utilizes a rice endogenous retrotransposon *Tos*17 has been found to be effective in functional characterization of rice genes (Hirochika 2001). Mutations due to *Tos*17 transposition are normally induced under tissue culture conditions and

are inherited in subsequent generations to facilitate analysis of the mutated gene. So far, a *Tos17* mutant panel with more than 50,000 insertional mutant lines carrying about 500,000 insertions has been generated. These resources would be very useful for forward and reverse genetic analyses. The Rice *Tos*17 Insertion Mutant Database (http://tos.nias.affrc.go.jp/) currently contains flanking sequences of *Tos*17 insertion sites from 5,000 lines and associated phenotypes. By performing a simple BLAST search against these flanking sequences, any gene of interest can be easily found if the collection contains a mutation in that gene.

The disruption of gene function by insertional mutagenesis is generally applicable for rice because it can be easily transformed and regenerated. The insertion of *Tos17* into the coding region can induce partial or complete inactivation of the gene that may therefore lead to identification of the function of the gene.

Genetic analysis materials

Analysis of agronomically useful genes and complex traits including QTL have been effectively accomplished using a number of resources including a high-density linkage map, appropriate crosses and subsequent generations of segregating populations to allow the mapping of the traits of interest. A strategy that involved developing of specific mapping populations and fine mapping of many traits proved effective in map-based cloning. The genetic populations currently available include backcross inbred lines (BILs), chromosome segment substitution lines (CSSLs) and doubled-haploid lines (DHLs) from japonica / indica crosses (Table 1). The genotype of each line was determined using RFLP markers distributed along the 12 rice chromosomes. These genetic materials have been successfully used in QTL mapping of many agronomic traits including heading date, seed dormancy and regeneration ability.

Many traits in rice such as flowering time, seed development, disease resistance etc. have been extensively studied in the context of germplasm diversity. Several QTLs associated with such traits have been identified (Yano et al. 2003). However there are many key agronomic characteristics that remain to be analyzed in rice. In addition to a wide array of molecular markers for rice, the availability of genetic populations with well-characterized chromosomal segments will be very useful for fine mapping of QTLs.

Table 1. Biological materials from the Rice Genome Resource Center

Resources	Stocks	No of clones/lines
Rice full-length cDNA	DNA	32,000 clones
Tos17 insertion mutant lines	seeds	5,000 lines
Nipponbare/Kasalath BILs	seeds	98 lines
Nipponbare/Kasalath CSSLs	seeds	54 lines
Koshihikari/Kasalath BILs	seeds	182 lines
Koshihikari/Kasalath CSSLs	seeds	39 lines
Sasanishiki/Habataki BILs	seeds	85 lines
Sasanishiki/Habataki CSSLs	seeds	39 lines
Akihikari/Koshihikari DHLs	seeds	212 lines

High-throughput technologies for functional genomics

Much of the researches in the post-genome sequencing era will depend on high-throughput

technologies. The Rice Genome Resource Center is providing access to microarray technology that will allow a much larger number of investigators to apply global expression profiling into their specific research programs on rice. A rice oligonucleotide microarray with 22,000 non-redundant probes was constructed in collaboration with Agilent Technologies (http://www.agilent.co.jp). A 2-day protocol for microarray analysis was also developed to allow researchers to explore expression profiling. After RNA extraction, sample labeling and hybridization can be performed on the first day. Subsequently, washing and data spot analysis can be performed on the second day. It is a highly reliable protocol eliminating most of the limiting factors that affect microarray expression analysis such as sensitivity of the quantity of RNA and background intensity. Imaging analysis using MFP-3D atomic force microscopy was performed to determine the quality of each DNA spot in this microarray. At 90 μm 5 90 μm scale each DNA spot in this array is nearly circular in shape. Magnification of a 3μm 5 3μm section as well as 1μm 5 1μm section showed a clear boundary between the glass surface and the DNA spot. This suggests that hybridization of target genes can be performed with high reproducibility and enhanced sensitivity. This microarray chip is also available commercially through Agilent Technologies.

A platform for rice functional genomics initiative

The effort of the Rice Genome Resource Center in providing access to genome resources is a major step towards developing a genetic platform for functional genomics. The international community is responding as well as reflected in the formation of the International Rice Functional Genomics Consortium, which was patterned after the International Rice Genome sequencing Project (Hirochika et al. 2004). The collective goals of the consortium include sharing of genomic materials, integration of databases, bilateral or multilateral partnerships, implementing initiatives for the cooperative elucidation of gene function and accelerating delivery of research results to benefit rice production (http://www.iris.irri.org:8080/IRFGC/). Specifically, the consortium has set a timeline for various functional genomics programs as follows: 1) making genome-wide oligo chips publicly available by 2005; 2) tagging 90% of rice genes using a variety of mutant collections by 2005; 3) establishing a global internet network of rice functional genomics databases by the end of 2005; 4) developing a high-throughput verification system by 2007; and 5) characterizing the function of 50% of rice genes by 2010. With the rapid accumulation of genomics resources and the growing interest of researchers in rice, these goals could be easily achieved. The current progress on how the rice scientific community is moving towards these goals can be reflected from the resources available from various groups worldwide (Table 2).

Table 2. Rice genome resources available worldwide

Resources	Description	Access
Complete genome sequence	Japonica var. Nipponbare	IRGSP
Draft genome sequence	Indica variety	Beijing Genomics Inst.
Draft genome sequence	Japonica var. Nipponbare	Syngenta
Gene clone	Full-length cDNA	RGRC
Mutant lines	*Tos*17insertion lines	RGRC
Mutant lines	T-DNA lines	Pohang Univ. Sci. Tech.
Mutant lines	T-DNA lines	IRRI
Genetic populations	BIL, CSSL, DHL	RGRC

Gene array	22,000 full-length cDNA	RGRC
Gene array	60,000 EST / genomic sequence	Beijing Genomics Inst.
Gene array	20,230 TIGR gene models	Univ. California Davis

The Rice Genome Resource Center will continue to take part in this initiative by providing access to more biological materials as they become available and developing more high-throughput technologies to assist rice researchers.

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References

The Rice Full-length cDNA Consortium, 2003. Collection, mapping, and annotation of over 28,000 cDNA clones from *japonica* rice. Science 301:376-37

Hirochika, H., 2001. Contribution of the *Tos*17 retrotransposon to rice functional genomics. Curr. Opin. Plant Biol. **4**:118-122.

Hirochika, H. et al., 2004. Rice mutant resources for gene discovery. Plant Mol. Biol. 54:325-334.

International Rice Genome Sequencing Project, 2005. The map-based sequence of the rice genome. Nature **436**:793-800.

The Rice Full-length cDNA Consortium, 2003. Collection, mapping, and annotation of over 28,000 cDNA clones from japonica rice. Science **301**:376-379.

Yano, M. et al., 2003.Marker-assisted dissection and pyramiding of complex traits in rice. Rice Science: Innovations and Impact for Livelihood (edited by Mew,T.W. et al.) p257-263. IRRI.

FUNCTIONAL ANALYSIS OF GENES INVOLVING CULM ELONGATION IN RICE

Motoyuki Ashikari and Makoto Matsuoka
Bioscience & Biotechnology Center, Nagoya University, Furouchou, Chikusa, Nagoya, Aichi, 464-8601, Japan.
Corresponding author: **makoto@agr.nagoya-u.ac.jp**, Tel: 81-52-789-5225,Fax: 81-52-789-5226,

In the 1960s, the rapid acceleration of the world population growth rate and dramatic decrease in cultivated-lands raised fears that food production would not meet the growing demand, leading to a global food crisis. To address the problem, in 1966, the International Rice Research Institute (IRRI) bred a semi-dwarf and high yielding variety IR8, well known as "miracle rice", by the crossing between a Taiwanese native semi-dwarf variety Dee-geo-woo-gen, which carries the *semi dwarf 1* (*sd1*) gene, and an Indonesian good taste variety, Peta. The yield improvement of IR8 was mainly due to the reduction of plant height caused by the *sd1* allele. In general, nitrogen fertilization is essential to increase grain production, but it also induces culm elongation, resulting in an overall increase in the height of crop plants. Such tall crop plants are easily damaged by wind and rain and consequently yield losses occur. The IR8 semi-dwarf rice variety dramatically resolved this problem, mainly because it responded to fertilizer inputs to produce an increased yield without culm elongation. Widespread adoption of IR8 led to major increases in rice grain production and a famine was averted. It was the so-called rice "green revolution". Like IR8, the high yielding varieties Taichung Native 1 in Taiwan and Tongil in Korea, which also contained the *sd1* allele from Dee-geo-woo-gen, contributed to food security in these countries. Similarly, a Japanese native semi-dwarf variety Jikkoku, and γ-ray induced variety Reimei in Japan, and the γ-ray induced variety Calrose 76 in the United States of America, also carried different *sd1* alleles and were widely used in the rice breeding programs in these countries. The fact that such different *sd1* alleles have been used as cross parents and contributed to numerous rice breeding programs for both indica and japonica species demonstrates that the *sd1* locus is the most suitable for controlling the height of the rice plant.

There are various reasons for the dwarf phenotype in plants, but one of the most important factors for determining plant height are gibberellins (GAs) known as one of the plant hormones. GAs are a large family of tetracyclic diterpenoids and are associated with a number of plant growth and developmental processes such as seed germination, stem elongation, flowering and fruit development. To date, we have studied several rice GA-related mutants and isolated the genes involved in GA biosynthesis and signal transduction in the rice plant. Through these studies on rice GA-related mutants, we recognized that the plant morphology of the *sd1* mutants is similar to that of GA-deficient mutants with weak allele. To investigate the possibility that the *SD1* gene is related to GA, we examined the GA response in the *sd1* mutant. As a first step, we compared the second leaf sheath elongation in the *sd1* and wild type plants in response to various amounts of GA_3 treatments. The *sd1* seedlings responded better than the wild type and the sheath length of *sd1* was recovered to that of the wild type at 10^{-6} M of GA_3. This result suggests that *sd1* is GA –deficient mutant.

To identify which enzyme the *SD1* gene encodes, we directly examined the intermediate GA levels in the *sd1* mutant. The amounts of GA_{20}, GA_1, GA_8 and GA_{29} in the *sd1* mutants were lower than that of the wild type plants, whereas the amounts of GA_{44} and GA_{19} in the mutants were equal level those of wild type. According to GA_1 is mainly used as active GA in rice, these results

suggest that the activity of GA 20-oxidase (GA20ox), which catalyzes the 3 steps from GA_{53}-GA_{44}-GA_{19} to GA_{20}, is weaker in the mutants than in the wild type, and therefore the *SD1* gene may encode GA20ox.

So far, one gene encoding a GA20ox (*GA20ox-1*) has been isolated from the rice plant, but it does not correspond to the *SD1* gene since *GA20ox-1* was mapped on chromosome 3 of the rice genome whereas *sd1* was mapped on the long arm of chromosome 1. With this in mind, we suspected that *SD1* may encode another GA20ox since the *Arabidopsis* genome carries four *GA 20-oxidase* genes and the products function in a redundant manner. To isolate other *GA20ox*-related genes in rice, we designed degenerated primers based on the conserved domain between the rice and *Arabidopsis GA20ox* genes. Two amplified DNA fragments were obtained from the rice genome (japonica cultivar, Taichung 65); one corresponds to the previously identified *GA20ox-1* and the other is a novel *GA20ox* gene (*GA20ox-2*). The *GA20ox-2* was located on the long arm of chromosome 1, tightly linked to the *sd1* locus. We isolated the full length of the *GA20ox-2* gene and the cDNA clones. The deduced amino acid sequence of *GA20ox-2* showed 47.8% or 49.5% identity to that of *GA20ox-1* or *Arabidopsi GA5,* respectively. We cloned and sequenced the *GA20ox-2* genes from four *sd1* mutants and found that one *sd1* allele contained 383 bp deletion (Dee-geo-woo-gen), and other three *sd1* alleles had single nucleotide exchanges to induce amino acid exchange respectively (Jikkoku, Reimei and Calrose 76). Various mutations in the gene of *sd1* mutants strongly indicate that *SD1* encodes GA20ox-2. The introduction of the *GA20ox-2* gene from the wild type plant rescued the dwarf phenotype of *sd1,* confirming that *SD1* encodes GA20ox-2. To demonstrate that *GA20ox-2* encodes an active GA20ox, we subcloned the coding region in an expression vector and expressed its product in *E. coli.* The recombinant protein catalyzed the conversion of GA_{53} to GA_{20}. These results show that *GA20ox-2* encodes an active GA20ox.

Recently, the wheat green revolution gene, *Rht,* was identified and revealed that it encodes a transcriptional factor that works as a negative regulator of GA signaling. As wheat has a hexaploid genome, recessive alleles such as *sd1* in rice are not available for producing a semi-dwarf strain of wheat. Actually the *Rht* gene was a gain-of-function allele caused by an N-terminal truncated product which is specific region related to the perception of GA signaling. Even though the genetic and biochemical function of the rice SD1 and wheat RHT proteins are completely different, that is, recessive and dominant, loss-of-function and gain-of-function events, enzyme and transcriptional factor, respectively, the products of both genes are related to GA. The fact that both green revolution genes are related to GA may not be a coincidence, but may be a natural consequence. Consequently, manipulation of GA biosynthesis or perception may be a good target for regulation of crop height.

The rate of world population growth has now once again exceeded the rate of growth in food-grain production, and prompt measures and action for a second green revolution have been called for to avoid widespread food scarcity in the future. As our present study revealed that modulating active GA levels during the vegetative stage can produce a suitable plant architecture for high crop yield, the genetic manipulation of GA-biosynthesis using a molecular biological approach may provide us with an opportunity to address food security concerns as was the case for the previous green revolution.

References

Ashikari M, Sasaki A, Ueguchi-Tanaka M, Itoh H, Nishimura A, Datta S. K, Ishiyama K, Saito T, Kobayashi M, Khush G. S, Kitano H, Matsuoka M. 2002 Loss-of-function of a rice gibberellin biosynthetic gene, GA20 oxidase (*GA20ox-2*), led to the rice green revolution.

Breeding Science.52: 143-150.
Khush G. S. 1999 Green revolution: preparing for the 21st century. Genome 42: 646-655.
Peng J, Richards D. E, Hartley N. M, Murphy G. P, Devos K. M, Flintham J. E, Beales J, Fish L. J, Worland A. J, Pelica F, Sudhakar D, Christou P, Snape J. W, M. Gale D, Harberd N. P. 1999 'Green revolution' genes encode mutant gibberellin response modulators. Nature. 400: 256-261.
Sasaki A. Ashikari M, Ueguchi-Tanaka M, Itoh H, Nishimura A, Datta S. K, Ishiyama K, Saito T, Kobayashi M, Khush G. S, Kitano H, Matsuoka M. 2002 A mutant of gibberellin-synthesis gene in rice. Nature. 416: 701-702.

MOLECULAR MECHANISM OF BROAD-SPECTRUM RESISTANCE MEDIATED BY NBS-LRR PROTEIN IN RICE

Guo-Liang Wang[1,4,*], Shanghong Qu[1], Bo Zhou[1], Guifu Liu[1], Bin Han[2], Hajime Sakai[3]

1. Department of Plant Pathology, Ohio State University, Columbus, OH 43210 USA
2. National Center for Gene Research, Chinese Academy of Sciences, Shanghai 200233, China
3. DuPont Crop Genetics, Delaware Technology Park 100/200, DE, USA
4. Rice Genomics Laboratory, Hunan Agricultural University, Changsha, Hunan, 410128 China

* Corresponding author: Guo-Liang Wang, Department of Plant Pathology, The Ohio State University, 201 Kottman Hall, 2021 Coffey Rd, Columbus OH 43210 USA. Tel (614) 292 9280 or 1375, Fax (614) 292 4455, Email: wang.620@osu.edu

Abstract

To understand the molecular basis underlying the broad-spectrum resistance to rice pathogens, we cloned and characterized the rice blast resistance genes *Pi9* and *Pi2*. Both of the resistance genes confer high level of resistance to diverse isolates of *Magnaporthe grisea* collected from different rice growing areas. Sequence analysis indicated that the two resistance genes are allelic to each other and encode highly homologous NBS-LRR proteins. Each locus contains multiple family members spanning about a 100 kb region. Comparative sequence analysis of five haplotypes revealed that the LRR region is under diversification selection. The orthologous region of the *Pi9* and *Pi2* genes in three wild species is being sequenced which will provide important information about the origin and evolution of the complex resistance gene cluster in the genus *Oryza*.

Introduction

Broad-spectrum disease resistance is defined as a type of resistance that is effective to many strains of the same pathogen or to strains of two or more unrelated pathogens. It not only provides a useful resource for breeding of durable resistance in crop plants but also an excellent material for the understanding of the molecular basis of resistance specificity in plants. To date, several broad-spectrum resistance genes have been cloned in plants. *Xa21* is the resistance gene cloned with a high level of resistance to many *Xanthomonas oryzae* pv. *oryzae* strains (Wang *et al.* 1996). The recessive mutations (*mlo*) of the barley *Mlo* locus mediate a broad-spectrum resistance to all known isolates of powdery mildew fungus (*Blumeria graminis f. sp. hordei*) (Buschges *et al.* 1997). *mlo*–based disease resistance involves a spontaneous-lesion phenotype and cell-wall deposition in epidermal tissues preceding pathogen attack. The *Arabidopsis RPW8* gene (Xiao *et al.* 2001) belongs to a new family of broad-spectrum resistance gene and confers resistance to different isolates of the same powdery mildew pathogens as well as to different powdery mildew fungi. The protein it encodes is a small membrane protein with a putative coiled-coil domain that has limited homology to the N-terminus of an NBS-LRR gene (Xiao *et al.* 2001). *RPW8* is not involved in the gene-for-gene interaction and may interact with different *AVR* genes from different pathogens. Recently, the same resistance gene that was denoted *RB* by Song *et al.* (2003) and *RPI (Rpi-blb1)* by Van der Vossen *et al.* (2003) was cloned from an NBS-LRR gene cluster. *RB/RPI* confers a broad-spectrum resistance to all known races of the late blight pathogen *Phytophthora infestans.*

Rice blast, caused by the fungal pathogen *Magnaporthe grisea* is a major disease in rice production worldwide. To elucidate the molecular basis of broad-spectrum resistance to the

pathogen, we cloned the blast resistance genes *Pi9* and *Pi2* genes using a map-based cloning strategy. Both *Pi9* and *Pi2* confer high level of resistance to diverse isolates collected from many countries and have been widely used in rice breeding programs. Detailed characterization of the resistance cluster provides new insight into the molecular basis of broad-spectrum resistance to plant pathogens.

Results and Discussion

An NBS-LRR gene cluster was identified by sequence analysis of a 76-kb genomic region from the *Pi9* locus: A BAC (bacterial artificial chromosome) was constructed using the DNA isolated from the *Pi9* donor line 75-1-127. Using the *Pi9*-linked marker pB8 as the hybridization probe, three BAC clones spanning the locus was identified that were used to build a BAC contig (LIU *et al.* 2002). To obtain sequence information at the *Pi9* locus, we fully sequenced two of the BAC clones, 75-1-127BAC12 and 75-1-127BAC3. The gene prediction program GENSCAN was first used to identify the putative coding sequence (CDS) in the 76-kb region, and then the BLAST program was used for homology searches to confirm the gene prediction results. Seven putative genes were identified from the 76-kb sequence. The first gene in the 76-kb region (between 10489–12966 bp), located at the SP6 end in 75-1-127BAC12, is a homolog of the maize gene that encodes a putative nitrate-induced NOI protein. The other six genes, denoted *Nbs1-Pi9* to *Nbs6-Pi9*, were considered to be *Pi9* candidate genes because they all have high homology to the NBS-LRR disease resistance genes cloned from various plant species (BENT, 1996; DANGL and JONES, 2001; MARTIN *et al.* 2003).

Rice transformation with individual candidate genes determined that *Nbs2-Pi9* is *Pi9*. From the *Pi9* deletion mutant analysis, we narrowed down the *Pi9* gene to either *Nbs2-Pi9* or *Nbs3-Pi9*. To pinpoint the *Pi9* gene in the genomic region, we made two genomic constructs of the *Nbs2-Pi9* and *Nbs3-Pi9* genes. The pNBS3 construct contained a 14.6-kb (18395–33070 bp) genomic fragment comprising the *Nbs3-Pi9* gene. T_1 plants of 19 independently transformed lines were susceptible to PO-6-6, and all of the T_2 plants from nine transgenic lines were highly susceptible to PO-6-6. For rice transformation of *Nbs2-Pi9*, we developed the pNBS2 construct, which containing a 13.5-kb *Sal*I genomic fragment (32363–45848 bp). This 13.5-kb fragment contained 1362 bp of the *Nbs2-Pi9* 5'-UTR region (32962–33724 bp), the whole *Nbs2-Pi9* coding sequence (33,725–42,313 bp), and 1804 bp of the 3'-UTR sequence (42314–44117 bp). Of the 19 T_1 transgenic plants we inoculated with rice blast isolate PO6-6, 13 were resistant and six were susceptible. We subsequently evaluated T_2 plants from three resistant T_1 lines (#10, #12, and #77) and one susceptible T_1 line (#8) for resistance to rice blast isolate PO6-6 and observed the segregation of resistant and susceptible plants in all three resistant lines.

Co-segregation of the transgene and blast resistance was confirmed in transgenic line #12 using Southern blot analyses. Genomic DNA was extracted from ten T_2 plants of the #12 line, digested with *Eco*RI, and probed with a 928-bp fragment from *Nbs2-Pi9* (40350–41278 bp). The 928-bp fragment was found to be located within the second exon of the putative *Nbs2-Pi9*-coding sequence and corresponds to the sequence encoding the last 151 amino acids of the NBS domain and the first 158 amino acids of the LRR domain. All seven resistant T_2 plants (#12-13, #12-14, #12-18, #12-20, #12-21, #12-29, and #12-30) contained the expected 11-kb *Nbs2-Pi9* band, while the three susceptible T_2 plants (#12-5, #12-17, and #12-23) did not, suggesting that the resistance in the transgenic lines is truly due to the *Pi9* transgene.

***Pi9* encodes a putative NBS-LRR protein containing 1,032 amino acids:** To isolate the cDNA fragment of *Pi9*, we prepared total RNA using leaf tissue from 75-1-127 collected 24 h following blast inoculation. The *Pi9* cDNA was 4009 bp, including 3099 bp of *Pi9* coding sequence and 910 bp of 3'-UTR. Protein translation of the cDNA sequence revealed that the *Pi9* gene encodes a predicted 1032-amino acid polypeptide with a molecular weight of 117.05 kDa and an isoelectric point (pI) of 7.55. The deduced *Pi9* protein belongs to the NBS-LRR class of R protein. The three sequences in the NBS domain, GMGGLGKT (positions 193–200), KRYFVILDDLW (positions 277–287), and GSRIVITTRNVDL (positions 307–319), correspond to kinase 1a (P-loop), kinase 2, and kinase 3a, respectively. At the C-terminal region is the LRR domain, which is composed of 17 imperfect LRR repeats.

***Pi2* is allelic to *Pi9* and encodes an NBS-LRR protein:** Using the markers tightly linked to *Pi9*, a high-resolution map at the *Pi2* locus was established. A BAC/TAC contig that covered the *Pi2* region was constructed and subsequently sequenced completely. Sequence analysis showed that there is a gene family in the *Pi2* region that comprises nine NBS-LRR resistance gene-like sequences (named *Nbs1-Pi2 to Nbs9-Pi2*). The *Pi2* candidate gene was delimited into both *Nbs1-Pi2* and *Nbs2-Pi2* based on results from the high-resolution mapping in the *Pi2* region. Molecular analysis of the *Pi2* susceptible mutants further narrowed the candidates to *Nbs2-Pi2*, which was finally confirmed by gene complementation test. *Pi2* encodes a novel nT-NBS-LRR protein and has a unique gene structure in respect to the intron position. *Pi2* was constitutively expressed at low levels in both resistant and susceptible rice after blast infection. Physical mapping and sequence analysis revealed that *Pi2* and *Pi9* are allelic although they were introgressed from different donor lines. Cloning and characterization of the *Pi2* gene provide us additional material to elucidate the molecular mechanisms of host-pathogen interaction, evolution, race-specificity and signaling pathway of broad-spectrum resistance to rice blast.

Reference

Bent, A. F., 1996 Plant disease resistance genes: function meets structure. Plant Cell **8:** 1757-1771.

Buschges, R., K. Hollricher, R. Panstruga, G. Simons, M. Wolter *et al.*, 1997 The barley *Mlo* gene: a novel control element of plant pathogen resistance. Cell **88:** 695-705.

Dangl, J. L., and J. D. G. Jones, 2001 Plant pathogens and integrated defense responses to infection. Nature **411:** 826-833.

Liu, G., G. Lu, L. Zeng and G. L. Wang, 2002 Two broad-spectrum blast resistance genes, *Pi9(t)* and *Pi2(t)*, are physically linked on rice chromosome 6. Mol. Genet. Genomics **267:** 472-480.

Martin, G. B., A. J. Bogdanove and G. Sessa, 2003 Understanding the functions of plant disease resistance proteins. Annu. Rev. Plant Biol. **54:** 23-61.

Song, J., J. M. Bradeen, S. K. Naess, J. A. Raasch, S. M. Wielgus, *et al.*, 2003 Gene *RB* cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. Proc. Natl. Acad. Sci. USA **100:** 9128-9133.

Van Der Vossen, E., A. Sikkema, B. L. Hekkert, J. Gros, P. Stevens *et al.*, 2003 An ancient *R* gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. Plant J. **36:** 867-882.

Wang GL, Song W, Ruan D, Sideris S, Ronald PC. 1996 The cloned gene, *Xa21*, confers resistance to multiple *Xanthomonas oryzae* pv. *oryzae* isolates in transgenic plants. Molecular Plant-Microbe Interactions **9**:850-855.

Xiao, S., S. Ellwood, O. Calis, E. Patrick, T. LI *et al.*, 2001 Broad-spectrum mildew resistance in *Arabidopsis thaliana* mediated by *RPW8*. Science **291:** 118-120.

ISOLATION AND FUNCTIONAL ANALYSIS OF GENES CONTROLLING SI UPTAKE IN RICE

Jian Feng Ma[1*], Naoki Yamaji[1], Kazunori Tamai[1], Namiki Mitani[1], Saeko Konishi[2], Masahiro Yano[3]

[1] Research Institute for Bioresources, Okayama University, Chuo 2-20-1, Kurashiki 710-0046, Japan

[2] Institute of Society for Techno-innovation of Agriculture, Forestry and Fisheries, Kamiyokaba, Tsukuba, Ibaraki 305-0854, Japan

[3] Department of Molecular Genetics, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan

*Corresponding author: maj@rib.okayama-u.ac.jp (Tel: +81 86 434 1209)

Beneficial effect of silicon on plant growth

Silicon (Si) is the second most abundant element in soil. In soil solution, Si occurs mainly as monosilicic acid (H_4SiO_4) at concentrations ranging from 0.1 to 0.6 mM and is taken up by plants in this form (Epstein 1994; Ma and Takahashi 2002). After the uptake, Si accumulates on the epidermis of various tissues mainly as a polymer of hydrated amorphous silica. All terrestrial plants contain Si in their tissues although the content of Si varies considerably with the species, ranging from 0.1% to 10% Si on a dry weight basis (Ma and Takahashi 2002). Silicon has not been recognized as an essential element for plant growth from viewpoint of physiology, however, a lot of beneficial effects of Si on plant growth have been observed in a wide of plant species. The beneficial effects of Si reported include increased photosynthetic activity, increased insect and disease resistance, reduced mineral toxicity, improvement of nutrient imbalance, and enhanced drought and frost tolerance (for reviews, see Ma and Tahakashi, 2002, Savant et al., 1997). Overall, the beneficial effects of Si show two characteristics. One is that the beneficial effects vary with the plant species. Beneficial effects are usually obvious in plants that accumulate high levels of Si in their shoots (Ma et al. 2001). One typical example is rice, which accumulates Si up to 10% Si on a dry weight basis in the shoot. High accumulation of Si in rice has been demonstrated to be necessary for healthy growth and high and stable production. For this reason, Si has been recognized as an "agronomically essential element" in Japan and silicate fertilizers have been applied to paddy soils. The other characteristic is that the beneficial effects of Si are usually expressed more clearly when plants are subjected to various abiotic and biotic stresses. Silicon is probably the only element which is able to enhance the resistance to multiple stresses.

Silicon uptake mechanism in different plant species

The accumulation of silicon (Si) in the shoots varies considerably among plant species, and this variation has been attributed to the different capacity of the roots to take up Si. The uptake system of Si was recently investigated in terms of radial transport from external solution to root cortical cells and release of Si from cortical cells to xylem in rice, cucumber and tomato, which differ greatly in the shoot Si concentration (Mitani and Ma, 2005). The concentrations of Si in the root-cell symplast in all species were higher than that in the external solution, although the concentration in rice was 3- and 5-fold higher than that in cucumber and tomato, respectively. A kinetic study showed that the radial transport of Si was mediated by a transporter with a K_m value of 0.15 mM in all species, but with different V_{max} values in the order of rice> cucumber >tomato. In the presence of the metabolic inhibitor 2,4-dinitrophenol and at low temperature, the Si concentration in the root-cell symplast decreased to the similar level of apoplasmic solution.

These results suggest that both transporter-mediated transport and passive diffusion of Si are involved in the radial transport of Si and that the transporter-mediated transport is an energy-dependent process (Fig. 1).

The Si concentration of xylem sap in rice was 20- and 100-fold higher than that in cucumber and tomato, respectively. In contrast to rice, the Si concentration in the xylem sap was lower than that in the external solution in cucumber and tomato (Mitani and Ma, 2005). A kinetic study showed that xylem loading of Si was also mediated by a kind of transporter in rice (Tamai and Ma, 2003), but by passive diffusion in cucumber and tomato (Fig. 1). These results indicate that a higher density of transporter for radial transport and the presence of a transporter for xylem loading are responsible for high Si accumulation in rice.

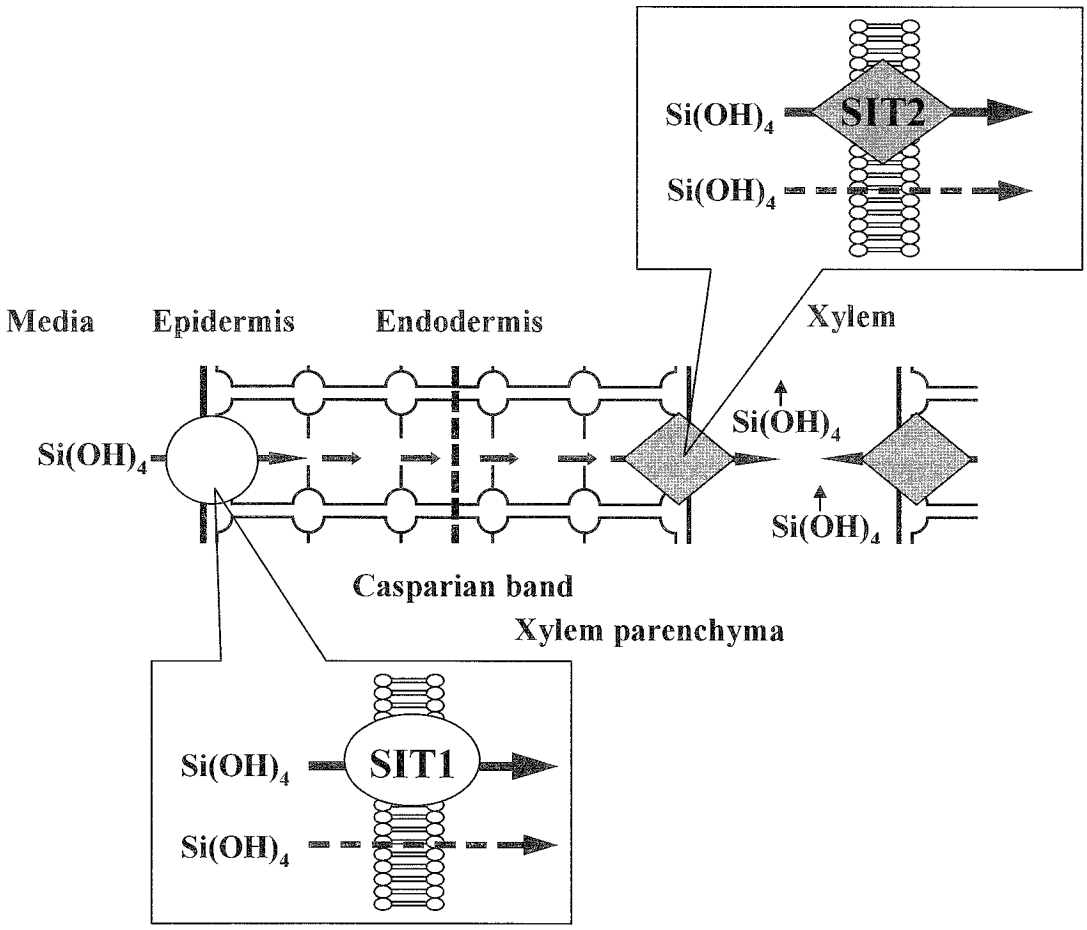


Fig. 1 Schematic representation of Si uptake system in different plant species. Radial transport of Si includes transporter-mediated transport and passive diffusion in rice, cucumber and tomato. Xylem loading of Si is mediated by a kind of transporter in rice, but by diffusion in cucumber and tomato. SIT1, Si transporter from external solution to cortical cells; SIT2, Si transporter for xylem loading. From Mitani and Ma (2005).

Isolation and characterization of a gene controlling Si uptake in rice

To identify the genes encoding Si transporter in rice, a rice mutant (*lsi1*) defective in Si uptake has been isolated, by using Ge tolerance as an index (Ma et al., 2002). This mutant had a plant type similar to the wild type except that the leaf blade of *lsi1* remained droopy when Si was

supplied. The Si concentration of the tops was much lower in the mutant than in the wild type, while that of the roots was similar. A short-term uptake experiment showed that the Si uptake by the mutant was significantly lower than that by the wild type (Fig. 2), while there was no difference in the uptake of other nutrients such as P and K. Further, Si uptake by the wild-type rice was inhibited by metabolic inhibitors including NaCN and 2, 4-dinitrophenol and by low temperature, whereas Si uptake by *lsi1* was not inhibited by these agents. The Si concentration in the xylem sap of the wild-type rice was also much higher than that of *lsi1*. These results suggest that an active transport system for Si uptake is disrupted in the mutant (Ma et al., 2002). Analysis of F_2 populations between *lsi1* and WT showed that roots with high Si uptake and roots with low Si uptake segregated at a 3:1 ratio, suggesting that *lsi1* is a recessive mutant of Si uptake.

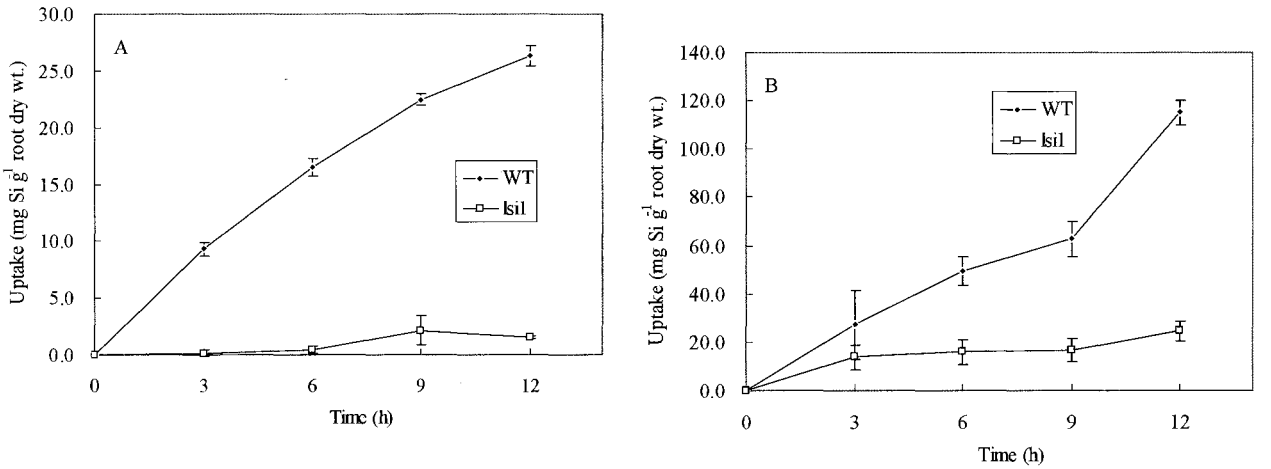


Fig. 2 Uptake of Si by WT rice (cv Oochikara) and a mutant (lsi1) defective in Si uptake. Twenty-day-old seedlings were placed in a nutrient solution containing 0.15 (A) and 1.5 mM (B) Si as silicic acid. Error bars represent ±SD (n=3).

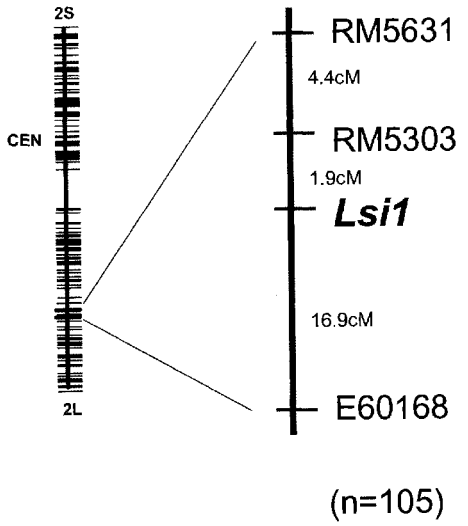


Fig. 3 Linkage relationship between EST-based PCR and microsatellite markers and a gene for Si xylem loading (*Lsi1*) on chromosome 2 in rice.

To map the responsible gene, we performed a bulked segregant analysis by using both microsatellite and EST-based PCR markers in a mapping population derived from a cross between *lsi1* and Kasalath,. As a result, the gene was mapped to chromosome 2, flanked by microsatellite marker RM5303 and EST-based PCR marker E60168 (Ma et al., 2004) (Fig. 3). Fine mapping resulted in the isolation of this gene. The cDNA was 1409 bp long and the deduced protein consisted of 298 residues, and the program PSORT predicts that the *Lsi1* encodes a membrane protein. Real-time PCR analysis showed that this gene was not induced by Si and constitutively expressed mainly in the roots. To investigate cellular localization of Lsi1, *Lsi1* with its promoter was fused with GFP and introduced into rice. Microscopic observation showed that LSI1 was localized in endodermis and exodermis, where Casparian strips exist. Furthermore, staining with anti-Lsi1 polyclonal antibody revealed the localization of Lsi1 in the plasma membrane of the endodermis and exodermis.

References

Epstein E 1994: The anomaly of silicon in plant biology. *Proc. Natl. Acad. Sci.*, **91**, 11-17

Ma JF, Miyake Y, and Takahashi, E 2001a: Silicon as a beneficial element for crop plants. *In* Silicon in Agriculture. Ed. LE Datnoff, GH Snyder, and GH Korndorfer, p. 17-39, Elsevier Science, Amsterdam

Ma JF and Takahashi 2002: Soil, Fertilizer, and Plant Silicon Research in Japan. Elsevier Science, Amsterdam.

Ma JF, Tamai K, Ichii M, and Wu K 2002: A rice mutant defective in active Si uptake. *Plant Physiol.*, **130**, 2111-2117

Ma JF, Mitani M, Nagao S, Konishi S, Tamai K, Iwashita T and Yano M 2004：Characterization of Si uptake system and molecular mapping of Si transporter gene in rice. *Plant Physiol.* **136**, 3284–3289

Mitani M, Ma J F 2005: Uptake system of silicon in different plant species. *J. Exp. Bot.* **56**, 1255-1261

Savant NK, Snyder GH, and Datnoff LE 1997: Silicon management and sustainable rice production. *Advan. Agron.*, **58**, 151-199

Tamai K and Ma JF 2003: Characterization of silicon uptake by rice roots. *New Phytol.*, **158**: 431-436

Corresponding author: **Jian Feng Ma** Research Institute for Bioresources, Okayama University, Chuo 2-20-1, Kurashiki 710-0046, Japan. maj@rib.okayama-u.ac.jp; Tel/Fax 086-434-1209

TILLING: WHERE FUNCTIONAL GENOMICS MEETS CONVENTIONAL BREEDING

Vanavichit A[1/*]., S. Sasoo[2/] ,T. Amorn [2/], S. Ruengphayak[2/] , A. Plabpla[2/], T. Toojinda[2/], S. Tragoonrung[2/]
[1/*]Agronomy Department, Faculty of Agriculture, Kasetsart Univeristy, Kampangsaen, Nakhon Pathom, 73140, Thailand, apichart@dna.kps.ku.ac.th, Tel: 66-34-355-192
[2/]Rice Gene Discovery, National Center for Genetic Engineering and Biotechnology at Kasetsart University, Thailand

Introduction

In a new challenging world, rice has now been the focal point as a medium for alleviating poverty and a hidden hunger. Taking one example, a new paradigm for rice breeding is to improve overall grain nutritional status that have been overlooked since the green revolution. In this situation, genetic diverisity for grain quality may not be available to drive a successful rice breeding program. Mutation breeding has been successfully used to generate new genetic variations from any genetic background. However, the limitation of any mutation breeding is the lack of efficient and high throughput screening. TILLING, Targeted Induced Local Lesion in Genome, a new reverse genetics strategy to discover allelic series of induced mutations in genes of interest was first developed in Arabidopsis http://tilling.fhcrc.org:9366. The original TILLING was induced by ethyl methane sulfonate (EMS) followed by PCR-based screening of coding regions of interest (Colbert et al., 2001; McCallum et al., 2000a,b). A target 1 kb region of a coding fragments are amplified using 5'-end-labeled primers on DNA extracted from EMS-mutagenized M_2 plant. A mismatch occurs at the site of the point mutation, which can be detected using mismatch-specific endonucleases such as CELI from celery (*Apium graveolens*; Yang et al., 2000) and detection of cleaved fluorescent products on denaturing polyacrylamide gel electrophoresis (Kulinski et al., 2000; Oleykowski et al., 1998). Genetic mutation is a powerful tool that establishes a direct link between the biochemical function of a gene product and its phenotypes. This TILLING project allows the identification of allelic variation in a target gene in a high-throughput manner. Any useful mutants can readily be used to add new traits to a target cultivar.

Materials and Methods

Plant materials

JHN is a non-photosensitive/purple-seed rice cultivar developed for nutrition purposes. Its grain contains up to 1.5 mg/100 g of Fe, 2.5 mg/100 g of Zn and 12.5% of protein, and highly enriched in antioxidants, vitamins B and E. Several mutations observed naturally when grown in large production, reflecting its mutable nature of this rice. JHN matures approximately 105 days and grown to about 65 cm to the panicle top.

Radiation mutagenesis

JHN was irradiated with fast neutron at 33 Gy. The total of 400,000 seeds was allocated into 26 vacuum aluminum vessels containing approximately 300 g or 15,000 seeds. Each vessel was then soaked into a radioactive pond for 30 min and then air-dried for 1 day before germination. The output seeds were irradiated up to 0.1 Krad as measured by a Giger counter. The mutagenized population consisted of approximately 125,000 M_1 and M_2.

Chemical mutagenesis

JHN was also treated with 1.0 mM Sodium aside (NaN_3) for 8 hr in the dark. This treatment was selected from pre-testing chemical mutagen that had four treatment: 0.5 mM NaN_3 for 2 hr., 0.5 mM NaN_3 for 8 hr., 1 mM NaN_3 for 2 hr., and 1 mM NaN_3 for 8 hr. The mutagenized population consisted of 8,000 M_1 (Maintenance of the $M_{2\text{-}3}$ populations).

For each M_1 plant, all panicles were harvested, dried and refrigerated for long-term storage. In the M_2 generation, a single panicle from each M_1 was seeded and thinned out leaving eight plants for observation.

Genomic DNA extraction

All rice plants were grown as a 96-well-plate format in the experimental field. Leaves from two-week-old plants were randomly collected, following the 1D and 3D protocols, for the isolation of genomic DNA. The original CTAB extraction procedure (Rogers and Bendich, 1994) was modified slightly

DNA pooling strategies

The 3D pool: Plants were grown in a 96-well Falcon plate style. Each plot (plate) consists of 96 hills arranged in 8 rows of 12 M_1 plants (or 12 columns of 8 M_1 plants). Each sample unit consisted of 10 plots (plates). Leaves were excised from two-week old M_1 plants were pooled as three dimentions in 8 rows (120 plants each) and 12 columns (80 plants each), and 10 plates (960 plants each). To equalize the contribution of each genotype in the pool, cares were taken to excise equal amount of leaf tissue from each plant. The Genomic DNA was isolated from each pooled leaf and stored at -20^{0}C freezers as references. In total, there were 4,050 pools representing the 135,000 M_1 lines.

The 1D pool: For the M_2 generation, leaves were collected from 24 M1-derived M_2 sublines to increase the probability of detecting mutation in the M_2. This ratio was determined from a sensitivity test using different mixtures of known mutation in JHN wild type. Five mixtures of the wild type to a mutant were 95:1, 47:1, 23:1, 11:1 and 1:1. Based on the outcome of this sensitivity test, the 23:1 ratio was used for developing genomic pools for the M_2 generation. In total 208 genomic pools were isolated from 40,000 M_2 sublines.

Mutation detection

SSCP analysis: PCR products denatured at 95°C were separated on 8% polyacrylamide gels. Allelic strands were dissociated under specific temperature profiles conditions according to their conformation characteristics. Gels were stained with silver staining according to the protocol recommended by the manufacturer.

DHPLC analysis: Immediately prior to DHPLC analysis, PCR products were denatured by heating at 96°C for 5 min and then gradually cooling to 25°C over a period of 30 min. The samples were kept at 4°C, 3 ul was applied to a preheated reversed-phase column (Eclipse,dsDNA preparative column; 4.6x75mm; Hewlett Packard). DNA was eluted with the following gradient consisting of buffer A (0.1 mol/L triethyammonium acetate) and buffer B (0.1 mol/L triethyammonium acetate containing 250 mL/L acetonitrile) : 60% A-40% B for 30s; 50% A-50% B for 5.5 min; 25% A-75% B for 10 s; 5% A-95% B for 1 min; and 60% A-40% B for 1.33 min. The mobile phase was heated to the respective denaturing temperatures. The gradient start and end points were adjusted according to the size of the PCR amplicon. Melting temperatures were analyzed by DHPLC Melt Program that multiple temperatures were tested for optimal resolution.

Sequencing

Overlapping PCR strategy was applied to sequence the whole gene. The PCR products were sequenced from both directions using ABI377 XL with Big-dye terminator chemistry (ABI). The fragments were assembled by program on web (http://rice.kps.ku.ac.th/h_connect.html). The multiple sequence alignment and clustalW programs were used comparing the assembled sequences.

Amplified Fragment Length Polymorphism (AFLP)

The AFLP proceduce was practiced by following the protocol of VOS et al.(1995) with some minor modifications. The best combinations of AFLP primers for mutation detection in the FA population were CT-CAG (72%), CT-CTG (2%) and CT-GCC (2%).

Phenotyping

Phenotypic data was collected on specific M_2 generation while mutation is detected in M_1. M_2 seeds of each M_1 plants stored systematically and supplied upon request by collaborators. Phenotypic data collected from collaborators was recorded in a relational database to facilitate public access. Characterization of phenotype in M_2 generation, were collected on database, correlated with candidate gene approach that is the important quality traits. We recorded a characteristic phenotype as 7 major group: awn, rice grain, culm angle (tiller), panicle, glume (hull), stem, and leaf. In addition, we can be inspected a characteristic phenotypes by indirect methods. For example, measurement of phosphorous content was inspected by HIP method. Other phenotypes can be measured with lipid, protein, and antioxidant content.

HIP (High Inorganic Phosphorous) testing

Example extraction, to measure an each seed grain. Next step, aliquot HCL solution 0.4 M per 10-fold of each seed grain weight content. Seed grain was incubated in HCL solution at 25 °C for 5 hr and grinned seed rice, and then incubates seed was ground at 4 °C for 8 hr. Extraction solution was taken 20 ul. Put 80 ul of dH2O in the sample. Added 100 ul of colorimetric reagent (Component: 3 M H_2SO_4: 2.5 % (w/v) ammonium molybdate :10 % (v/v) ascorbic acid (incubation at 4 °C) and dH2O ratio as 1 : 1 : 1 : 2 respectively) into sample solution. Incubated mixer solution at 25 °C for 1 hr. Then, compare color of mixer solution with standard solution. Standard solution is a component of 1 mM K_2HPO_4 : level 0 = 0.0 ug/ml Pi, level 1 = 0.15 ug/ml Pi, level 2 = 0.46 ug/ml Pi, level 3 = 0.93 ug/ml Pi and level 4 = 1.39 ug/ml Pi (freshly prepared).

Results and Discussion

Mutant library construction

To construct a mutant library , JHN wild type was treated with Fast neutron radiation at 33 Gy and NaN_3 mutagen at 1 mM. M_1 seeds were planted. These pooled seeds (materials and methods) were planted on a 96-well-plate format, and whole leaf rice was collected between 20 - 30 days according to 3D method. Genomic DNA was isolated from the rice tissue. M_2 seeds were collected from individual M_1. In M_2 plant were pooled as 1D. Because the limited sensitivity of detection method 1D adapted pooling was in M_2. All mutant lines were represented by DNA samples into pools of complexity DNA samples were inspected by PCR-base method, SSCP and DHPLC strategy. The mutant libraries consist of 136,000 M1 and corresponding M2 lines.

Mutation Screening

Several pooling strategies of genomic DNA were implemented to facilitate the rapid screening of the targeted coding sequence. Three screening methods for mutation detection

including DHPLC, SSCP, PAGE, and AGE, were compared. The DHPLC is the most sensitive mutation detection. This method of mutation detection was 2.76 folds more efficient when genomic DNA is pooled as 24-fold (1-D) than 96-fold (3-D).

Estimation of Mutation Rate

A mutation rate was estimated by using sequencing in M_2 as 1.54×10^{-7} per nucleotide. In addition, frequency of mutation was estimated as 8.04×10^{-4} per plant basis while frequency of mutation analyzed by AFLP technique was 17%. When compared frequency of mutation in Fast neutron radiation (FA) populations, AFLP technique has proven to be an efficient technique for estimating frequency of mutation in the rice TILLING project.

The Thai TILLING project is the largest TILLING project so far established world-wide (Table 1). In the first year, at least six target mutant genes were identified in the Thai TILLING project (Table 2). This is considered a good start because all mutants are viable and will play important roles in understanding nutritional blocks in rice breeding. From the breeding point of view, these productive mutants are invaluable genetic materials for gene pyramiding aiming for improving nutritive values. For examples, crossing between original JHN and low phytic acid, Fe chelate mutants are underway to improve Fe density and bioavailability in rice.

TILLING is useful for isolating QTLs. We demonstrated herein the case of high inorganic phosphate (HIP) mutants that AFLP tagging in M3 can be used to isolate the responsible genes.

Two successful cases for the isolation of mutants were reported for myo-inositol phosphate synthase (IPS) and ketoacyl-ACP-synthase (KAS III). For the IPS gene, two amino acids were changed that affect the gene functioning. For KasIII on the other hand, large number of single nucleotide changes had no effects on functioning of Kas III. Therefore,

Table 1 Rice mutant resources developed by the International and National Rice Research Community.

Institution[a]	Variety	Type	Vector/mutagen	Population size (current)	Population size (target)	Mutated loci per genome	Mutated sites in total collection
IRRI	IR64	Deletions and point mutation	Fast neutron γ-ray diepoxybutane EMS	40 000 at M3 or M4	50 000	10	500 000
National Institute of Agrobiological Sciences	Nipponbare	Deletion mutations	γ-ray ion beam	15 000 M2 7000 M2	50 000	nd	nd
Kasetsart University	JHN	Deletions and point mutation	Fast neutron EMS	136,000 M2	400,000	nd	nd

Table 2 Gene specific primer were designed for mutation screening

TYPE	CANDIDATE GENE	No. of primer	pathway	Target fragment (kb)	No. of Exon	No. of Intron	Type of gene
NUTRITION							
Phytic acid (RGP, KOME)	*Myo-inositol phosphate synthase (IPS)*	7	Phytic acid biosynthesis	3.8	11	10	Mutigene family
	Inositol phosphatase	5		3.1			
	Phytase	5		2.8	10	9	Mutigene family
					7	6	Mutigene family
Fatty acid (NCBI)	*ACCases (Homomeric acetyl-CoA carboxylase)*	1	Fatty acid biosynthesis	0.3	-	-	Mutigene family
	Acyl carrier protein	1		0.65	-	-	
	Ketoacyl-ACP synthase (KASIII)	1		0.3	-	-	Mutigene family
	Acyl-ACP thioesterase (Fat B)	1		0.25	-	-	Mutigene family
	Stearoyl-ACP desaturase	1		0.75	-	-	Mutigene family
Iron density (NCBI, KOME, RGP)	*Ferritin*	6		2	8	7	Duplicate gene
	Frataxin	6		2.5	5	4	Single gene
	Aconitase	1		0.7	-	-	Mutigene family
	Phytosiderophore	1		0.4	-	-	Mutigene family
	Ferric chelate reductase	3		4	4	3	Mutigene family
	Metallothionein	3		1.68	2	1	Mutigene family
Beta-carotene (NCBI, KOME)	*Beta carotene hydroxylase*	3	Carotenoid biosysthesis	0.77	6	5	Single gene
	Lycopene beta cyclase	2		0.41	1	-	Single gene
	Lycopene epsilon cyclase	3		0.64	10	9	Single gene

Table 2 Gene specific primer were designed for mutation screening (continued)

TYPE	CANDIDATE GENE	No. of primer	Pathway	Genome size (kb)	No. of Exon	No. of Intron	Type of gene
SEED QUALITY *Seed color* (RGP, NCBI)	*Anthocyanin synthase*	1	Flavanoid biosynthesis	4.9	-	-	Mutigene family
	OSB1 (Orysa sativa. Booster1)	1		3.1	-	-	Mutigene family
	DFR (Dihydroflavonol reductase)	3		3.4	-	-	Mutigene family
	CHS (Chalcone synthase)	3		3.3	-	-	Mutigene family
Aroma	*Dynamin*	1		9.4	-	-	Mutigene family
	Os2AP	1		0.4	1	-	Duplicate gene
Cooking quality, Waxy, (NCBI, RGP, KOME)	*Waxy Variation region First exon (23 bp deletion)*	2	Starch synthesis	0.3	-	-	Single gene
	ADP glucose phytophosphorylase	7		5.49	-	-	
	UDP-glucose phytophosphorylase	6		3.91	-	-	
Submergence	*Ras*	1		0.6	-	-	
Photoperiod	*Hd1*	1		0.3	-	-	

Table 3 Types of mutation detected in the Thai TILLING project

Candidate gene	No. of fragment Screened	Plants screen		Target fragment (kb)	No. of mutations		Truncation	Missense	Silent	SNPs	
		M1 (3D)	M2 (1D)		In/del	SNPs				Transition	Tranversion
Ferric chelate reductase*1	3	9600	1728	4	1	7	0	2	6	6	1
Myo-inositol phosphate synthase *2	9	4000	-	3.8	1	7	0	2	6	2	5
ADP glucose phytophosphorylase*1	7	9600	5000	5.49	1	0	1	0	0	0	0
KasIII, 3-ketoacyl carrier protein synthase III*2	1	2000	-	0.25	3	8	0	0	11	7	1
Lipoxygenase*1	7	9600	23,040	2.83	1	1	0	0	2	1	0
Lycopene epsilon cyclase*1	3	9600	18,432	6.4	1	0	0	0	1	0	0
Methallothionein*1	3	-	13,824	1.68	-	-	-	-	-	-	-
Total	30			22.77	8	23	1	4	26	16	7

*1 was found only 1:23 ratio pool.

*2 was found both 1:95 and 1:23 ratio pools.

FUNCTIONAL ANALYSIS OF THE GENES CONTROLLING EATING AND PROCESSING QUALITY OF RICE

Takayuki Umemoto[1]*, Noriaki Aoki[1], Tetsuya Horibata[2], Naoyoshi Inouchi[2], Masahiro Yano[3]
1 National Institute of Crop Science, Tsukuba, Ibaraki 305-8518, Japan
2 Dept. of Applied Biological Science, Fukuyama University, Fukuyama, Hiroshima 729-0292, Japan
3 National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan
*Corresponding author: ume@affrc.go.jp (Tel: +81 29 838 8952)

Introduction

In the rice breeding program, eating quality is the one of the most important targets. However, the sensory evaluation of cooked rice quality is time consuming and need a large amount of rice samples. Therefore, DNA marker-aided breeding is favorable strategy to select the eating quality. To perform marker-aided breeding, we have to know the genetic factors that control eating quality of rice. Since the major component of rice is starch, variations in starch composition, amylose to amylopectin ratio, or molecular structure of amylose and amylopectin could affect cooked rice quality (Juliano, 1998). Thus, the genetic factors that control starch variations also could control eating quality of rice. In this study, we examined effects of natural variations in the three starch synthase genes on the starch properties and cooked rice quality.

Amylose and amylopectin are both glucose polymers. Amylose is basically a linear molecule with tiny amount of branched chains. On the other hand, amylopectin is a highly branched molecule. Three sorts of enzymes; starch synthase, branching enzyme, and debranching enzyme are indispensable to form starch (Fig. 1). Each enzyme has several isoforms (Hirose and Terao, 2004). Among them, this study deals with granule-bound starch synthase I (GBSSI, Wx protein) working in amylose synthesis, Starch synthase I (SSI) and starch synthase IIa (SSIIa, Alk) those elongate short chains of amylopectin (Nakamura, 2002). In the later half of this study will focus on the natural variations in *SSIIa* of rice to identify the responsible SNPs important for the enzyme's starch-binding function and starch properties.

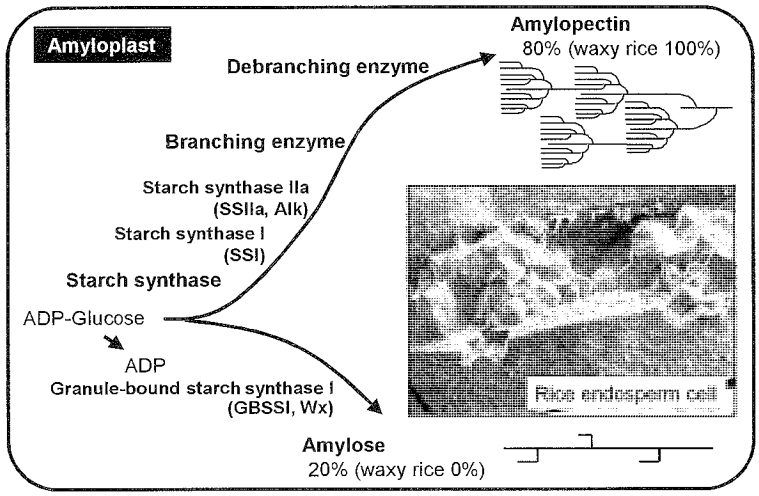


Figure 1. Starch biosynthesis pathway in rice endosperm

Effects of starch synthase variations on starch properties and cooked rice quality

We used near isogenic lines for each starch synthase locus (Fig. 2). The three starch

synthase genes are all on the short arm of chromosome 6. These NILs have chromosomal segments from an indica cultivar Kasalath with a japonica cultivar Nipponbare genetic background. NIL(Wx^a) produces several times more Wx protein compared to Nipponbare with Wx^b allele. NIL(SSI^k) has starch synthase I with slightly stronger activity and higher mobility on the native-gel containing glycogen. NIL(*Alk*) has tolerance against alkaline solution of potassium hydroxide. Recently, *starch synthase IIa* gene was proven to be the *alk* gene that controls alkali disintegration property of rice grain by map-based cloning (Gao *et al.* 2003).

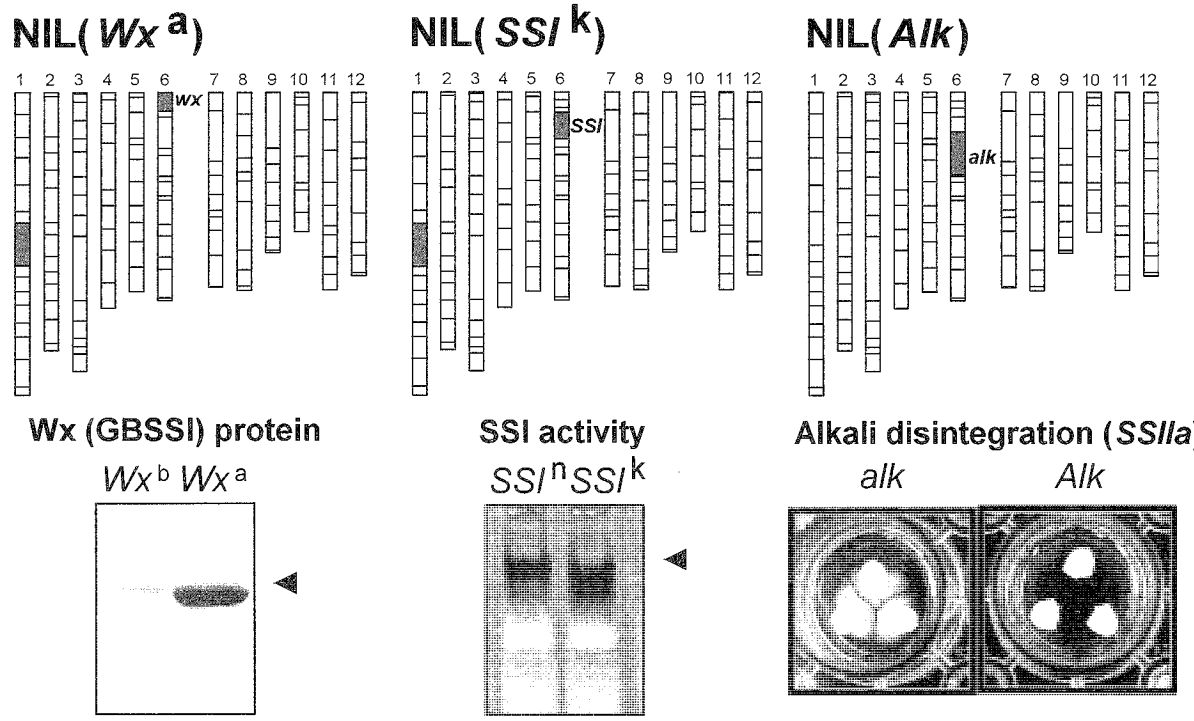


Figure 2. Near isogenic lines for three *starch synthase* loci
The NILs have *indica* Kasalath chromosomal segments (closed rectangle) with *japonica* Nipponbare genetic background.

Amylose content was measured by gel permeation chromatography after starch samples were debranched with isoamylase. The amylose content of Nipponbare was 19.9%, while Kasalath was 31.5%. The NIL(Wx^a) inherited the *Wx* allele from Kasalath and the amylose content was similar to that of Kasalath (20.1%). Thus, the natural variation in *SSI* between Nipponbare and Kasalath did not largely affect amylose content. The NIL(*Alk*) had slightly lower amylose content (17.4%) than Nipponbare. Using the NILs, we confirmed that the Wx^a allele had the largest effect on amylose content among the natural variations in the three starch synthase genes.

Chain-length distribution of amylopectin was analysed by an HPAEC-PAD after the amylopectin branches were released by isoamylase. The chain-length distributions of amylopectin of Kasalath and NILs were compared that of Nipponbare (data not shown). Kasalath has less short chain of degree of polymerisation (dp) around 8 and more middle chains dp around 17 compared to Nipponbare. Very similar profiles was observed between NIL(*Alk*) and

Nipponbare. This means that the differences in chain distribution of amylopectin between Kasalath and Nipponbare are almost explained by the *alk* locus or the chromosomal region close to the *alk*. The chain distribution of NIL(*Wx*^{a}) was also differed from that of Nipponbare. However, this difference is probably duo to the differences in temperature during grain filling. The relative amount of short chains dp around 6 to 13 was increased while that of dp around 20 to 27 was decreased when the temperature during grain-filling decreased (Umemoto et al. 1999). Actually, flowering period of this NIL was about one week later than Nipponbare, and matured under cooler temperature compared to Nipponbare.

How do starch synthase variations affect cooked rice quality?

Cooked rice of NILs was evaluated in the stickiness, softness, and overall preference with Nipponbare as the standard just after cooking (Fig. 3). NIL(*Wx*^{a}) with high amylose was less sticky and had hard texture and overall preference for Japanese testers were very low. These were similar to the evaluation of Kasalath. The natural variation in *SSI* between Nipponbare and Kasalath did not largely affect eating quality judging from the evaluation of cooked NIL(*SSI*^{k}). Interestingly, *Alk* variation clearly affected chain-length distribution of amylopectin and alkali disintegration of rice grain, however, eating quality of NIL(*Alk*) did not differ from those of Nipponbare.

We also conducted the evaluation after the cooked rice has been cooled at 5°C over night (Fig. 3). Compared to Nipponbare after cooling, Kasalath, NIL(*Wx*^{a}) and NIL(*SSI*^{k}) showed similar textures and preference to the results just after cooking. However, the results of NIL(*Alk*) greatly differed from those of just after cooking. Once cooled NIL(*Alk*) was less sticky and had harder texture, and overall preference was clearly decreased.

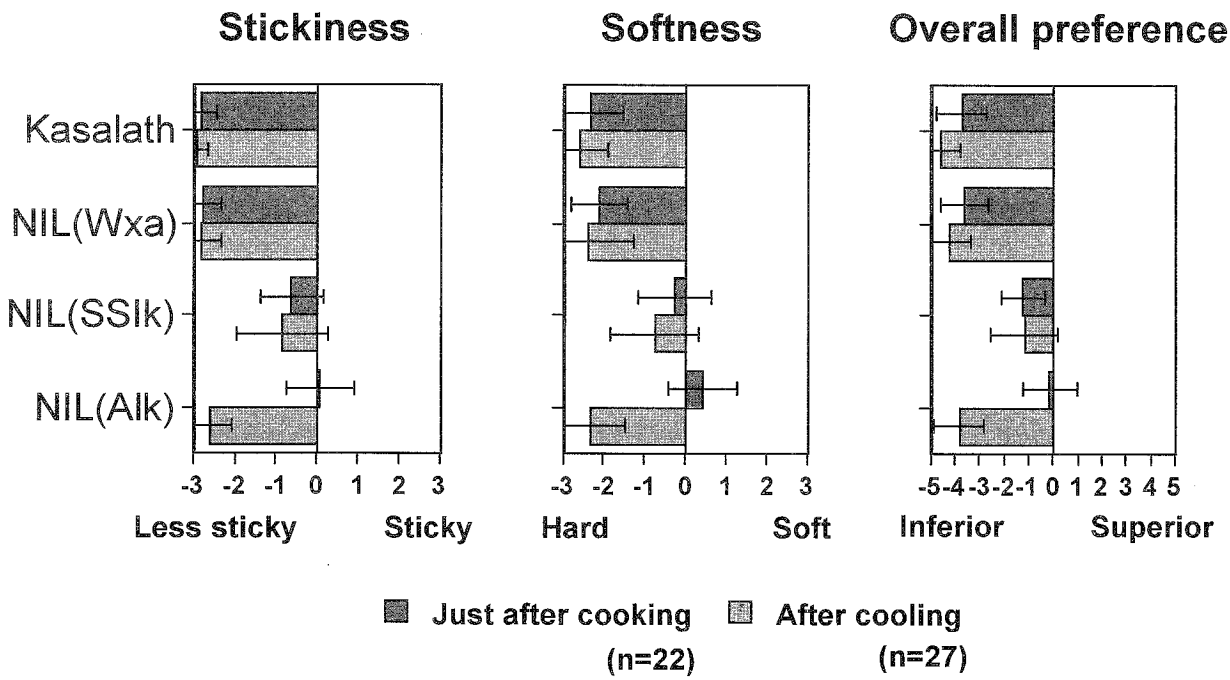


Figure 3. Sensory evaluation of NILs
Cooked NILs were evaluated with Nipponbare as the standard.

Functional analysis of *starch synthase IIa* (*SSIIa*, *Alk*)

It was reported that *SSIIa* is the gene responsible for the varietal differences in chain-length distribution of amylopectin and alkali disintegration of rice grain (Umemoto et al., 2002; Gao et al., 2003). SSIIa in the alkali tolerant cultivars has the function bind to starch granule *in vivo*, but SSIIa in alkali susceptible cultivars lacked this function even though SSIIa was produced in the endosperm (Umemoto et al., 2004).

Which specific variation in *SSIIa* gene is critical one for the enzyme and starch properties? We compared *SSIIa* sequences available at that time and found four single nucleotide polymorphisms predicting amino acid substitution (Umemoto and Aoki, 2005). These SNPs were genotyped with 60 rice cultivars world wide, and five haplotypes were found. We conducted an association study between the *SSIIa* haplotypes and the starch binding of SSIIa protein, short chain ratio of amylopectin, alkali spreading score, and pasting temperature of rice starch (Fig. 4). It was very clear that haplotypes 1, 2, 3a were functional haplotypes producing SSIIa protein that can bind to starch granule, had fewer short chains of amylopectin, more alkali tolerant, and had higher pasting temperature compared to nonfunctional haplotypes, haplotype 3b and 4. Then, which specific SNP is really important? Compared to haplotype 3a, haplotype 3b only differ at SNP4. Thus, SNP4 is critical one for the enzyme function and starch properties. Compared to haplotype 3a, hapotype 4 only differ SNP3. This shows that SNP3 is also a critical SNP. As a result, we identified two responsible SNPs for the enzyme function and starch properties. These SNPs in rice *SSIIa* are probably responsible for the eating and processing qualities of rice.

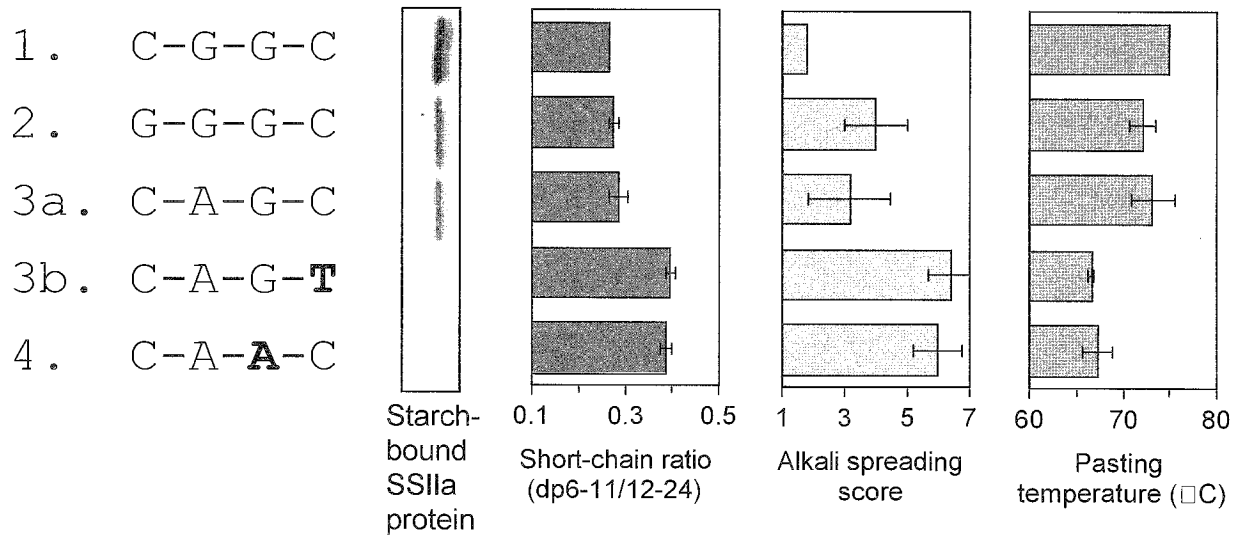


Figure 4. Association of the *SSIIa* haplotypes with the starch-binding of SSIIa protein, short chain ratio of amylopectin, alkali spreading score, and pasting temperature of rice starch.

Conclusion

Using NILs, we have confirmed the effects of natural variations in the three starch synthases on starch properties and cooked rice quality. The variation in *Wx* (*GBSSI*) gene (Wx^a and Wx^b) affected amylose content and cooked rice quality both just after cooking and after cooling. Effects of *SSI* variation between Nipponbare and Kasalath on starch and eating properties were not clear in this study. Currently, we are conducting precise evaluation of the *SSI* variation on rice quality using Koshihikari/Kasalath recombinant lines. Variation in *Alk* affected

the chain-length distribution of amylopectin and the eating quality of rice, but only after the cooked rice became cool. Okamoto et al. (2002) revealed that this variation in *SSIIa* also affected the hardening speed of rice cakes, one of the important characters in waxy rice processing. Finally, we have proposed two functional SNPs for SSIIa and starch properties, which in turn alter the eating and processing qualities of rice.

References

Gao Z, Zeng D, Cui X, Zhou Y, Yan M, Huang D, Li J, Qian Q (2003) Map-based cloning of the ALK gene, which controls the gelatinization temperature of rice. *Science in China* (Series C) **46,** 661-668.

Hirose T, Terao T (2004) A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa* L.). *Planta* **220**, 9-16.

Juliano BO (1998) Varietal impact on rice quality. *Cereal Foods World* **43**, 207-222.

Nakamura Y (2002) Towards a Better Understanding of the Metabolic System for Amylopectin Biosynthesis in Plants: Rice Endosperm as a Model Tissue. *Plant and Cell Physiology* **43**, 718-725.

Okamoto K, Kobayashi K, Hirasawa H, Umemoto T (2002) Structural differences in amylopectin affect waxy rice processing. *Plant Production Science* **5**, 45-50.

Umemoto T, Nakamura Y, Satoh H, Terashima K (1999) Differences in amylopectin structure between two rice varieties in relation to the effects of temperature during grain filling. *Starch* **51,** 58-62.

Umemoto T, Yano M, Satoh H, Shomura A, Nakamura Y (2002) Mapping of a gene responsible for the difference in amylopectin structure between japonica-type and indica-type rice varieties. *Theoretical and Applied Genetics* **104**, 1-8.

Umemoto T, Aoki N, Lin HX, Nakamura Y, Inouchi N, Sato YI, Yano M, Hirabayashi H, Maruyama S (2004) Natural variation in rice *starch synthase IIa* affects enzyme and starch properties. *Functional Plant Biology* **31,** 671-684.

Umemoto T, Aoki N (2005) Single-nucleotide polymorphisms in rice *starch synthase IIa* that alter starch gelatinisation and starch association of the enzyme. *Functional Plant Biology* **32,** 763-768.

Topic 2

Global and Regional Strategies for Conservation and Sustainable Use of Plant Genetic Resources

INTERNATIONAL PLATFORMS FOR SUSTAINED COLLABORATION IN PLANT GENETIC RESOURCES: PROSPECTS AND CHALLENGES

Percy E. Sajise
Regional Director
International Plant Genetic Resources Institute
Asia, Pacific and Oceania (APO)
P O Box 236, UPM Post Office
43400 Serdang, Selangor, Malaysia
Tel : 603-8942-3891
Fax : 603-8948-7655
p.sajise@cgiar.org

Brief Background

Plant genetic resources before the 1980's was considered as a common good and heritage of humankind and were exchanged freely. The International Undertaking on plant genetic resources (IU) of the Food and Agriculture Organization (FAO) which served as basis for this paradigm was adopted in 1983 (Hawtin 1999). During this same period, the application of Intellectual Property Protection (IPP) on plant genetic resources was also starting. For example, in 1961, seven countries in Europe signed an agreement to protect new plant varieties. This group became recognized as the Union for the Protection of New Varieties of Plants (UPOV). The UPOV agreement was revised in 1978 to allow each member state to legislate its own Plant Variety Protection Act (PVPA) that provides protection of new plant varieties that meet the requirements of novelty, distinctiveness, uniformity and stability.This was again revised in 1991 and received independent status from UPOV-1978 because many developing countries resisted changes made to the original agreement, demanding a continuation of UPOV-1978. In addition, there is the Treaty on Trade-Related Aspects of Intellectual Property Rights (TRIPS) under the purview of the General Agreement on Tariffs and Trade (GATT) which is the most comprensive, multilateral treaty which deals with types of IPR with the minor exceptions of breeders rights and utility models or petty patents. In the context of PGRFA, the most important clause of TRIPS is article 27.3(b) under which "any WTO member may exclude plants and animals other than micro-organisms and essential biological processes for the production of plants or animals other than non-biological and microbial processes" from patentability under TRIPS. However, "members shall provide for the protection of plant varieties either by patents or by an effective *sui generis* system or by any combination thereof "(Bragdon and Downes, 1998).

These conflicting IPR regimes on PGRFA reached a climax in 1993 when the Convention on Biological Diversity (CBD) came into force and Agenda 21 was put into place. Both the CBD and Agenda 21 stress the importance of developing and strengthening the capacity of countries to benefit fully from the biological resources available to them within their borders. They also promote access to these resources to others on mutually agreed terms. In return for gaining access to genetic resources, parties are required to provide access to new technologies, as well as training, information and financial resources, to enable developing countries to better conserve and use their biodiversity and strengthen their capacity to reduce hunger and poverty (Hawtin *et al* 2000). In the face of these conflicting international protocols and platforms on access and benefit appropriation on PGRFA which slowed down the needed exchanges of PGR materials, information and technology, FAO in 1994, initiated an intergovernmental negotiating

process to revise the IU of 1983 and to make it a legally binding agreement, harmonized with the provisions of the CBD.

The new and CBD-harmonized version of the IU took shape in the form of the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) which was ratified in June 2004. The Treaty involves a multilateral system to facilitate access to key genetic resources with minimal procedural and administrative costs. The multilateral system is intended to be efficient, effective and transparent. It aims to ease access not only to plant genetic resources for food and agriculture but also to information about those resources, and to share fairly and equitably any benefits that may arise from the use of these resources (Frison 2005). Those benefits go beyond the merely financial to include information exchange and access to and transfer of technology, as well as facilitated access to the genetic resources themselves. Under the benefit sharing mechanism, the "owners" of a commercialized product that incorporates materials from the multilateral system will pay a royalty into a specially designated fund. The payment is mandatory if the products are not available for further research and breeding, as a result of the application of measures to protect intellectual property. It is voluntary when the product can be freely used for breeding and research. While details of the level of royalties, the organization and governance of the fund, and disbursements from it have yet to be agreed, the Treaty clearly envisages that benefits will flow primarily to farmers in developing countries who conserve and use crop diversity (Frison 2005). Another funding support for the Treaty will be coming from the Global Crop Diversity Trust (GCDT) which is an independent global body set up to generate the funds in perpetuity to provide support to secure the most important ex situ collections of Annex 1 crops of the Treaty. The Establishment Agreement for the Trust was signed in early 2005 and it is now in the process of funding the development of crop and regional conservation strategies as well as selectively providing funds to some important *ex situ* collections which require urgent support in some parts of the world.

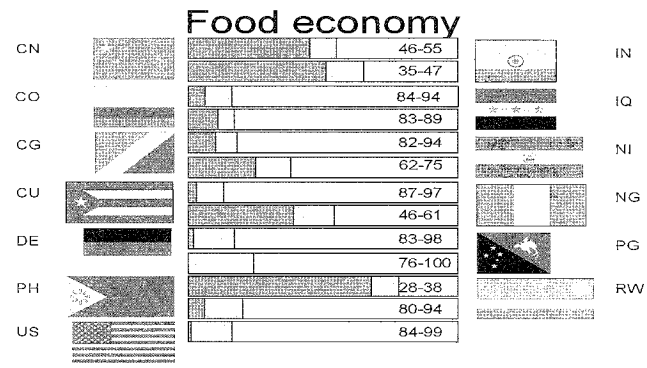
Another landmark platform on PGRFA is the Global Plan of Action (GPA) for the conservation and sustainable use of PGRFA which was adopted during the International Technical Conference on Plant Genetic Resources in Leipzig, Germany in June 1996. The objectives of the GPA are to ensure the conservation of PGRFA for food security, promote sustainable use of PGRFA and to promote a fair and equitable sharing of the benefits arising from the use of PGRFA. It comprises 20 priority activities and is based on the principle that only through the use of PGRFA can the social and economic benefits of the conservation of PGR be realized (Cooper *et al* 1998). Currently, a monitoring and evaluation system on the implementation of the GPA in some selected countries is going on through the development of an information sharing system on PGRFA. This will be implemented on a global basis in the coming months.

In summary, the international platforms for PGRFA has been oscillating from these resources being considered as a common heritage in the IU to a mixed platform of mixed property rights, i.e., protected to common. These changes in the property rights regimes for PGRFA has been primarily due to both scientific advancements in the field of molecular biology especially on how traits are transferred to the science of "biological engineering" as well as a greater recognition of the importance that biodiversity materials can provide in the face of dwindling natural resources. It has also been a result of declining public investments in this biological area of research and the increasing assumption of investments by the private sector.

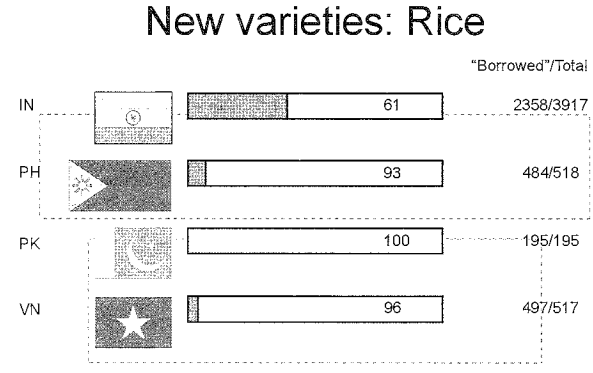
The Need for Sustained PGRFA Collaboration

The continuous evolution of living organisms and ecosystems, manifesting in one form as the existing biodiversity, is a basic phenomenon that provides the requirements of human existence for today and in the future. Inherent in this process is the exchange of plant genetic materials through natural or human-mediated processes, which allow not only the use of the genetic diversity but also its enhancement which in turn becomes the source of variability for crop improvement, as a natural defense mechanisms against genetic vulnerability and even providing a buffer for environmental risks (Council 1972; Brown 1983; Prescott-Allen and Prescott-Allen 1990). For example, in 1993, taro blight destroyed 95% of the taro crop in Samoa where it is a staple crop. The only reason for the recovery of the taro crop in Samoa is the availability of leaf blight resistant taro varieties from the Philippines and Palau which provided the genetic resources which enabled Samoa to recover the production of their staple crops. New threats are constantly emerging; among the newest is Asian soybean rust, which arrived in the United States in November 2004. Projected losses is around US 2B and it is already costing Brazil and China millions of dollars in fungicides and lost harvests (Frison 2005). Changes in global climate will require new materials for more adaptable species to biotic and abiotic stresses. Historical records are replete with similar examples that befall important crops illustrating this important rationale and emphasizing that PGR is a common heritage of humankind (Lebot 1992).

The global interdependence of countries in terms of crops for their food economy was reported by Palacios (1998). For example the following countries have been dependent on outside sources of genetic materials for their food crops; China 46% to 55%, Colombia, 84% to 94%, Republic of Congo, 82% to 94%, Cuba, 87% to 97%, Germanay, 83% to 98%, India, 35% ti 47%, Iraq, 83% to 89%, Nicaragua, 62% to 75%, Nigeria, 46% to 61%, Papua New Guinea, 76% to 100%, Philippines, 28% to 38%, Rwanda, 80% to 94% and the United States, 84% to 99%. The same pattern can be seen in the improvement of individual crop species. For example, with modern rice varieties released in 15 countries, the landraces used in breeding to produce the variety overwhelmingly comes from other countries (Frison 2005).



Food Economy Dependency of Countries on External PGR



In summary, the agriculture of today has been made possible through substantial exchanges of PGRFA in the past until now. It is also a fact that no single country is self-sufficient for its present and future needs of plant genetic resources in general. Additionally, centres of

diversity of plants and animals do not recognize country boundaries and important food crops and animals are already widely distributed. It is, therefore, important that the exchanges of genetic resources should continue in a more rational and acceptable way, through various international, national and local platforms to ensure food security while at the same time recognizing the creativity and innovativeness of stakeholders and the fair and equitable sharing of benefits for these stakeholders and users including the farmers and local communities who are custodians of these resources and knowledge system.

Challenges and Prospects of Current International Platforms

The Convention on Biological Diversity and Agenda 21

Article 3 of CBD recognizes the sovereign rights of nations over their biological resources. While the CBD recognizes these rights, it also places certain responsibilities and obligations on each party. The onus for conserving biodiversity is on the countries themselves. It recommends use of biological resources sustainably and the equitable sharing of benefits. Prior informed consent is a requirement in obtaining access to these materials and the knowledge and technology associated with them. Exchanges of PGRFA can take place under this platform but will be inefficient and less transparent compared with the provisions in the ITPGRFA. It can take place most practically on bilateral basis instead of a multilateral system of arrangements. Both the CBD and Agenda 21 stress the importance of developing and strengthening the capacity of countries to benefit fully from the biological resources available to them within their borders. CBD offers perpetual protection to the country's biological resources not covered by other international treaties which exempts some species otherwise.

Trade-Related Property Protection Platforms

UPOV 1978 and 1991 as a platform offers definite periods of protection to products of formal breeders and has both full exemption (1978 UPOV) and limited exemption (1991 UPOV) to farmers right. It give strong protection and IPR's over improved PGRFA to formal breeders.

TRIPS, on the other hand, both under the Utility Patent Act and *sui generis* system, provide strong IPR protection to formal breeders and their products of improved PGRFA. It has either conflicting or opposite provisions as well as options for maneuvering with regards to informal farmer breeders as well as recognizing community and individuals ownership over local PGRFA.

Overall, these platforms provides protection and incentives to formal breeders and others who produce novel and commerciable plant varieties but have been criticized for monopoly control, lack of recognition of materials produced by farmers and communities over generations and its effect in hindering exchanges of PGRFA for agricultural development in general and greater marginalization of low input farmers in particular.

The International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) and the Global Plan of Action (GPA)

The ITPGRFA aims to both simplify the procedures for access to and exchange of genetic resources, and to ensure that benefits that may accrue from the use of such resources is equitably shared through an original multilateral mechanism. The objectives of the Treaty parallels those of the CBD. However, while CBD deals with biodiversity in general, the Treaty

applies only to "plant genetic resources for food and agriculture" which is specified in Annex 1 of the Treaty. The Treaty, through the Material Transfer Agreement (MTA), governs access and binds recipients including the in trust collection of almost 600,000 accessions of the Future Harvest Centres. The ITPGRFA recognizes the most important food and forage crops as a common heritage which should be exchanged among countries through an agreed arrangement, the MTA, and just like the CBD, its period of protection is perpetual. It offers exemptions to Farmer's and Breeder's varieties and specifies benefit sharing and assurance for access to new technology.

Challenges and Prospects

Today, there exist several international platforms of cooperation which provides a range of property regimes to PGRFA from being a common heritage to a strictly property protected regimes (Figure 1). What is needed is to be able to balance the need for public goods, especially for the most important and basic food and human welfare necessity crops and plant genetic resources and the protection of private rights. The combinations of these platforms must be able to balance the rights and recognition to rights of various stakeholders especially the guardians and stewards of these genetic resources, i.e., plant breeders, farmer-breeders, traditional knowledge holders and others. At this point in time, these platforms of cooperation has not even defined what constitute distinctively farmer's rights. There are even fewer countries which have attempted to define and implement these farmer's rights. The biggest challenge of all for the global society is to swing the pendulum in favor of granting more easier access on a fair and equitable manner to more crops and plant genetic resource needed for basic human needs for food and health security as well as ensuring a functional ecological systems to provide the needed common services to ensure human welfare such as clean air, water and landscapes/seascapes (Figure 2).

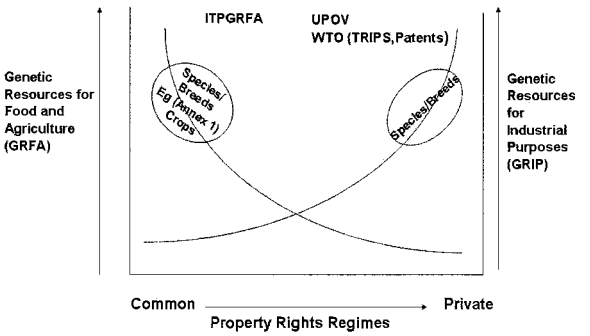


Figure 1. Current international platforms in terms of species and spectrum of property rights regimes

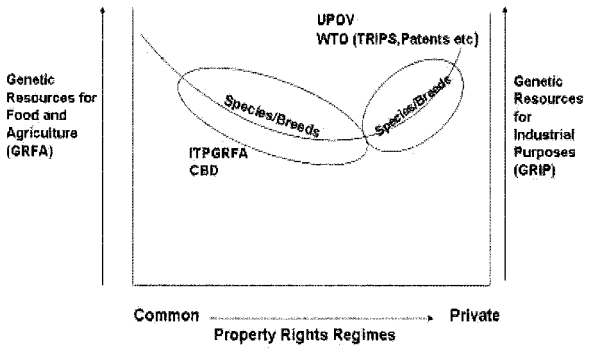


Figure 2. Ideal international platforms in terms of species and spectrum of property rights regimes

This would mean expanding the list of species covered by the ITPGRFA while cautiously "opening up" the provisions of the CBD on what species can be delegated to the Treaty. This will also mean that the parties to the Treaty must quickly resolve the arrangements for the MTA and the mechanisms for benefit sharing which are at the heart of the Treaty. The parties to the Treaty must also resolve the scope of article 12.3d "in the form received from the multilateral system". As of now if it is interpreted to exclude the genes and materials of the genes in the materials received. In this context, the door to an open access to the most important crops listed in Annex 1 of the Treaty can be closed as this can be interpreted that genes and materials

derived from the modification can be patented even if it is with crops belonging to Annex 1 of the Treaty received in genebanks.

Conclusion

Biodiversity, particularly plant diversity for food and agriculture, still offer one of the best ways of meeting present and future needs of humankind for basic food and nutritional security as well as ecosystem services. Diversity within and among plant species remains perhaps as our best insurance that we will be able to continue to meet these basic needs in the future. As we are continously losing these valuable plant diversity, there is an urgent need to conserve them but conserving them alone is nothing without our ability to use it. A food secure future will be necessary for the attainment of global peace and security.

A food secure future for all demands not only that the world's plant heritage is accessible to all but that all have an equal abiity to use and benefit from it. Global platforms by which these basic principles will be allowed to be nurtured and applied is needed and will probably be a combination of what international platforms already exist but should evolve more in terms of specific group of crop species and a set of more effective, efficient and transparent multi-lateral systems of access and benefit sharing.

References

Brown, W.L. 1983. Genetic diversity and genetic vulnerability- An appraisal. Economic Botany 37,4-12.

Council, National Research. 1972. Genetic vulnerability of major crops. National Academy of Sciences. Washington D.C. 1996. Report on the State of the World's Plant Genetic Resources- International Technical Conference on Plant Genetic Resources, Leipzig, Germany, 17-23 June 1996. FAO, Rome. pp 56.

Cooper, H.D., C. Spillane, I. Kermali and N. Murthi Anishetty. 1998. Harnessing plant genetic resources for sustainable agriculture, PGR Newsletter 114:1-8.

Frison, E. 2005. Agricultural biodiversity and sustainability of agriculture. Paper presented during the International Symposium on "Human Centered Sustainable Development" to commemorate 80th birth anniversary of MS Swaminathan, Chennai, India, 7 August 2005.

Hawtin, G. 1999. Management of plant genetic resources in the CGIAR: problems, prospects and quest for equity. Paper presented to the World Trade forum, 27-28 August 1999, Berne, Switzerland.

Hawtin, G., P.E. Sajise and V. Ramanatha Rao. 2000. The conservation and use of plant genetic resources for a food secure future. Introductory address to the International Symposium on Oil Palm Genetic Resources and Utilization, 8 June 2000, Hilton, KL, Malaysia.

Lebot, V. 1992. Genetic vulnerability of Oceania's traditional crops. Experimental Agriculture 28:309-323.

Palacio, XF. 1998. Contribution to the estimation of countries' interdependence in the area of plant genetic resources. Rep. 7, Rev.1, UN Food and Agriculture Organizaing Committee on Resources, Food and Agriculture. Rome, Italy.

Prescoot-Allen, R. and C. Prescott-Allen. 1990. How many plants feed the world. Conservation Biology 4:365-373.

GENOMIC-BASED CROP GERMPLASM RESEARCH

Jizeng Jia[1] and Qifa Zhang[2]

1. Key Lab of Crop Germplasm & Biothechnology, MOA, China; Institute of Crop Sciences, CAAS, Beijing, 100081, China
2. National Key Lab of Crop Genetic Improvement; Huazhong Agricultural University, Wuhan, China

Abstract

Plant genomics is one of the fields in which new developments emerge in endlessly. Genomics-based germplasm research, known as"Genoplasmics", is a new intersecting discipline resulting from the hybridization between plant genomics and germplasm research. Genoplasmics refers to germplasm research at the global genomic level, which include the five topics: (1) genoplasmics platform; (2) studies on genetic diversity and core collections; (3) gene discovery in germplasm resources; (4) gene cloning; (5) germplasm enhancement. The rise and development of genoplasmics will promote germplasm research to a new stage through determining structures and functions of genes carried in germplasm and making"germplasm banks" worthy of the name of "gene banks".These endeavors will greatly accelerate the establishment of foundations for agriculture and other related industries.

A technology strategy of genomics-based germplasm research has been development, in which, the groundwork is the core collection, the approach is to develop genomic stocks, and the key target is gene discuvery. This strategy combined crop genomics, germplasm and crop breeding together. Following this research route, we have obtained primary achievements:

1) Established core collection and mini-core collections of rice, wheat and soybean, and investigated genetic diversity of germplasm resources for above three crops;
2) Established a new strategy for genomic stock development, germplasm enhancement and breeding. By using the strategy and the core collection, hundreds of recombination (RIL), thousands of near isogenic lines (NIL), introgression lines (IL) were generated. These materials have become important germplasm resources in the new era. Some of them have been used as breeding parents, even as new varieties to be released. The results proved that this strategy is a efficient strategy to combine genomics, germplasm research and plant breeding.
3) Based on the above mapping population, hundreds of novel genes/QTLs have been discovered by using genetic mapping approach.
4) More than forty agronomic important genes have been cloned by using map-based cloning and comparative genomics. Detection of natural allele variation is on the way.

The above results have proved that this strategy is a high efficient to germplasm research. We anticipate that there will be great achievement in the future.

Corresponding author: Jizeng Jia, Key Laboratory of Crop Germplasm & Biotechnology, Institute of Crop Sciences, CAAS, Beijing, 100081, Chine, jzjia@mail.caas.net.cn

GENETIC DISSECTION OF RESISTANCE TO RICE BLAST FOR RICE GERMPLASM ENHANCEMENT

Shuichi Fukuoka[1], Norikuni Saka[2], Satoru Kudo[2], Takehiko Shimizu[3], Masahiro Yano, [1] Kazutoshi Okuno[1]

1 National Institute of Agrobiological Science (NIAS) Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602 Japan

2 Aichi Agricultural Research Center (AARC), Mountainous Region Agricultural Research Institute, Inabu, Toyota, Aichi 441-2513, Japan

3. Institute of Society for Techno-Innovation of Agriculture, Forestry and Fisheries(STAFF), Ippaizuka, Kamiyokoba ,Tsukuba, Ibaraki 305-0854 , Japan

*Corresponding author: fukusan@affrc.go.jp (Tel: +81-29-838-7458)

Introduction

Rice has a great diversity that is recognized as morphological and physiological traits. Beneficial traits found in germplasms have been introduced into desirable genetic background through cross breeding. Since most agronomic traits are controlled by multiple loci, genetic improvement is time consuming especially when introducing the traits from exotic germplasms. High-density genetic linkage map and physical map are powerful tools for targeting genes for agronomic traits. DNA marker-based analysis makes us possible to determine genotype at each locus that was difficult in conventional method. Recently, complete genome sequence information of Nipponbare, gene annotation database and cDNA libraries have been developed as the tools that will accelerate the genetic studies for practical breeding as well as establishing its connection with basic science. These rice genome resources have broadened the use of the rice gene pool.

Rice blast is a destructive disease of rice worldwide and improvement of resistance to blast is an important breeding objective. Two types of resistance to blast, complete resistance and field (partial) resistance, have been described in rice (Ezuka 1972). Complete resistance induces a hypersensitive reaction and is characterized by a resistant infection type. More than 20 loci for complete blast resistance have been identified in rice (McCouch et al. 1994). In spite of their significant effect against rice blast, complete resistance genes have been overcome by compatible races of the pathogen several years after the resistant varieties with this type of resistance were released. Field resistance is usually incomplete and characterized by a susceptible infection type, although it does limit the proportion of diseased leaf area. Field resistance is considered to be durable, but the genetic basis and mechanism is not fully understood. Present study was initiated to understand genetic control of field resistance of the germplasm containing high level of field resistance for enhancement.

Detection of chromosomal regions conferring field resistance to rice blast

Japanese upland rice is in the primary gene pool of Asian cultivated rice, being used as a source of the genes for field resistance to rice blast. Previous studies have suggested that field resistance in Japanese upland rice is under complex genetic control (Higashi and Saito 1985), and conventional genetic analysis did not allow researchers to identify the genes and their chromosomal locations for field resistance. Major part of the resistance in Japanese upland rice was not used in irrigated rice varieties, possibly due to the close linkage between the gene for field resistance and certain undesirable characteristics. Quantitative trait loci (QTL) analysis

using DNA markers allows for improved understanding of the genetics of the traits controlled by multiple genes (Tanksley 1993). To determine the chromosomal regions involved in field resistance to blast in Japanese upland rice, QTL analysis was carried out using 146 F_4 lines of a cross between Japanese irrigated rice cultivar Nipponbare and the upland cultivar Owarihatamochi (Fukuoka and Okuno 2001). Nipponbare and Owarihatamochi have no gene for complete resistance to rice blast, based on the screening using Japanese differential blast race. Nipponbare has a lower level of field resistance than does Owarihatamochi. We assessed field resistance in F_4 lines based on the diseased leaf area (DLA). of 40- to 50-day-old plant, scored using a rating from 0 (highly resistant) to 10 (highly susceptible). The scores for Owarihatamochi and Nipponbare were 2.8 and 8.0, respectively. The frequency distribution of the field resistance score in the F_4 lines was continuous and ranged from 1 to 10. We used 111 restriction fragment length polymorphism (RFLP) and 5 single sequence repeat (SSR) marker loci to detect QTLs by using software MAPMAKER/QTL ver. 1.0 in a logarithm of the odds (LOD) threshold of 2.0. The analysis revealed four QTLs located on three chromosomes (Figure 1). The resistant allele on chromosome 9 is derived from Nipponbare, while the resistant alleles on chromosomes 4 and 12 come from Owarihatamochi. The two QTLs on chromosome 4, close to RFLP marker loci *G271* (*pi21*) and *G177* (qBR4-2), explained 45.7% and 29.4% of the phenotypic variation, respectively, while the QTLs on chromosome 9 (*qBR9-1*) and 12 (*qBR12-1*) explained 7.9% and 13.7% of the total phenotypic variation, respectively. All together, the four QTLs explained 66.3% of the total phenotypic variation.

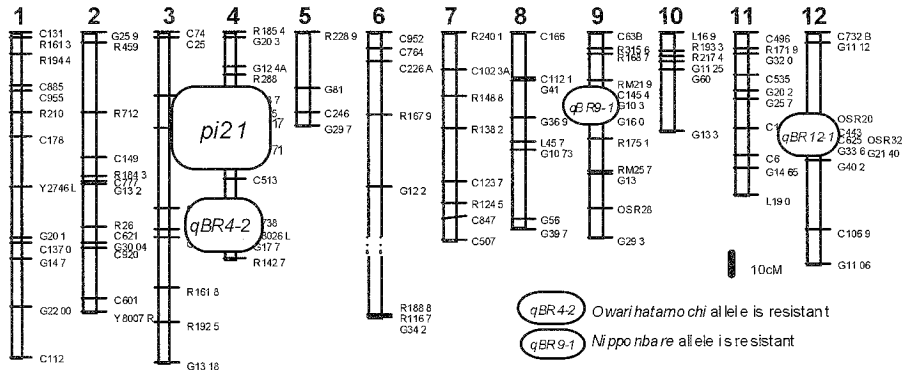


Figure 1. Location of the QTLs for field resistance to rice blast in F_4 lines of the cross between irrigated rice Nipponbare and upland rice Owarihatamochi.

Development of backcrossed lines to confirm the effect of putative QTLs

One strategy for analyzing QTL as single Mendelian factors and for characterizing each QTL is the development of backcrossed progeny lines for each QTL by marker-assisted selection (Yano and Sasaki, 1997). To confirm the effect of putative QTLs, backcrossed progeny lines were developed from three F_3 plants identified as having resistant alleles from upland rice based on the result of QTL analysis. Aichiasahi, a highly susceptible cultivar, was used as the recurrent parent. The same set of DNA markers in QTL analysis was used to select relevant chromosomal regions. During backcross and selection the proportion of upland rice chromosomes in these plants decreased to less than 6% based on the genotypes of DNA markers. We selected three BC_2F_1

plants that contain just one out of three blast resistance alleles from Owarihatamochi. The field resistance was assessed in 98, 44 and 46 BC2F3 lines for *pi21*, *qBR4-2* and *qBR12-1*, respectively. We compared the levels of field resistance among the lines with Owarihatamochi-homozygous alleles and Aichiasahi-homozygous alleles. Genotypes at each QTL were estimated based on the genotype of DNA markers around QTLs.The average DLA of the lines with the Owarihatamochi allele was significantly higher than those with the Aichiasahi alleles at all three QTLs (Figure 2). The difference between two genotypes was largest at the QTL on chromosome 4 and smallest at the QTL on chromosome 12, in good accordance with the result of QTL analysis.

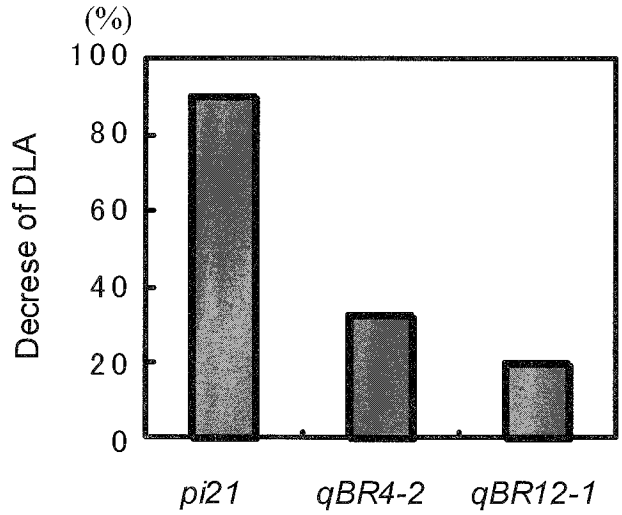


Figure 2. Proportion of the decrease of the diseased leaf area in the plants with resistant allele compared with those with susceptible alleles at the three QTL for blast field resistance. The ratio from the average DLA of the lines with the Owarihatamochi allele to those with the Aichiasahi alleles is indicated.

Map-based cloning of field resistance gene, *pi21*

Map-based cloning of target genes is one of the strategies for identifying gene. As a preliminary survey, 82 BC_1F_3 lines in which *pi21* was segregated were subjected to genetic linkage analysis to determine the map location of *pi21*. The genotypes at the *pi21* were readily determined based on the difference in diseased leaf area and its segregation among plants within a line. A resistance gene for this QTL, was mapped as a single recessive loci between RFLP marker loci *G271* and *G317* at a distance of 5.0 cM and 8.5 cM, respectively (Fukuoka and Okuno 2001). Further analysis using expressed sequence tag (EST) clones developed by the RGP (Rice Genome Research Program, Japan) identified RFLP marker locus *RA3591* between RFLP marker locus *G271* and *pi21*, at a distance of 0.6 cM to *pi21*. To develop DNA markers around the *pi21* locus, P1 artificial chromosome (PAC) clones were screened from a Nipponbare PAC library constructed by the RGP using the sequence-tagged-site (STS) for *RA3591* and *C975*, which is proximal to *G317*. A polymorphism survey of twelve PAC-end fragments or subclones by RFLP and SSCP (single strand conformation polymorphism) analysis identified eleven DNA

markers around the *pi21* locus. We developed a mapping population consisting of 1014 lines for fine genetic mapping of *pi21* and selected 182 recombinants between RFLP marker locus RA3591 and G317 for a field resistance test to determine genotypes at the *pi21* locus. Linkage analysis revealed that *pi21* is located between the two RFLP marker loci *14T1* and *13S1*. The analysis narrowed downed the *pi21* region as 29kb where six genes are predicted in the annotation database. Further genetic analysis using the mapping population consisting 2703 plants successfully identified one predicted gene as candidate gene for *pi21*. The sequence of *pi21* gene does not have homology with genes of known function but had partial homology with the genes in sugarcane and sorghum. These observations imply that field resistance gene *pi21* is a novel type of resistance gene. To characterize field resistance, histological study and expression analysis were carried out by using near-isogenic line for *pi21*. The response of the plant cell against fungus invasion was different from that in hypersensitive reaction. The expression of *pi21* gene is constitutive and the levels of expression did not change after fungus inoculation in RT-PCR analysis. These results provide useful information for the mechanism of the field resistance.

Conclusion

Genetic analysis using DNA markers efficiently identified the chromosomal regions associated with beneficial trait under complicated genetic control. The genetic analysis using backcrossed lines are useful to limit the regions for QTLs and identify DNA markers for their selection. Characterization and evaluation of these lines allow us to collect information to eliminate undesirable characteristics as well as for understanding the mechanism of trait expression. Map-based cloning of the gene for beneficial traits has a great impact on not only understanding the gene function but also allowing us to screen allelic variation,, enhancing the use of the wide range of germplasm. Selecting the lines with beneficial traits under complicated genetic control is difficult by conventional rice breeding. After selecting the lines with each QTL in desirable genetic background, combining them in one plant is the strategy to enhance rice germplasm. Rice genome resources will accelerate to discover novel and beneficial genes and contribute to efficient use of rice gene pool.

Reference

Ezuka A (1972) Field resistance of rice varieties to rice blast disease. Rev Plant Prot Res 5: 1-21

Fukuoka S and K Okuno (2001) QTL analysis and mapping of *pi21*, a recessive gene for field resistance to rice blast in Japanese upland rice. Theor Appl Genet. 103: 185-190

Higashi T, Saito S. 1985. Linkage group of field resistance genes upland rice variety Sensho to leaf blast caused by Pyricularia oryzae CAV. Japan J Breed 35 : 438-448

McCouch SR, Nelson RJ, Tohme J, Zeigler RS (1994) Mapping of blast resistance gene in rice. In Rice Blast Disease (edt by Zeigler RS, Leong SA, Teng PS) pp167-186, IRRI, Manila

Tanksley SD 1993 Mapping polygenes. Annual Rev Genet 27:205-233

Yano M and Sasaki T. 1997. Genetic and molecular dissection of quantitative traits in rice. Plant Mol Biol 35: 145-153

GENOMICS AND BIOINFORMATICS APPROACHES FOR EFFCTIVE UTILIZATION AND ENHANCEMENT OF PLANT GENETIC RESOURCES IN RDA-NIAB GENEBANK

Yong-Jin Park[1,2*], V. Ramanatha Rao[2], Jae-Gyun Kwag[1], Tae-San Kim[1]
[1] National Institute of Agricultural Biotechnology, Suwon 441-707, R. of Korea
[2] IPGRI-APO, PO Box 236, UPM Post Office, Serdang 43400, Malaysia
*Corresponding author: y.park@cgiar.org (Tel: +603 8942 3891)

Abstract

With the efforts of International Rice Genome Sequencing Project (IRGSP), complete sequence information of whole genome of *Oryza sativa* L cv. Nippon bare is publically available now, which can be exploited for allele mining of diverse germplasm to identify useful variants for many important traits such as resistance to biotic stresses, tolerance of abiotic stresses, enhanced yield and improved quality. Identification of allelic series of variants of such genes and their association with phenotypic or biochemical characters will provide a potential tool for crop improvement. The database of DNA stocks and profiles covers 15 crops and 19,000 accessions such as 7,500 rice, 5,000 soybean, 600 barley, 500 wheat, 700 mungbean, 600 apple, 500 *Vigna* species, 500 sesame, 500 perilla, 500 Italian millet, 500 buckwheat, 500 pear, 400 maize, 400 red pepper and 300 Job's tears accessions. The DNA stock along with tissue bank provides the base for developing their DNA profiles and molecular characterization in the RDA gene bank. For DNA profiling of under-utilized crops (UUCs) such as sesame, perilla, Job's tears, buckwheat, amaranth, finger millet, mungbean and Chinese cabbage, around 30-50 marker sets of simple sequence repeats (SSR) have been developed for each crop by the RDA gene bank. This marker development work is going to be extended to other UUCs including fruit species such as zinger, several millets, *Zoysia*, *Allium*, persimmon, Korean plum, *Citrus* etc. A web-based software has also been developed in order to provide DNA profile information with passport and characterization data for germplasm. It includes several functions such as facility to calculate genetic distances using standard algorithms, generating matrix tables of target accessions and clustering analysis using the DNA profile data. This DNA profile database has potential uses in identification of duplicate accessions and for management of large germplasm collections. Using genomic and bioinformatics tools, the RDAGB attempted a case study on gene mining of useful traits from the rice accessions of the RDA gene bank. We are targeting the genes involved to grain quality of rice for association analysis between phenotypic and allele variation, more focused on Korea originated landrace and weedy collection. For the association analysis between alleles and amylose contents in 394 accessions of Korean landrace using the combination of alleles of microsatellite markers within the genic regions of GBSS (Granule-Bound Starch Synthase), SBE (Starch Branching Enzyme) and SSS (Soluble Starch Synthase) was sought with the other physiochemical properties including amylopectin composition. In addition, the association analysis by highly saturated DNA profiling with microsatellite and SNP markers selected and designed from the 82 genes associated to embryo & endosperm development, screened from the various database of rice genome including TIGR and NCBI, is undergoing for increasing germplasm uses.

Key words

Gene bank, high-throughput genotyping, SSR markers, core collection, allele mining

I. Introduction

Plant genetic resources of agriculture include landraces, wild species, bred lines & varieties and their wild relatives. The release of new varieties and changing to monoculture of cropping system led drastic replacement of landraces. Rapid industrialization and expansion of civil areas also destroyed habitats of wild species and landraces of many crops. Therefore, there is an urgent need to collect and conserve landraces and wild species together with other important artificial lines and varieties for future uses in plant breeding including bio-industries. The RDA (Rural Development Administration) gene bank has 151,287 seed accessions of 1,939 species or sub-species. About 30,000 accessions are landrace of diverse crops collected from local farms or farmers' houses before 1986. As for the vegetatively propagating crops, the gene bank has 22,625 accessions of 996 species on the fields of 15 different research stations or institutes.

With the availability of genomic information and new tools of biotechnology, the development of DNA stocks of seed & clone germplasm is needed for their characterization using DNA markers and gene-based uses of germplasm (Ortiz 1998; Uyoh et al. 2003). The information of each accession at DNA level may be

compounded with the phenotype data. Recently, the RDA gene bank has started to develop the DNA bank system and took molecular characterization for diversity assessment, development of core collection, allele mining of useful characters and gene discovery within germplasm collections. These biotechnological approaches will provide a better management and uses of germplasm.

II. A new approach for developing core sets with maximized genetic diversity and minimized redundancy using a heuristic algorithm in rice (*Oryza sativa* L.)

Since the development of a concept for core collection by Frankel (1984), many strategies for sampling and developing method of core set using cluster analysis have been studied. But skew population and unequal diversity among accessions makes the difficulty in constructing diverse and representative core collection. To facilitate the management and to analyze the genetic resources in RDA gene bank, the method for maximizing genetic diversity and minimizing genetic redundancy had been studied. As a result, an advanced M strategy, that uses admissible heuristic algorithm, was designed. In brief, after designing the classified matrix according to the descriptions of the characters, each accession was stacked into matrix by means of evaluation of A* algorithm. Admissible heuristic algorithm evaluates the path at each node by the sum of the node number and the corresponding value to be filled with the expected accession.

168 accessions were selected from 10,368 characterized accessions using 11 qualitative and 27 quantitative characters by this algorithm. This core set showed 22% higher genetic diversity value (the results of Shannon-Weaver Diversity Index value) than that of the initial collection due to the diverse constitutes with minimum accessions. Also, the heuristic core set showed the highest diversity in comparison with core sets developed by using clustering analysis and different sampling strategies. There was significant difference in coincidence rate (CR %) between the entire collection and core sets developed by the method of clustering analysis. But, heuristic core set showed 100% CR value, which represented a full retention of the entire collection's characters. But, even distribution of characters at heuristic core set resulted in the difference from the entire collection by t-test and $\chi 2$-test.

These results showed that Advanced M strategy designed by heuristic algorithm provides the efficient solution for reducing the redundancy and effective representing character of the collection with small number of core set. Especially, since enormous SSR DNA profile data in various crops was accumulated in RDA database, Advanced M strategy will be useful to develop the core set. Although it remains to study the further adaptation of various data like genotyping data, the advanced M strategy of designing admissible heuristic method might be an efficient way to retain the genetic diversity and reducing the redundancy of the entire collection. And if combined with cluster analysis, it maybe offers a good explanation of characteristics of resources.

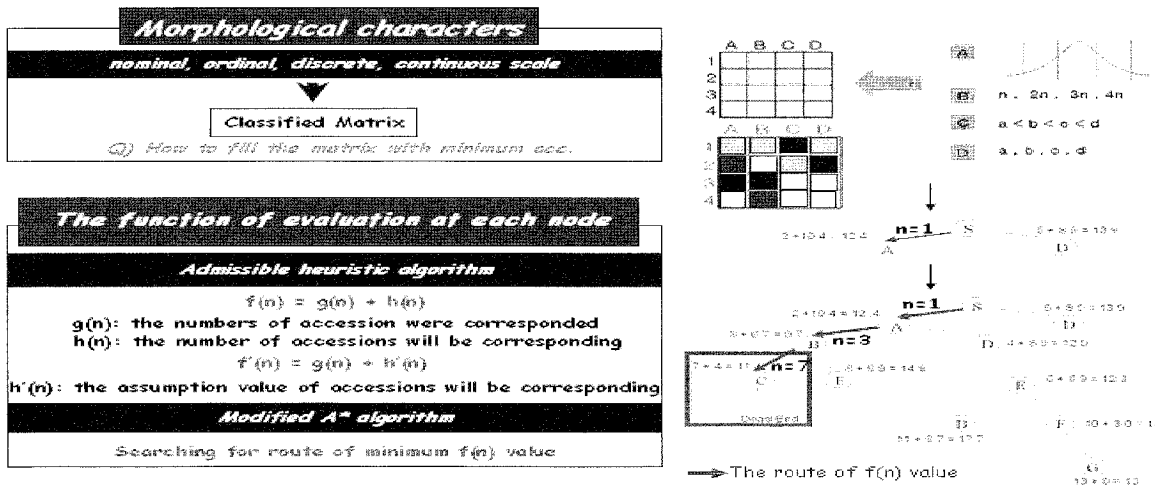
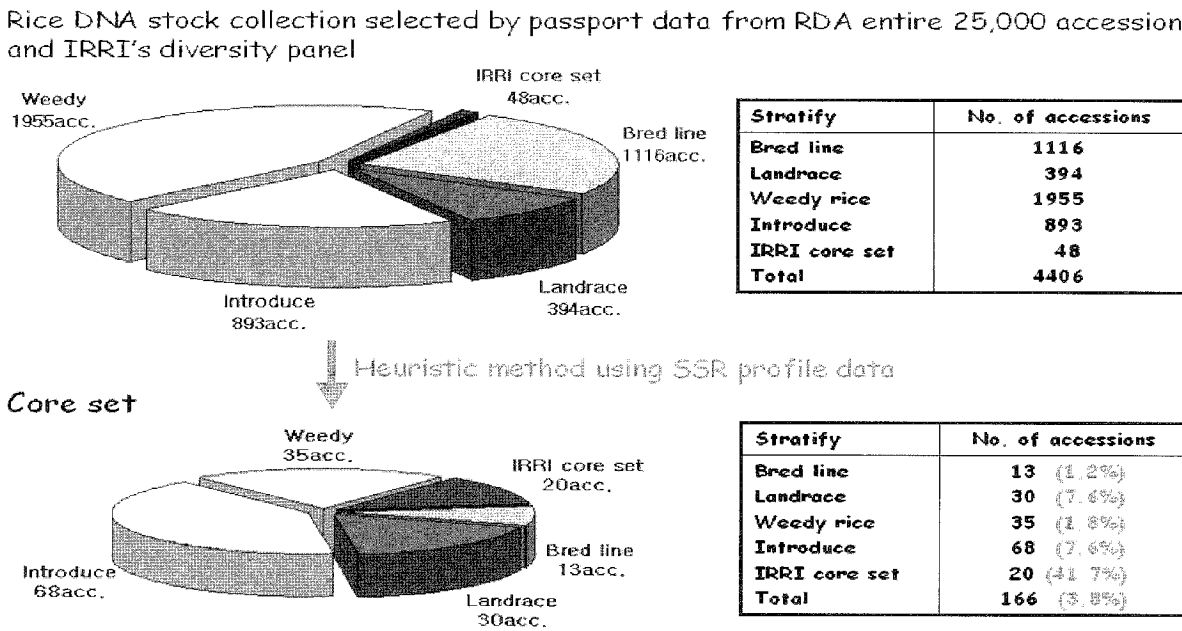


Fig 1. Diagrammatic illustration for development of heuristic program to select the entries of core collection.



Stratify	No. of accessions
Bred line	1116
Landrace	394
Weedy rice	1955
Introduce	893
IRRI core set	48
Total	4406

Stratify	No. of accessions
Bred line	13 (1.2%)
Landrace	30 (7.6%)
Weedy rice	35 (1.8%)
Introduce	68 (7.6%)
IRRI core set	20 (41.7%)
Total	166 (3.8%)

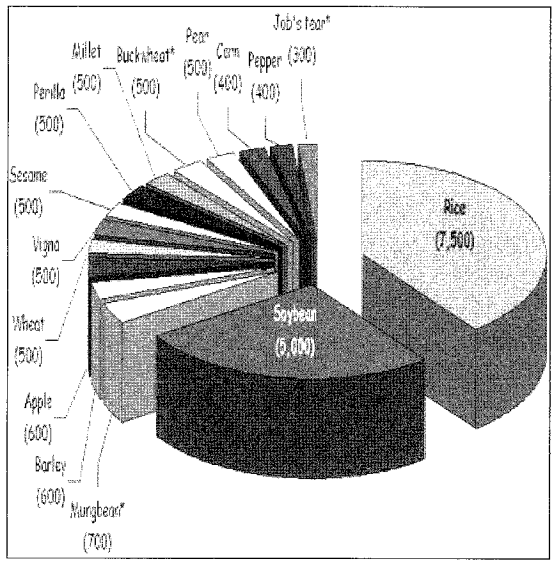
Fig 2. Status of accession composition of rice core collection of rice developed using SSR profile data and newly developed heuristic program by the RDA genebank.

III. High-throughput DNA profiling of Gene bank accession and development of SSR markers on neglected/under-utilized crop germplasm

The database of DNA stocks and profiles covers 19,000 accessions of 15 crops. The crops species, which are currently being subjected to the DNA profiling are, rice, soybean, barley, wheat, maize, cowpea, sesame, perilla, sorghum, mungbean, buckwheat, Italian millet, pear, apple, persimmon, red pepper, *Brassica* spp. and *Allium* spp. For providing DNA profile information on the web-site, the RDA gene bank developed a new web-based program to analyze genetic similarity and clustering of accessions, which are based on DNA profile data. The digital data of DNA profiles can be visualized by their fragment size like gel image with different colors by primer sets.

Realizing the importance of many under-utilized crops for their use as significant complements to the major cereals, we have initiated a program to evaluate levels of genetic diversity within germplasm collections of many UUCs by developing new microsatellite markers. In this regard we have developed around 30-50 microsatellite marker sets for eight different UUCs namely, *Amaranthus, Brassica,* fingermillet, Job's tears, mungbean, *Perilla,*radish and sesame.

Screening of microsatellites from huge sequence data is very laborious and time consuming. Several microsatellites finding programs were already developed, but most of them has no function for screening interrupted repeat units or inefficient and inconvenient to use. To overcome these problems, a new program 'ARGOS' was designed for detecting microsatellites including interrupted or split SSRs in cloned sequences and can also design primers from the flanking sequences of repeat units for supporting SSR marker development program.



Crop	Method	No. of using marker	Ave. of Diversity Index (%)	Number of accessions
Rice	SSR	18	71%	7500
Soybean	SSR	9	75%	5000
Barley	SSR	6	53%	600
Apple	SSR	6	74%	600
Wheat	SSR	9	74%	500
Vigna	SSR	6	71%	500
Sesame	SSR	19	56%	500
Perilla	SSR	9	42%	500
Millet	SSR	6	82%	500
Pear	SSR	6	86%	500
Corn	SSR	12	61%	400
Pepper	SSR	6	43%	400
Mungbean	AFLP	6[a]	95.8%*	700
Job's tears	AFLP	10[a]	95.8%*	300
Buckwheat	AFLP	7[a]	37.2%*	500

( ): number of accessions

a : Number of AFLP Primer combination
* : Percent of polymorphic fragment

Fig 3. The current status of high-throughput DNA profiling work carried out at RDA-NIAB gene bank. So far, total 19,000 accessions of main as well as under-utilized crops were analyzed with DNA markers (SSRs & AFLPs).

IV. Allele mining and gene discovery of rice collection

The association analysis of rice germplasm using microsatellite markers associated with starch-synthesizing genes of GBSS, SBE and SSS was successfully applied for characterization of grain quality in rice (Bao et al. 2002). This analysis was conducted with 394 Korean landraces. The original type of allele combination in 3 genes is deduced as $(CT)_{18}$ in GBSS, sbe c in SBE and sss c in SSS. The various recombinations on these genes had been studied regarding changes in amylose contents and ADV (Akali Digestibility Values). The association analysis on these genes is undergoing with the 1,955 rice accessions including Korean weedy collection and selected world collection. The association analysis using microsatellite markers will be extended with 100 SSR makers within 2005 and with expected 500 SSR markers in 2006. The selection based on grain quality and disease resistances for blast and bacterial blight is sought for the Korean originated accessions through core collection strategy.

According to amylose contents in related to microsatellite in Landrace rice

waxy	G/T	gbss	sbe	sss	NO.	Amylose	ADV
		(CT)8	sbe c	sss a	1	24.4	4.0
		(CT)8	sbe a	sss a	2	30.4	2.8
		(CT)11	sbe a	sss b	1	30.1	5.2
	G	(CT)11	sbe a	sss a	1	23.0	4.8
		(CT)16	sbe c	sss c	3	24.1	3.7
		(CT)17	sbe c	sss c	10	25.5	4.7
		(CT)18	sbe c	sss c	2	22.7	6.1
non		(CT)19	sbe c	sss c	3	23.2	5.4
waxy		(CT)19	sbe b	sss c	2	23.9	5.0
		(CT)19	sbe a	sss c	2	22.0	7.0
		(CT)17	sbe c	sss c	59	19.1	6.2
		(CT)17	sbe b	sss c	9	17.7	6.6
	T	(CT)17	sbe a	sss c	3	17.7	6.9
		(CT)18	sbe c	sss c	167	18.8	6.3
		(CT)18	sbe b	sss c	2	17.2	5.5
		(CT)18	sbe a	sss c	1	20.7	5.0
		(CT)19	sbe c	sss c	6	19.4	6.1

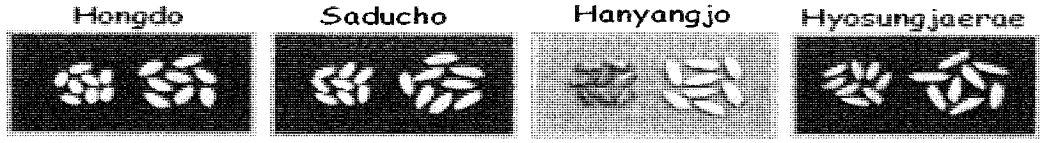


Fig 4. The example of association analysis of the alleles with amylose/ADV (Akali Digestibility Value) in the

non-waxy accessions of Korean landraces. The above photos show long-size grains from 4 different accessions of Indica type Korean landraces, which are rarely found in Korea with very high amylose contents.

Allele mining is exploitation of DNA sequence of one genotype to isolate useful alleles from related genotypes. With the efforts of International Rice Genome Sequencing Project (IRGSP), complete sequence information of whole genome of *Oryza sativa* L cv. Nippon bare is publically available now, which can be exploited for allele mining of many important traits including starch quality. Identification of allelic series of variants of starch biosynthesis genes and their association with quality of starch will provide a potential tool for crop improvement. Single nucleotide polymorphisms (SNPs) are the most common type of sequence differences between alleles. These polymorphisms could be used as simple genetic markers, which may be identified within or in the vicinity of virtually every gene. There is a great potential for the use of SNPs in the detection of associations between allelic forms of a gene and phenotypes. In this regard, several SNPs have been identified within different genes responsible for grain quality in rice including a granule-bound starch synthase I (GBSS- I) or waxy gene, which plays a critical role in the synthesis of amylase in all the cereal crops including rice. Based on these SNPs, we have developed CAPS/ dCAPS markers and demonstrated their successful utilization in DNA profiling of different rice accessions.

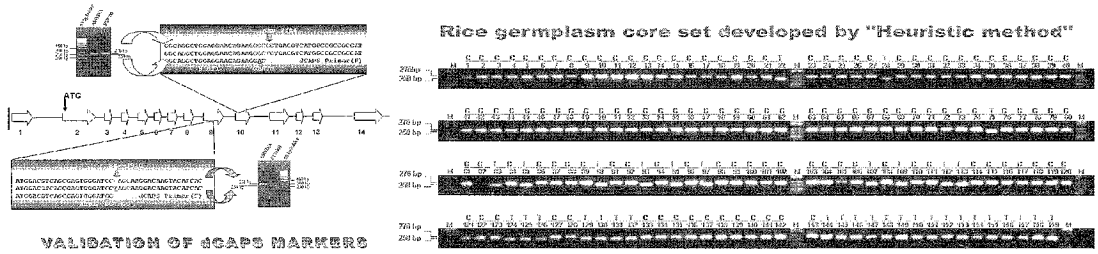
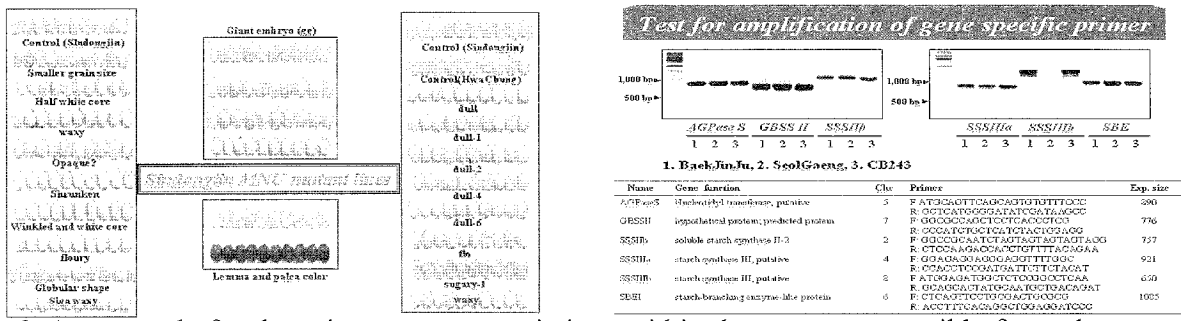


Fig 5. An example of development of SNP- based dCAPS markers and their use in genotyping

Endosperm and embryo mutants with different genetic backgrounds were selected after the chemical or radiation mutagenesis using MNU (M-Methyl-N-Nitroso Urea) and γ-ray. So far, mutants composed of 5,800 lines of 'Sindongjin', 837 lines of 'Ilpum', 293 lines of 'Aranghyangchal', 1,066 lines of 'Vandana' and 314 lines of 'Hyangnambyeo'.



Name	Gene function	Chr	Primer	Exp. size
AGPase S	Nucleotidyl transferase, putative	5	F ATGCAGTTCAGCAGTGTGTTTCCC R: GCTCATGGGGATATCGATAAGCC	290
GBSSII	hypothetical protein; predicted protein	7	F GGCGCCAGCTCCTCACCCTCG R: GCCATCTGCTCATCTACTGGAGG	776
SSSIIb	soluble starch synthase II-3	2	F GGCCGCAATCTAGTAGTAGTAGTAGG R: CTCCAAGACCACCTGTTTTACAGAA	757
SSSIIIa	starch synthase III, putative	4	F: GGAGAGGAGGGAGGTTTTGGC R: CCACCTCCGATGATTCTTCTACAT	921
SSSIIIb	starch synthase III, putative	8	F ATGGAGATGGCTCTCCGGCCTCAA R. GCAGCACTATGCAATGCTGACAGAT	650
SBEI	starch-branching enzyme-like protein	6	F: CTCAGTTCCTGCGACTGCGCG R: ACCTTTCACAGGCTGGAGGATCCC	1005

Fig.6. An example for detecting sequence variations within the genes, responsible for endosperm and embryo development from mutants of rice.

For analyzing association of phenotype to genetic variation in mutants, 82 candidate genes have been identified after searching the TIGR database by the criterion of genes responsible for embryo and endosperm development including starch. These genes code for the enzymes related with carbohydrate synthesis, starch synthesis (amylose and amylopectin), enriched proteins during embryo development, and dehydrins related to dehydration of seeds in Rice. Each primer set was designed from the 5' flanking region including exons and introns of the selected genes based on rice BAC sequences and verified the location of primer sequences by e-PCR in Rice genome sequences.

We have to determine the sequence variations in these genes' and to identify the SNPs or indels within the sequences among mutations. After the designing of markers such as dCAPS and STS, each F_2 population will be screened the relation of phenotypes with genes.

V. Conclusion

A new trial has been conducted to develop an integrated system for utilizing information of the conserved germplasms. This integrated system includes passport information, characterization data, GIS information, web-based core-set development, DNA profile data and their compounding data. This system is more focusing on the utilization of PGRs. The RDAGB is going to provide a processed data of each crop as a case study for increasing utilization of stored germplasm. Mutant accessions as a new type of germplasm such as T-DNA or As/ Ds insertional mutants need managing system for flanking sequences as passport data in gene banks. The temporary system for mutant germplasm has already been developed for managing 10,000 accessions of mutants.

The development of software program to provide better information on characterization including DNA profile data for germplasm users has been attempted through developing core collection and web-based genetic analysis system. Using genomics and bioinformatics tools, the RDAGB has initiated program for gene mining of useful traits from the gene bank accessions. Also, the association analysis by highly saturated DNA profiling is planed during next 5 years. To develop DNA profile databases for under-utilized crops (UUCs) such as sesame, *Perilla*, Job's tears, buckwheat, amaranth, finger millet, mungbean, Chinese cabbage, around 30-50 marker sets of simple sequence repeats for each crops have been developed by the RDAGB now. This marker development work is going to be extended for other UUCs and fruits such as zinger, several millets, *Zoysia*, *Allium*, persimmon, Korean plum, *Citrus* etc. Some case study through molecular characterization in rice collection provided a new type of database.

The need for capacity building in the RDAGB is to secure properly educated researcheres and to provide proper training to the staffs on molecular technology and bioinformatics. The development of a mutant gene bank needs hands on experience at functional study. The bioinformatics may help in providing network system of gene bank materials for the purpose of biotechnological research. The RDAGB is preparing for development of functional data of the preserved accessions such as DNA micro array analysis including protein profiling of specific tissues or those expressed during specific conditions such as cold and salt in future.

References

Bao S, Corke H, Sun M 2002. Microsatellites in starch-synthesizing genes in relation to starch physiochemical properties in waxy rice (*Oryza sativa* L.). TAG 105: 898-905.

Dixit A et al. 2005. Development of polymorphic microsatellite markers in sesame (*Sesamum indicum* L.). Mole cular Ecology Note. Published on- line at http://www.blackwell-synergy.com/toc/men/0/0; jsessionid=kH-9JyeaPD_9

Kim YC, Park YJ et al.2004. Screening of Bacterial Leaf Blight Resistance Genes (*xa5, xa13, Xa21*) using Sequ ence Tagged Site (STS) Marker in Korean Varieties and Landraces. Korean J. of Plant Resources 7(3): 187-194.

Kwon SJ et al. 2005. Isolation and characterization of microsatellite markers in *Perilla frutescens* Britt. Technical Note in Molecular Ecology Notes 5: 454-456.

Ortiz R 1998. Critical role of plant biotechnology for the genetic improvement of food crops: perspectives for the next millennium. EJB Electronic Journal of Biotechnology 1(3): 152-159.

Rao NK 2004. Plant genetic resources: Advancing conservation and use through biotechnology. African Journal of Biotechnology 3(2): 136-145.Uyoh EA, Nkang AE, Eneobong EE 2003. Biotechnology, genetic conservation and sustainable use of bioresources. African Journal of Biotechnology 2(12): 704-709.

RESEARCH AND CONSERVATION OF THE AZUKI BEAN AND SOYBEAN CROP COMPLEXES

D. A. Vaughan, A. Kaga, N. Tomooka, Y. Kuroda, T. Isemura
National Institute of Agrobiological Sciences (NIAS)
Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan
Corresponding author: Duncan Vaughan. Kannondai 2-1-2, Tsukuba 305-8602, Ibaraki, Japan. Tel: 81-298-38-7474, Fax:81-298-38-7408, Email: duncan@affrc.go.jp

Abstract

Crop complexes occur where wild, cultivated and intermediate forms are sympatric and represent the primary gene pool of crops. Crop complexes are the principal genetic diversity for crop evolution and breeding. Each crop has a unique crop complex. The conservation of crop complexes poses particular problems as the wild and weedy components are found in ruderal habitats subject to frequent, usually unpredictable, disturbances. Over the past decade we have been studying the azuki bean crop complex and more recently the soybean crop complex. In this paper three questions are addressed in relation to these two crop complexes. (a) What are the components of the crop complex and where are they found? (b) What genetic variation exists in the components of the crop complex and what are their genetic relationships? (c) What are the implications of crop complex research for conservation of the crop complex?

The azuki bean crop complex is distributed across much of East Asia but wild and weedy components seem to be most common today in Japan. A distinct center of diversity for this crop complex exists in the Himalayan region and adjacent areas. While the azuki bean complex consists of three recognizable components wild, weedy and cultivated forms many intermediate types exist suggesting continuous variation. The inter and intra-population diversity of this crop complex in Japan are greatest in central parts of Honshu. In this region the various components of the crop complex show a high level of genetic diversity and complex patterns of pollen and seed flow. Currently the components of this crop complex in Japan are well represented in *ex situ* genebank collections. However, pollen flow during regeneration means that isolation is necessary to maintain accession genetic integrity. Overall in natural environments in Japan this crop complex is not threatened but populations in some areas are isolated and thus in danger of local extinction.

The soybean crop complex is confined to East Asia and Far Eastern Russia Despite the global importance of soybean little is known about the components of this crop complex particularly weedy forms. In China weedy forms are reported but no good morphological characters can be used to accurately distinguish between wild and weedy types. In Japan a weedy ecotype of soybean has not been recognized although recently hybrid individuals or small populations have been found. It appears that geneflow among components of the soybean crop complex does occur but that in natural habitats hybrids quickly revert to wild type habit. Wild soybeans in Japan are more common than wild azuki and they are only locally threatened such as where there are development projects. In China, however, the threat to wild/weedy populations is high. The components of the soybean crop complex are well represented in *ex situ* genebank collections. As with the azuki crop complex regeneration of this crop complex requires attention to the potential for pollen flow among different accessions.

Introduction

Crop complexes constitute all or part of the primary gene pool of a crop (Harlan and DeWet, 1971). Components of the primary gene pool can generally form fertile hybrids with good chromosome pairing and normal gene segregation. The crop complex is therefore

essentially equivalent to a biological species. For most crop complexes there are three morphologically recognisable components – the cultigen, the close wild relatives of the cultigen and weedy forms. Where two or more of these components grow in the same region they exist as a complex. The importance of crop complexes lies in the fact that most crop evolution occurs within them and plant breeders can readily use the genes from crop complexes.

In this paper we focus on two important East Asian legumes and their crop complexes the azuki bean crop complex and soybean crop complex. Both azuki bean and soybean belong to the Phaseoleae that includes many hot weather legumes of importance to agriculture. While they are phylogenetically closely related they have different chromosome number (azuki bean 2n=2x=22 and soybean 2n=2x=40). Both azuki bean and soybean were domesticated in East Asia where they are important for human nutrition.

The azuki bean crop complex

a. What are the components of the crop complex and where are they found?

The azuki bean crop complex consists of the cultigen *Vigna angularis* var. *angularis* and wild form *V. angularis* var. *nipponensis* and intermediate or hybrid forms between these two varieties. In addition, a recently described species from the Himalayan region, *V. nepalensis*, is considered a member of the azuki bean complex (Tomooka *et al.*, 2005). There are several other species in the azuki bean primary gene pool these species do not grow in the same region as *V. angularis* hence are not part of the azuki bean complex (Vaughan *et al.*, 2005) (Fig. 1a). The main production areas of azuki bean are China, Japan, the Korean peninsula, Nepal and Bhutan. In Japan azuki beans are grown mainly in Hokkaido where the wild form does not occur. In the rest of Japan azuki bean is grown primarily on a small scale for home consumption. In Japan a weedy form has been described (Yamaguchi, 1992). This weedy type seems to form a stable ecotype in some parts of Japan. However, detailed observations of weedy type plants reveal that there is continuous variation from the wild type var. *nipponensis* to the cultigen var. *angularis* (the authors, personal observations).

b. What genetic variation exists in the components of the crop complex and what are their genetic relationships?

Several studies have been conducted using different molecular techniques to determine the genetic relationships among components of the azuki bean crop complex. Analysis of the azuki bean complex accessions from across its entire range has been performed using AFLP (Zong *et al.*, 2003) and SSR analyses (Xu *et al.*, in preparation). These analyses indicated that geographic origin, primarily, and status wild, weedy or cultivated, secondarily, are the major basis for genetic variation. These studies reveal a high level of genetic diversity in germplasm of the Himalayan region.

It is not known where azuki bean was first domesticated or whether it was domesticated more than once. The process of domestication can be deduced from the way in which wild Asian *Vigna* are gathered today. Pod picking is rather easy for Asian *Vigna* since the pods are relatively large and clustered (Tomooka *et al.*, 2003). In Japan the intermediate (or weedy) type has been harvested in the recent past (Egawa *et al.*, 1990).

The *Vigna angularis* complex in Japan

Vigna angularis in Japan forms a true crop complex with three types of natural population readily recognisable – wild, weedy and mixed or "complex" populations that are composed of wild, weedy and intermediate type plants. Complex populations tend to cover a larger area than wild or weedy populations and sometimes evidence of hybridisation among members of the population can be observed based on variation in plant morphology.

Analyses of germplasm accessions do not provide information on the diversity within populations at a single site. Morphological and molecular (SSR) studies of many individuals and

populations in Tottori prefecture have enabled the site-to-site variation in the azuki bean complex to be dissected in this prefecture (Miranda-Jonson *et al.*, in preparation; Wang el al., 2004). Population size, spread, gene cluster (patch) size in populations and introgression from cultivated to wild or complex populations are influenced by the time of population establishment and local ecology of sites. Thus a wild azuki population that established on a steep landslip established a patch size similar to populations established on non-sloping land for a longer time. These studies suggest that seed flow is more important than pollen flow in explaining the genetic diversity in this crop complex center of diversity.

c. What are the implications of this crop complex research for conservation of the crop complex?

Ex situ conservation of the azuki bean complex was reviewed by Tomooka *et al.* (2002). The wild and weedy relatives of azuki are poorly conserved for most areas except Japan and collections of cultivated azuki from the Himalayan region are deficient.

Genetic analysis of the azuki bean complex population types suggests that particular attention should be placed on *in situ* conservation of populations that consist of mixed populations (complex populations) of wild and weedy types in proximity to the cultigen. These complex populations have a higher level of genetic variation than populations of wild or weedy azuki bean. To understand the changes occurring in populations scientific monitoring both at different times during the year and from year to year is necessary. Monitoring of the immediate environment of the population and its general vicinity are important components to observe.

The soybean crop complex

a. What are the components of the soybean crop complex and where are they found?

The taxonomy of the soybean complex has a rather confused history. Two species are widely recognized the cultigen (*Glycine max* Merr.) and its presumed wild progenitor *G. soja* Sieb. & Zucc. (formerly called *G. ussuriensis* Regal & Maack). In addition, there is a type of wild soybean in northern Taiwan, China that has been called *G. formosana* Hosokawa. *G. formosana* has been show by RAPD analysis to be more diverged from *G. max* than *G. soja* (Thseng *et al.*, 2000) and to have a heat soluble protein profile different from *Glycine max* (Hsieh *et al.*, 2001). In addition, to these species an intermediate type has been named *G. gracilis* that is presumed to result from hybridization between *G. soja* and *G. max* (Hymowitz and Newell, 1977). *G. gracilis* falls within the range of variation of *G. max* based on RAPD polymorphism (Xu and Gai, 2003). While these four species names have commonly been used for members of the soybean complex they are one biological species. Soybean workers have not reached a consensus on the taxonomic rank or status of these taxa (Fig. 1b).

The distribution of wild soybean is restricted to East Asia and adjacent parts of Russia along the Amur River. In China *G. soja* is reported from as far west as Tibet (Dong *et al.*, 2001; Lu, 2004). Thus the soybean crop complex is only found in this region. *Glycine soja* is widely distributed across Japan from southern Hokkaido in the north to Kyushu in the south (Shimamoto *et al.*, 1998). In Japan wild soybeans occur in either naturally or human disturbed habitats. The natural habitat appears to be river sides. These are disturbed sites as a result of flooding.

Chinese and Korean populations of intermediate or weedy soybean occur but it is not clear how common they are (Oka, 1983; Lu, 2005). In Japan a weedy type of soybean has not been described but individual plants that are presumed hybrids between cultivated soybean and wild soybean have been reported (Kuroda *et al.*, 2005a). The habitat of presumed wild soybean/soybean hybrids were with wild soybean populations that were adjacent to cultivated soybean fields.

b. What genetic variation exists in the components of the crop complex and what are their relationships?

The present day diversity of and similarity between wild and landrace soybean germplasm based on cytoplasmic genome diversity suggests the Yangtze River valley of southern China is where soybean most likely was domesticated (Shimamoto, 2001). Vavilov suggested that the greatest diversity for a species can correspond with its center of domestication. Based on analysis of 15 morphological and biochemical traits in 22,695 accession Zhou *et al.* (1999) proposed a diffuse area from southwest to northeast China as the likely region of soybean domestication.

It is clear that in common with other crops soybean underwent a genetic bottleneck during domestication as several studies show much higher diversity in wild soybean than cultivated soybean (Xu *et al.*, 2002; Maughan *et al.*, 1995, 1996, Powell *et al.*, 1996).

Compared to other crops of Chinese origin, such as rice, soybean appears to be a relatively recent domesticate with first dates related to soybean appearing in 1100 BC literature (Hymowitz and Newell, 1980). It is not known how soybean was domesticated from wild soybean, particularly how wild soybeans were initially harvested. The pods of wild soybeans are small and difficult to pick suggesting plants may have been cut or uprooted, piled up and dropped seeds gathered after the pods shattered. Alternatively wild soybeans may have been cultivated as forage and then dropped seeds around farmhouses gathered. There are many forage types of cultivated soybean all having small seeds.

There are no crossing barriers between soybean and wild soybean, *G. soja*. However, recognizable intermediate types are rare in Japan. In China they are many reports of intermediate types (Oka, 1983; Lu, 2005) and germplasm from China in the USDA soybean collection are recognized as intermediate types. In experimental crosses it is very difficult to obtain progeny that are similar to *G. max* in F_2 populations derived from *G. max* and *G. soja* crosses (Carter *et al.*, 2004a).

Molecular techniques enable cryptic gene flow from cultivated soybean to wild soybean to be estimated. Results of SSR analysis of 77 wild populations (616 individuals deliberately selected for small seed size) and cultivated soybean (53 cultivars) showed that a small percentage of samples (about 6%) appeared to have introgressed genes from cultivated soybean. However, variation from population to population was high. In most populations outcrossing could not be detected due to small population size, however, 23 populations had variable outcrossing rates some in excess of 10%. These accessions with presumed introgression from soybean were found in all regions of Japan where wild soybeans occur. Thus gene flow between cultivated and wild soybeans in the field in Japan appears to be occurring. The results of this study suggest intermediate types quickly revert to wild type habit even though retaining some cultivated soybean genome fragments (Kuroda *et al.*, 2005b).

c. What are the implications of this crop complex research for conservation of the crop complex?

The current status of *ex situ* conservation of the soybean crop complex has recently been reviewed (Carter *et al.*, 2004b). This reveals that there are about 156,000 accessions of soybean and 10,000 accessions of wild soybean in the world's genebank collections. The maintenance of the genetic integrity of wild soybean populations *ex situ* is difficult as the plants can grow over a large area and thus require much land and wide spacing and cross pollination can occur where their natural pollinators are found.

There has been a proposal for *in situ* conservation of wild soybeans in China (Lui Liang personal communication). In Japan wild populations are very common even in heavily built up areas such as wasteland between newly built roads. In Japan this species is not threatened and is only likely to suffer from local extinct on small populations.

Conclusions

Azuki bean and soybean crop complexes overlap to a large extent in their distribution in Asia. Below is a summary of the main similarities and differences between these two crop complexes.

1. The azuki bean complex in Japan consists of the cultigen, wild progenitor and a readily recognisable weedy type and mixed populations of wild, weedy and intermediate types. The soybean complex consists of the cultigen and wild populations but intermediate types are apparently not common.
2. Azuki bean has complex primary, secondary and tertiary gene pools that seem to reflect a rather recent and continuing evolution of the Asian *Vigna*. Soybean has a simple primary gene pool consisting of the soybean complex only. There is no secondary gene pool and the tertiary gene pool consists of subgenus *Glycine*.
3. Wild azuki bean seems to be less aggressive than wild soybean since it is rarely found in highly disturbed built up areas. Wild soybeans are common all across Honshu, Japan, but wild azuki bean are less common than wild soybeans north of the Kansai region (Osaka/Kyoto).
4. The haploid chromosome number of azuki bean is 11 and that of soybean is 20.
5. Reported outcrossing rates among components of both crop complexes while usually very low includes a high degree of variation. Some high levels of outcrossing have been reported among members of both crop complexes (>10%).
6. The process of domestication for azuki bean can be inferred from present day harvesting of wild *Vigna* species. The process of domestication of soybean is unknown.

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References

Carter, T.E., T. Hymowitz and R.L. Nelson. 2004a. Biogeography, local adaptation, Vavilov, and genetic diversity in soybean. Pages 47-59 in D. Werner (ed.) Biological resources and migration. Springer, Berlin.

Carter, T.E., R.L. Nelson, C.H.Sneller and Z. Cui. 2004b. Genetic diversity in soybean. In: Boerma,H.R., Specht, J.E. (eds.) Soybean monograph, 3rd. ed. Am. Soc. Agron, Madison, WI pp 303-416

Dong, Y.S., B.C. Zhuang, L.M. Zhao, H. Sun, M.Y. He. 2001. The genetic diversity of annual wild soybeans grown in China. Theor. Appl. Genet. 103:98-103

Egawa, Y., D. Siriwardhane, K. Yagasaki, H. Hayashi, M. Takamatsu, M. Saito, Y. Nomura, Y. Okabe, F. Idezawa, S. Miyazaki. 1990. Collection of millets and grain legumes in Shinoina district of Nagano prefecture. 1989. Annual report on exploration and introduction of plant genetic resources (NIAR, Tsukuba, Japan) 6:1-22

Harlan, J.R. and J.M.J. DeWet. 1971. Towards a rational classification of cultivated plants. Taxon 20:509-517

Hsieh,J.S., K.L. Hsieh, Y.C. Tsai and Y.I.Hsing. 2001. Each species of Glycine collected in Taiwan has a unique seed protein pattern. Euphytica 118:67-73

Hymowitz, T. and C.A. Newell. 1980. Taxonomy, speciation, domestication, dissemination, germplasm resources and variation in the genus *Glycine*. Pages 251-264 in Advances in Legume Science R.J. Summerfield and A.H. Bunting (eds.) Royal Botanic Gardens, Kew, London.

Hymowitz, T. and C.A. Newell. 1977. Current thoughts on origins, present status and future of soybeans. Pages 197-209 in Crop Resources, D.S. Siegler (ed.) Academic Press, New York.

Hymowitz, T. 2004. Speciation and cytogenetics. In: Boerma,H.R., Specht, J.E. (eds.) Soybean monograph, 3rd. ed. Am. Soc. Agron, Madison, WI pp 97 –136

Kuroda Y, Kaga A, Apa A, *et al.* (2005a) Exploration, collection and monitoring of wild soybean and hybrid derivatives between wild soybean and cultivated soybean: based on field surveys at Akita, Ibaraki, Aichi, Hiroshima and Saga prefectures. *Annual Report on Exploration and Introduction of Plant Genetic Resources*, in press. (in Japanese with English summary)

Kuroda, Y., A. Kaga, N. Tomooka and D.A. Vaughan 2005b. Population genetic structure in Japanese wild soybeans (*Glycine soja*) based on microsatellite variation. Molecular Ecology (submitted)

Lu, B.R. 2004. Conserving biodiversity of soybean gene pool in the biotechnology era. Plant Species Biology 19:115-125

Lu, B.R. 2005. Multidirectional gene flow among wild, weedy, and cultivated soybeans. Pages 137-147 in Crop Ferality and Volunteerism, J. Gressel (ed.) Taylor and Francis, Boca Raton, Florida.

Maughan, P.J., M.A. Saghai Maroof, G.R. Buss. 1995. Microsatellite and amplified sequence length polymorphisms in cultivated and wild soybeans. Genome38: 715-723

Miranda-Jonson, G., A. Kaga, N. Tomooka and D.A. Vaughan. 2005. Gene flow in the azuki bean complex. Genetics (in process)

Oka, H.I. 1983. Genetic control of regenerating success in semi-natural conditions observed among lines derived from a cultivated X wild soybean hybrid. J. Applied Ecology

Powell, W., M. Morgante, J.J. Doyle, J.W. McNicol, S.V. Tingey and A.J. Rafalski. 1996. Gene pool variation in genus *Glycine* subgenus *Soja* revealed by polymorphic nuclear and chloroplast microsatellites. Genetics 144: 793-803

Shimamoto, Y., H. Fukushi, J. Abe, A. Kanazawa, J. Gai, Z. Gao, D. Xu. 1998.RFLPs of chloroplast and mitochondrial and mitochondrial DNA in wild soybean, *Glycine soja*, growing in China. Genet. Resour. Crop Evol. 47:611-617

Shimamoto, Y. 2001. Polymorphism and phylogeny of soybean based on chloroplast and mitochondrial DNA analysis. JARQ 35(2)79-84

Thseng, F.S., T.K Lin and S.T. Wu. 2000. The relation of genus *Glycine* subgenus *Soja* and *Glycine formosana* Hosok. Collected from Taiwan: revealed by RAPD analysis. Journal of Japanese Botany 75:270-279

Tomooka, N., D.A. Vaughan, H. Moss and N. Maxted. 2002. The Asian *Vigna*: Genus *Vigna* subgenus *Ceratotropis* genetic resources. Kluwer Academic Publishers, Dordrecht, The Netherlands. 270 pages.

Tomooka, N., S. Thadavong, C. Bounphanousay, P. Inthapanya, D.A.Vaughan and A. Kaga. 2004. Field survey of *Vigna* genetic resources in Laos, November 15-26, 2003. Crop Collecting Reports 20:77-91

Tomooka, N., A. Kaga and D.A.Vaughan. 2005 The Asian *Vigna*: Biodiversity and evolution. In A.K. Sharma and A. Sharma (eds.) Plant Genome: Biodiversity and evolution. Vol. Science Publishers, Inc., Enfield, NH (submitted)

Vaughan, D.A., N. Tomooka and A. Kaga. 2005. Azuki bean. Chapter 11 in Singh, R. J. and Jauhar, P.P.(eds.) Genetic Resources, chromosome engineering, and crop improvement. Grain legumes. CRC Press, Boca Raton, FL page 341-353

Wang, X.W., A. Kaga, N. Tomooka and D.A.Vaughan. 2004. The development of SSR markers by a new method in plants and their application to gene flow studies in azuki bean [*Vigna angularis* (Willd.) Ohwi & Ohashi]. Theor. Appl. Genet. 109:352-360

Xu, D.H. and J.Y. Gai. 2003. Genetic diversity of wild and cultivated soybeans growing in China revealed by RAPD analysis. Plant Breeding 122: 503-506

Xu, D.H., J.Abe, J.Y. Gai and , D.H., J.Abe, J.Y. Gai and Y. Shimamoto. 2002. Diversity if chloroplast DNA SSRs in wild and cultivated soybeans: evidence for multiple origins of cultivated soybean. Theor. Appl. Genet. 105:645-653

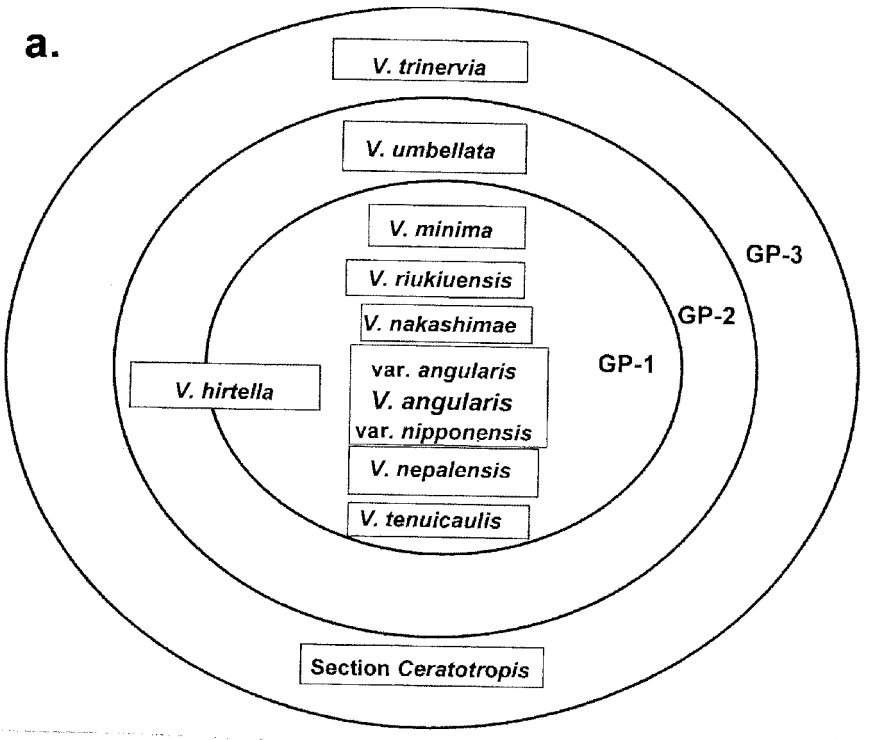
Xu, H.X., N. Tomooka, A. Kaga and D.A. Vaughan. 2005. Genetic diversity of cultivated and wild azuki bean [*Vigna angularis* (Willd.) Ohwi & Ohashi] as assessed by SSR markers. Genome (in preparation)

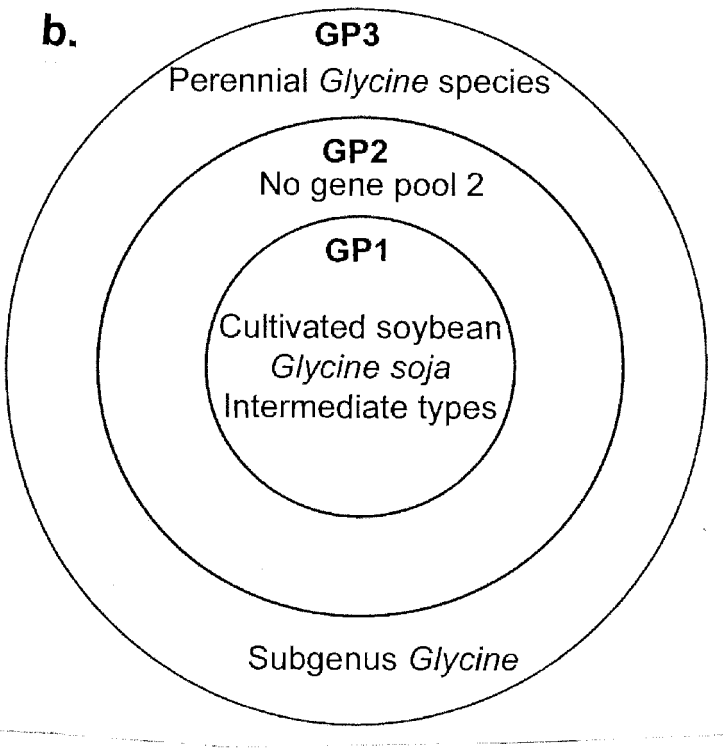
Yamaguchi, H. 1992. Wild and weed azuki beans in Japan. Econ. Bot. 46:384-394

Zhou, X., Y. Peng, G. Wang, R. Chang. 1999. Study on the center of genetic diversity and origin of cultivated soybean. In: Kauffman HE (ed.) Proc. World Soybean Res. Conf. VI, Chicago, IL, 4-7 Aug 1999. Superior Printing, Champaign IL, p510

Zong, X.X., A. Kaga, N. Tomooka, X.W.Wang, O.K. Han and D. A.Vaughan. 2003. The genetic diversity of the *Vigna angularis* complex in Asia. Genome 46:647-658

Fig. 1a. Gene pool system for azuki bean (*Vigna angularis*) (modified from Tomooka *et al.*, 2002); 1b. Gene pool system for soybean (*Glycine max*) (based on Hymowitz, 2004)





EX SITU CONSERVATION OF VEGETATIVELY PROPAGATED GENETIC RESOURCES: PRESENT STATUS AND STRATEGIES FOR TROPICAL AND SUB-TROPICAL SPECIES

Hiroko Takagi [1*] and Takao Niino [2]

1 Okinawa Subtropical Station, Japan International Research Center for Agricultural Sciences, Ishigaki, Japan

2 Genebank, National Institute of Agrobiological Sciences, Tsukuba, Japan

*Corresponding author: takagiw@affrc.go.jp (Tel: +81 980 88 6301)

Introduction

Ex situ conservation means the conservation of components of biological diversity outside their natural habitats (Engelmann & Engel 2001) and is generally used for safeguarding crop species and their relatives that are at risk of destruction, replacement, or genetic deterioration. *Ex situ* conservation activities at each genebank consist of collecting, handling and management of germplasm, conservation, regeneration, characterization/evaluation, documentation, and dissemination to users, including qualantine procedure. All of these activities, as routine operation, are under the responsibility of curators of genebanks, or breeders holding germplasm collections. To implement sustainable *ex situ* conservation, all the activities need to be well operated and managed. For the case of clonally preserved species, conservation techniques/systems of germplasm itself still remains as a basic problem to be ameliorated.

This paper presents a brief overview of present status and strategies of conservation techniques of *ex situ* collections of the species for which conservation in seed form is problematic, i.e. vegetatively propagated and non-orthodox seed species.

Present status of *ex situ* conservation of plant genetic resources

In a broad sense, *ex situ* conservation of germplasm is a practice that humans used since the beginning of agriculture. International united efforts were initiated in early 1960s. In 1963, FAO set international guidelines for the collection, conservation and exchange of germplasm to promote *ex situ* conservation of crop genetic resources (FAO 1997). Moreover, major advances in plant breeding brought about the 'Green Revolution' and continuous destruction of natural environments by developments made deterioration of plant genetic diversity as a common concern worldwide. Great emphasis was placed on global germplasm collecting during the 1970s and 1980s.

As a result, it is estimated that existing global *ex situ* collections contain approximately 6 million accessions at over 1,300 genebanks and germplasm collections. Of all the accessions in *ex situ* collections, some 600,000 are maintained within the Consultative Group on International Agricultural Research (CGIAR) system, and the remaining 5.5 million accessions are kept in national or regional genebanks (FAO 1997).

Ex situ collections of clonally preserved species

Collecting and conservation activities focused largely on cereals and some legumes and most conservation efforts were concentrated on *ex situ* conservation particulary seed genebanks. Approximately 90% of accessions in the *ex situ* collections are stored in form of orthodox seeds under dry condition combined with low temperatures. It is estimated that, worldwide, only 9% i.e. 527,000 accessions are stored in field genebanks, while fewer than 38,000 accessions are conserved *in vitro* (FAO, 1997).

The species which conservation in seed form is problematic are mainly vegetatively propagated species and species with reculcitrant seeds. Perennial species produce small amount of seeds and species with long life cycles can be also categorized into this group.

Major tropical species of roots and tubers (i.e. sweet potato, potato, cassava, yams, taro), banana/plantain and some export crops (i.e. rubber, sugarcane, oil palm, coffee, coconut) are relatively well collected and maintained by the CGIAR centers and national programs. For these crop species genomic based studies need to be strengthened to understand genetic diversity, to establish core collections and to organize additional collecting to complement the existing collections. For tropical fruit and forest tree species, collecting is much behind and systematic collecting should be emphasized. In addition, land races of minor and underutilized crops and non-cultivated species for local use should be given more attention by international community.

Conservation methods of germplasm collections of clonally preserved species

Traditionally, field genebanks has been the primary conservation method for the type of species. Field genebanks ensure the maintenance of genetic integrity of each genotypes immediately accessible for characterization, evaluation, multiplication and utilization. However, field collections are labour intensive, expensive and exposed to biotic and abiotic stresses.

In vitro storage by slow growth method is an important alternative and commonly used to complement field genebanks. Slow growth techniques allow clonal plant material to be stored under tissue culture conditions with extended subculture intervals. The techniques are now well developed and routinely applied to a range of species and across a range of genotypes within species (Ashmore 1997). The maintenance of *in vitro* collections is under reduced growth conditions (low light intensity, low temperature, modifications in media by adding osmotic inhibitors or growth retardants etc.) but still labor intensive and only available for midium-term conservation. There is always the risk of losing accessions due to microbial contamination or human error. Moreover, *in vitro* material of some species is subject to somaclonal variation and careful monitoring and investigation on stored materials are needed.

Pollen storage, true seeds populations as gene pools, conservation of DNA and *in situ* or on-farm conservation can be also used, depending on genetic feature of each crop, but only limited extent as supplemental methods. Therefore, development of techniques and systems for practical use of cryopreservation is keenly desired for long-term conservation of clonally preserved species.

Cryopreservation ; Current research status and utilization for plant germplasm

Cryopreservation is the only currently viable method for long-term storage of plant species for which conservation in seed form is problematic. Cryopreservation is a method to preserve materials at ultra low temperature, usually that of liquid nitrogen (-196°C). Under this temperature, biochemical and most physical processes are arrested. Therefore, the plant materials can be stored theoretically in stable condition for a unlimited period time and require low cost and limited maintenance.

Orthodox seeds or dormant buds go through natural dehydration process and show tolerance to dehydration or freezing. However, most of the materials for cryopreservation (cell suspension, shoot apices, embryos) are *in vitro* grown and contain high amount of water, thus sensitive to freeze injuries (Engelmann 2003). Therefore, technology development of cryopreservation for plant materials, it has been being the key issue to development of artificial dehydration techniques to protect materials from the damages caused by the crystallization of intracellular water into ice.

Development of Cryopreservation Techniques

First successful report on cryopreservation was reported for winter buds of cold hardy woody plants in 1960 (Sakai 1960), and techniques based on freeze-induced dehydration had been main approach to cryopreservation of plant materials. In the techniques, freezing tolerance is conferred by the addition of cryoprotectants and dehydration of cells by extracellular freezing, which is initiated by ice-inoculation and pursued during slow (-0.3 to -1°C/ min) cooling to -30 to -40°C (Sakai 1960, 1965, Kartha 1985).

In early 90's, some new procedures based on vitrification (Langis *et al.* 1990, Sakai *et al.* 1990, Fabre and Dereuddre 1990) were proposed and significant progress was made in cryobiological research and development of cryopreservation in the past 15 years. Vitrification, in this context, refers as a phase transition from a liquid into a non-crystalline solid (glass) while avoiding crystallization. Thus, vitrification is a freeze-avoidance mechanism that enables hydrated cells and tissues to survive in LN (Sakai & Yoshida 1967). In vitrification based techniques, cell dehydration is performed before plunging into liquid nitrogen by exposing materials to i) a concentrated cryoprotective media or ii) air dessication to induce glass transitions during rapid cooling (Engelmann 2003). Comparing with the classical techniques, these new techniques are less complex in operation and require only basic equipment. Moreover, the techniques have been successfully applied for more complex and larger organs (i.e. shoot apices and embryos) of both temperate and tropical species with higher post thaw survival rate (Sakai *et al.* 2003)

The following different vitrification-based procedures are currently well identified; i) vitrification (osmotic dehydration), ii) encapsulation-vitrification, iii) dehydration, iv) encapsuration-dehydration, v) droplet method (Sakai *et al.* 2003, Keller and Dreiling 2002, Panis *et al.* 2005) and most suitable procedure can be selected depends on species, tissue type and laboratory conditions etc. Basically, those procedures are specialized based on i) how dehydration is performed (osmotic dehydration by vitrification solution or by air-drying) and ii) whether samples are encapsulated into alginate beads or not. Details of these procedures were well reviewed in Sakai *et al.* (2003), Engelmann (2003), and Engelmann and Takagi (2000).

Key points of successful cyopreservation protocols

Among the above mentioned different procedures, the vitrification method (including encapsulation-vitrification) using osmotic dehydration with a highly concentrated vitrification solution was most widely applied procedure to different species. Especially, the protocols using a glycerol-based vitrification solution (7.8 M) named as PVS2 (contains 30%(w/v) glycerol, 15% (w/v) ethylene glycol and 15%(w/v) DMSO) (Sakai *et al.* 1990) were well adopted to around 200 species or cultivars of temperate and tropical species. Some examples of tropical species are cassava, banana , taro, yams, sweetpotato (Sakai *et al.* 2003).

Whatever cryogenic method is applied to cultured apices or cells, inducing higher levels of osmo- and dehydration-tolerance is the key for successful cryopreservation. Preculture with sucrose or sorbitol enriched medium for 1 to 3 days enhanced tolerance to PVS2 for some species. The treatment with a mixture of 2M glycerol and 0.4M sucrose (LS solution) following preculture with sucrose enriced media enhance the substantial increase in survival of vitrified meristems not only in temperate species but also in various tropical species (Sakai *et al.* 2003).

Beside the investigation of detailed conditions of protocols (i.e. appropriate time and temperature for LS solution treatment, dehydration by vitrification solution etc.), it should be also noted that optimized *in vitro* culture systems are very important for successful cryopreservation. Especially, a micropropagation system for producing a large number of uniform and suitable *in*

vitro plants makes the production of a high level of osmo-tolerance and vigorous recovery growth after cryopreservation realistic.

Practical applications of cryopreservation at genebanks

Cryopreservation techniques for shoot apices and embryos are now well developed. However, practical application to conservation at genebanks for large scale operation is still quite limited. Cryopreservation of vegetatively propagated species currently operated at genebanks are as follows but still limited to temperate species; mulberry (Japan), apple (USA), European elm (France), grape (USA) as dormant buds, and strawberry (Japan), Japanese horseraddish (Japan), garlic (Korea), potato(Germany, International Potato Center), pear (USA), grape (USA) as shoot apices.

Systematic approach has been being carried out for *Musa* germplasm at the transit center, INIBAP. Cryopreservation protocols in banana were developed for seeds, zygotic embryos, embryogenic cell suspensions, proliferating meristem cultures and apical meristems exised from rooted *in vitro* plants (Panis & Thinh 2001). Most recently, droplet-vitrification protocol was developed for shoot apices to be applied for wider range of germplasm accessions belong to *Musa* spp. The optimized procedure of droplet-vitrification protocol was applied to 56 accessions belonging to eight different genomic groups of *Musa* spp. and one *Ensete* spp., and an average of 52.9% post-thaw regeneration was obtained (Panis *et al.* 2005).

Future research needs in cropreservation

Although remarkable progress in cryobiological studies of plant materials and technology development was made in last decade, cryopreservation as a conservation method for plant genetic resources is still limited and cannot be routinely adopted in genebanks in contrast with microbial and mammalian culture collections.

Cryopreservation protocols for plant materials were mostly developed following an empirical, trial-and-error approach. Even if a good protocol is established, it cannot be adopted to other materials, since different species, varieties and tissue types tend to show different responses to cryopreservation protocol.

To further improve widly applicable protocol for large scale utilization of cryopreservation for germplasm conservation, basic research should be needed to understand the biophysical and metabolic processes underlying resistance/sensitivity of plant tissues to cryopreservation. An European Commission Research Project titled "Establishing cryopreservation methods for conserving European plant germplasm (CRYMCEPT project)" was initiated in 2003. The project involves basic researches on water thermal behaviour, analysis of proteins, sugars, membrane lipids, polyamines, oxidative stress and cytoskeletal changes. This type of approach could provide key information to understand the mechanisms involved in cryopreservation of plant tissues to develop cryopreservation protocols in a more rational, scientific and cost-effective manner.

Further research is also needed on i) genetic integrity of cryopreserved materials, ii) development of techniques for recalcitrant seeds which cryopreservation research is much less advanced.

Conclusion

This paper provides brief summary only on present status and problems of *ex situ* conservation from the technical and research aspects for the plant germplasm of clonally preserved. However, plant genetic resources conservation is not only developing the most

appropriate and effective technology, but also developing strategies and methods for proper management systems are also critical. Joint *ex situ* conservation efforts for better cordinated and synergetic activities between genebanks and botanical gardens at the institutional, national and international levels are essential.

References

Ashmore, S.E. (1997) Status report on the development and application of *in vitro* techniques for the conservation and use of plant genetic resources. IPGRI, Rome, Italy. 67 p.

Engelmann, F. and H. Takagi (eds.) (2000) Cryopreservation of tropical plant germplasm. Current research progress and application. JIRCAS, Tsukuba, Japan/ IPGRI, Rome, Italy. 496 p.

Engelmann, F. and J.M.M. Engels (2001) Technologies and strategies for *ex situ* conservation. In: J.M.M. Engels *et al.*(eds.) *Managing plant genetic diversities*. CAB International, Wallingford, UK, pp. 89-103.

Engelmann, F. (2003) Current research status and utilization of plant cryopreservation. Proceedings of the International Workshop on Cryopreservation of Bio-Genetic Resources. National Institute of Agricultural Biotechnology. Suwon, Republic of Korea.

Fabre, J. and J. Dereuddre (1990) Encapsulation-dehydration: A new approach to cryopreservation of *Solanum* shoot tips. Cryo-Letters 11:413-426.

FAO (1997) The state of the world's plant genetic resources for food and agriculture. Food and Agriculture Organization of the United Nations, Rome, Italy.

Kartha, K.K. (ed.) (1985) Cryopreservation of plant cells and organs. CRC Press, Boca Raton, Florida.

Keller, E.R.J. and M. Dreiling (2002) Potato cryopreservation in Germany-Using the droplet method for the establishment of a new large collection. Proceedings of the 25th International Horticultural Congress, Tront, Canada.

Langis, R., B. Schnabel-Preikstas, B.J. Earle and P.L. Steponkus (1990) Cryopreservation of carnation shoot tips by vitrification. Cryobiology 276: 658-659.

Panis, B., B. Piette and R. Swennen (2005) Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all *Musaceae*. Plant Science 168: 45-55.

Panis, B. and N.T. Thinh (2001) Cryopreservation of *Musa* germplasm. INIBAP Technical Guideline 5, J.V. Escalant and S. Sharrock(eds.). International Network for the Improvement of Banana and Plantain, Montpellier, France.

Sakai, A. (1960) Survival of twig of woody plants at -196°C. Nature 185:939-394.

Sakai, A. (1965) Determining the degree of frost-hardiness in highly hardy plants. Nature 206: 1064-1065.

Sakai, A. (2000) Development of cryopreservation techniques. In: Engelmann, F. and H. Takagi (eds.) Cryopreservation of tropical plant germplasm. Current research progress and application. JIRCAS, Tsukuba, Japan/ IPGRI, Rome, Italy. pp. 1-7.

Sakai, A., D. Hirai and R. Charoensub (2003) History and current issues of plant cryopreservation research. Proceedings of the International Workshop on Cryopreservation of Bio-Genetic Resources. National Institute of Agricultural Biotechnology. Suwon, Republic of Korea.

Sakai, A., S. Kobayashi and I. Oiyama (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. Var. *brasiliensis* Tanaka). Plant Cell Reports 9:30-33.

Sakai, A. and S. Yoshida (1967) Survival of plant tissue at super-low temperatures. VI. Effects of cooling and rewarming rates on survival. Plant Physiol. 42: 1965-1701.

STRATEGY FOR CONSERVATION AND ENHANCED USE OF CROP GENETIC RESOURCES

C. L. L. Gowda and H. D. Upadhyaya
International Crops Research Institute for the Semi-Arid Tropics,
Patancheru–502 324, Andhra Pradesh, India

Introduction

The plant genetic resources (PGR) are finite and vulnerable for loss due to introduction of new crop cultivars in agriculture, urbanization, and natural hazards, etc. The realization of this fact led to the drafting of Convention on Biological Diversity at the Earth Summit in Rio in 1992. The PGR contribute enormously towards achieving the Millennium Development Goals of food security, poverty alleviation, environmental protection, and sustainable development. PGR are critical components of crop improvement efforts aimed at increasing food security - both for short-term gains as well as for long-term increase in productivity. Over the years, genebanks have been established in a number of countries and the number of accessions conserved in genebanks now exceeds the six million mark (FAO 1998).

The mission of the Consultative Group on International Agricultural Research (CGIAR) is to achieve sustainable food security and reduce poverty in developing countries through research and development in the fields of agriculture, forestry, fisheries, policy, and environment. Exploration, exchange, and conservation of PGR is one of the main objectives of the CGIAR. The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT – one of the 15 CGIAR centers) has responded to this need by establishing a Genetic Resources Unit for assembly, characterization, evaluation, maintenance, conservation, documentation and distribution of germplasm of the sorghum, pearl millet, chickpea, pigeonpea, groundnut, finger millet, foxtail millet, barnyard millet, kodo millet, little millet and proso millet, and their wild relatives. In this paper we present a brief account of (i) ex situ germplasm assembly efforts, (ii) germplasm management in ICRISAT genebank (iii) germplasm utilization status and (iv) means to enhance use of germplasm in research and development.

1. Germplasm assembly in the ICRISAT genebank

Since the inception of ICRISAT in 1972, efforts were initiated to assemble the germplasm of the mandate crops from various research institutes around the world. The Rockefeller Foundation donated 11961 accessions of sorghum and 2000 accessions of pearl millet to ICRISAT. ICRISAT also obtained 2000 accessions of pearl millet collected by the Institut Francais de recherché Scientifique pour le Development en Cooperation (ORSTOM) in francophone West Africa. A chunk of chickpea and pigeonpea germplasm were the donations by the (former) Regional Pulse Improvement Project (RPIP). We also acquired over 1,200 chickpea accessions from the Arid Lands Agricultural Development (ALAD) program in Lebanon. Similarly, much of the groundnut germplasm was received from the Indian groundnut research programs and the USDA.

New germplasm of mandate crops was also collected from the priority areas. From 1975 to 2003, a total of 213 joint missions were launched in 62 countries securing 33,194 accessions of ICRISAT mandate crops. Besides genebank activities, ICRISAT also works on the genetic improvement of the mandate crops. A large number of improved breeding lines, stress resistant sources or the cultivars were developed, and 3905 such lines have been registered in the genebank for

future utilization. At present, a collection of 114,870 accessions of eleven crops is conserved in the ICRISAT genebank (Table 1).

2. Germplasm management in the ICRISAT Genebank

2.1. Phenotypic characterization and evaluation: Adequate characterization for agronomic and morphological traits is necessary to facilitate the utilization of germplasm by researchers. To achieve this, germplasm accessions of all the crops were sown in batches over the years and characterized for morphological and agronomic traits. Germplasm screening against biotic and abiotic stresses, and the estimations of grain food quality were conducted jointly with various disciplinary scientists. Germplasm sets were evaluated for agronomic performance over locations jointly with NARS scientists in India, Nepal, Thailand, Indonesia, Ethiopia, and Kenya and more intensively with the National Bureau of Plant Genetic Resources (NBPGR), India. The results of joint evaluations have led to better understanding of the germplasm material.

2.2. Regeneration: Regenerations were carried out to meet the seed increase requirements of (i) accessions that had reached a critical level of seed stock/viability; (ii) accessions required for medium-term storage (MTS) and/or long-term storage (LTS); and (iii) germplasm repatriation. During regeneration, all possible efforts are made to grow the accessions under healthy conditions and following the appropriate pollination control measures.

2.3. Conservation: Germplasm conservation requires cleaning the seed material, drying to minimal seed moisture content, storing in cool and dry conditions, and regular monitoring of seed health during storage. In the ICRISAT genebank, the seeds of the entire collection are stored in MTS (4^0C, 20–30% RH) in aluminum cans. The germplasm accessions are also conserved in LTS (-20^0C) after packing in vacuum-sealed aluminum foil pouches.

2.4. Safety back up: ICRISAT's agreement with FAO places the germplasm collections under the auspices of FAO, and requires safety back-up for long-term conservation in countries out side India. We have initiated efforts conserving 2000 chickpea at ICARDA (Syria) and 2006 groundnut and 4580 finger millet accessions at ICRISAT Regional Genbank at Niamey in Niger.

2.5. Documentation: The vast germplasm data gathered on chickpea and pigeonpea germplasm has been summarized and presented to the users in the form of catalogs (Pundir et al. 1988, Remanandan et al. 1988). During the last 15 years, we had a very purposeful collaboration with the NBPGR, India, on germplasm exploration, and evaluation at a number of locations, and the outcome were reviewed and discussed in a workshop 'Collaboration on Genetic Resources' held in 1988 at ICRISAT, Patancheru (ICRISAT 1989). Core and mini-core collections of ICRISAT mandate crops were established and the information was published through journal articles (Grenier et al. 2001; Bhattacharjee 2000; Upadhyaya and Ortiz 2001; Upadhyaya et al. 2001a; Upadhyaya et al. 2002; Upadhyaya et al. 2003; Upadhyaya et al. 2005; and Reddy et al. 2005) for the benefit of fellow research workers. A 'Manual of Genebank Operations and Procedures' has also been published (Rao and Bramel 2000) documenting the history of the collections, procedures for germplasm acquisition, maintenance, documentation, conservation, and distribution.

3. Germplasm utilization

3.1. Germplasm supply to users: ICRISAT Genebank supplies healthy, viable, and genetically pure seeds of genetic resources to the research workers. Scientists at ICRISAT are the largest users of the germplasm followed by the scientists in India. During 1973 to 2004, we have supplied over 660,000 seed samples to the scientists outside ICRISAT representing 143 countries.

3.2. Germplasm repatriation: The global collections held at ICRISAT serve an important purpose in restoration of germplasm to the source countries when national collections are lost due to some reasons. For example, we repatriated 362 sorghum accessions to Botswana, 1827 sorghum and 922 pearl millet to Cameroon, 1723 sorghum and 931 chickpea to Ethiopia, 838 sorghum, and 332 pigeonpea to Kenya, 1436 and 445 sorghum accessions respectively to Nigeria and Somalia and 71 pigeonpea accessions to Sri Lanka. The ICRISAT germplasm collections include 44,822 accessions received from or jointly collected with the Indian NARS. On request, these accessions have been restored to the NBPGR Genebank, New Delhi, India.

3.3. Impact of germplasm supplied to the NARS, worldwide: Besides the utilization of germplasm in ongoing research at other institutes, 66 germplasm accessions of seven crops from the ICRISAT supplied samples have been directly released as cultivars in 44 countries. These cultivars have greatly benefited countries where released.

4. Strategies to enhance germplasm utilization

ICRISAT genebank has supplied more than 660,000 germplasm samples to users outside the ICRISAT from 1975 to 2004. This figure could be considered as satisfactory use of germplasm. However, the use of basic germplasm in various breeding programs is scanty. We have adopted the following strategies to enhance utilization of the germplasm in research:

4.1. Germplasm evaluation for specific traits: Germplasm accessions are evaluated for traits of agronomic importance such as yellow endosperm trait in sorghum and pearl millet and large-seeded trait in chickpea and groundnut. Based on the emergence of new priorities, future plans will be worked out.

4.2. Developing core collections: One of the reasons that plant breeders are using less of basic germplasm in research is the lack of information on traits of economic importance, which often shows high genotype x environment interactions and requires replicated multilocational evaluations. This is a very costly and resource-demanding task owing to the large size of the germplasm collection. To overcome this, our research now focuses on studying the diversity of germplasm collection and developing "core collections," which are about 10% of the entire collection, but represent almost full diversity of the species. From the germplasm collection in the ICRISAT genebank, we have already developed core collection of seven of the 11 ICRISAT related crops.

4.3. Developing mini-core collection: When the size of entire collection is large, even a core collection size becomes unwieldy for evaluation by breeders. To overcome this, we developed a two-stage strategy to develop a mini-core collection that would consist of 10% accessions of the core collection (or 1% of entire collection). (Upadhyaya and Ortiz 2001). At ICRISAT, we have already developed mini-core collections of chickpea (211 accessions) (Upadhyaya and Ortiz

2001), groundnut (184 accessions) (Upadhyaya et al. 2002), pigeonpea (146 accessions) and finger millet (65 accessions).

4.4. Identification of sources for traits of economic importance using core and mini-core collections: Due to reduced size, the core collection can be evaluated extensively to identify useful parents for crop improvement. By evaluating core collection of chickpea, we identified new sources of early maturity (28 accessions), and large seeded kabuli (16 accessions) types. By evaluating groundnut core collection we found new sources of early maturity in 21 accessions, and tolerance to low temperature at germination in 158 accessions (Upadhyaya et al. 2001b). Also were found 15 Valencia, 20 Spanish, and 25 Virginia type germplasm lines in groundnut with high-yield, good shelling percentage and 100-seed weight through multilocational evaluation of the 'Asia region core collection' (Upadhyaya et al. 2005). These new sources performed better than or similar to the best control cultivars for particular trait (s) but were diverse from them. We have also identified 10 drought- and five salinity-tolerant accessions from the chickpea mini-core and 18 diverse accessions with high water use efficiency from the groundnut mini-core collection (Upadhyaya 2005) (Figure 1).

4.5. Multilocational evalutions of mini-core collections: The chickpea mini-core was evaluated at the Indian Institute of Pulses Research (IIPR), Kanpur, India during the 2002/03 and 2003/04. IIPR scientists were particularly excited with the large-seeded kabuli accessions. They selected 12 accessions for subsequent large plot evaluation and their use in the breeding program. Similarly, scientists in China, Vietnam, and Thailand have identified 8, 10 and 12 germplasm lines, respectively from evaluation of groundnut mini-core in their country.

5. Future outlook

- In future, we will have increased focus on assessment of the germplasm for their usefulness for crop improvement. The core and mini-core subsets of the germplasm will be evaluated at diverse locations to identify trait specific diverse parents.
- We have initiated developing composite sets of ICRISAT mandate crops under the Generation Challenge Program. Phenotypic and genotypic characterization of these sets will provide vast scope of identifying useful and unique germplasm resources for utilization in the crop improvement.
- Search for new and useful crop germplasm and try to secure and conserve them in the genebank.
- We need to secure new germplasm of wild *Arachis* from Peru, wild *Cicer* from Iran and pigeonpea from Myanmar and Uganda to fill the genetic gaps in the collections.

References

Bhattacharjee R (2000) Studies on the establishment of a core collection of pearl millet *(Pennisetum glaucum)*. Ph D Thesis, CCS Haryana Agricultural University, Hisar – 125 004, India 162 pp.

Convention on Biological Diversity (CBD) (1992) Convention signed by the International Community during the Earth Summit at Rio de Janeiro, Brazil, 1992.

FAO (1998) The state of ex-situ conservation. Page 90. *in* The state of the world's plant genetic resources for food and agriculture. FAO, Rome.

Grenier CPJ, Bramel PJ and Hamon P (2001) Core collection of the genetic resources of sorghum: 1. Stratification based on eco-geographical data. Crop Science 41: 234-240.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics) (1989) Collaboration on Genetic Resources: summary proceedings of a joint ICRISAT/ NBPGR (ICAR) workshop on germplasm exploration and evaluation in India, 14–15 Nov 1988, ICRISAT, Patancheru, India. Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics.

Pundir RPS, Reddy KN and Mengesha MH (1988) ICRISAT Chickpea Germplasm Catalog: Evaluation and Analysis. Patancheru, A. P. 503 324, India: International Crops Research Institute for the Semi-Arid Tropics. 94pp.

Rao NK and Bramel PJ (2000) Manual of Genebank Operations and Procedures. Technical Manual no. 6. Patancheru, A. P. 502 324, India: International Crops Research Institute for the Semi-Arid Tropics. 190 pp.

Reddy LJ, Upadhyaya HD, Gowda CLL, and Sube Singh (2005) Development of core collection in pigeonpea (*Cajanus cajan* (L) Millsp. Genetic Resources and Crop Evolution (in press).

Remanandan P, Sastry DVSSR and Mengesha MH (1988) ICRISAT Pigeonpea Germplasm Catalog: Evaluation and analysis. Patancheru, A. P. 503 324, India: International Crops Research Institute for the Semi-Arid Tropics. 89 pp.

Upadhyaya HD (2005) Variability for drought resistance related traits in the mini-core collection of peanut. Crop Science 45:1432-1480.

Upadhyaya HD, Bramel PJ, Ortiz R and Sube Singh (2002) Developing a mini core of peanut for utilization of genetic resources. Crop Science 42: 2150-2156.

Upadhyaya HD, Bramel PJ and Sube Singh (2001a) Development of a chickpea core subset using geographic distribution and quantitative traits. Crop Science 41: 206-210.

Upadhyaya HD, Gowda CLL, Pundir RPS, Reddy V Gopal, and Sube Singh (2005) Development of core subset of finger millet germplasm using geographical origin and data on 14 morpho-agronomic traits. Genetic Resources and Crop Evolution (in press).

Upadhyaya HD, Mallikarjuna Swamy BP, Kenchana Goudar PV, Kullaiswamy BY and Sube Singh (2005) Identification of diverse groundnut germplasm through multienvironment evaluation of a core collection for Asia. Field Crops Research 93:293-299.

Upadhyaya HD, Nigam SN and Sube Singh (2001b) Evaluation of groundnut core collections to identify sources of tolerance to low temperature at germination. Indian J. Plant Genet. Resources 14:165-167.

Upadhyaya HD and Ortiz R (2001) A mini core subset for capturing diversity and promoting utilization of chickpea genetic resources. Theoretical and Applied Genetics 102: 1292-1298.

Upadhyaya HD, Ortiz R, Bramel PJ, and Sube Singh (2003) Development of a groundnut core collection using taxonomical, geographical, and morphological descriptors. Genetic Resources and Crop Evolution 50: 139-148.

Table 1. Germplasm holdings in the Rajendra S Paroda Genebank, ICRISAT, Patancheru, December 2004.

Crop	Active collection	Base collection	Accessions held in-trust
Sorghum	36,774	31,669	35,780
Pearl millet	21,594	15,150	21,250
Chickpea	17,258	15,984	16,961
Pigeonpea	13,632	10,266	12,698
Groundnut	15,419	6,820	14,357
Finger millet	5,949	4,620	4,931
Foxtail millet	1,535	1,054	1,534
Proso millet	842	576	835
Little millet	466	384	460
Kodo millet	658	630	547
Barnyard millet	743	487	743
Total	114, 870	87,640	110,096

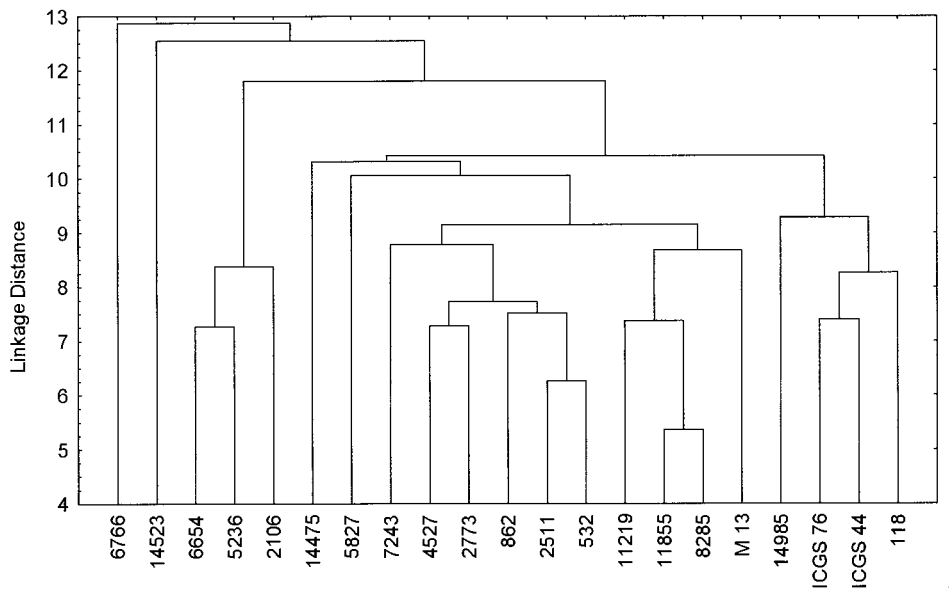


Figure. 1. Dendogram of 18 selected drought tolerant germplasm accessions and control cultivars of groundnut based on scores of first 12 principal components

COMPLIANCE, ACCOUNTABILITY AND ETHICS FOR TRUST-BUILDING PARTNERSHIPS IN INTERNATIONAL COLLABORATIVE RESEARCH ON PLANT GENETIC RESOURCES

Makoto Kawase
Plant Genetic Resources Laboratory, Genebank, National Institute of Agrobiological Sciences
1-2, Kan'nondai 2, Tsukuba, Ibaraki 305-8602, JAPAN
kawase@affrc.go.jp (Tel: +81 29 838 7458)

Introduction

Along with the technical development on molecular genetics and genomics, many new methods have been applied to evaluation, genetic analysis and use of plant genetic resources for food and agriculture (PGRFA). Different kinds of internaitonal research collaboration have been done and will be done on PGRFA in Asia. In this article, I would like to focus on field research. Still, field research is an important one in international collaboration on PGRFA: exploration for *ex situ* conservation, monitoring for *in situ* conservation, wild-weed-crop complex studies, and so on. PGRFA researchers are requested to have trust-building partnerships with compliance, accountability and ethics in international collaboration. Although some scientists have given warning on public awareness and intellectual property rights (IRP), there are many problems to solve, for example, a limitted number of legal specialists or patent attorneys on PGRFA, and shortage of recognition of the ownership of and benefit sharing from the PGRFA (Watanabe et al., 1998).

Present situation and prospect of cooperation on PGRFA

Generally speaking, we have a considerable amount of *ex situ* collection in CG centers nad national gene banks concerning major crops for which the major concerns are how to access and how to use them developing new strategies on allele mining, genomics-based germplasm enhancement, utilization of core collections, and so on rather than obtaining much more accessions as suggested by many researchers. With regard to minor crops or underutilized crops, still we have to consider how to cooperate to collect and conserve them systematically. We have to pay much attention to the traditional knowledge considering IPR. About wild relatives of cultivated plants, even in major crops, we have to study to dtermine what should be conserved *in situ* or on-farm, how to monitor genetic dynamics of populations, and wild-weed-crop complex. A broad range of studies on PGRFA including academic studies and pragmatic studies will have to be done, to which international trust-building partnerships will make good contribution.

PGRFA are attractive to various scientists working in different disciplines who will promote cooperation certainly. There are different types of access to PGRFA (Fig. 1). I dare to focus on collaborative field research, an long-established activity on PGRFA for the discussion on mutually beneficial international cooperation. Although exploration is unlikely to be needed for major crops, it is important for minor crops, for wild relatives, for activities for *in situ* conservation and for studies on wild-weed-crop complex. Field studies on PGRFA based on taxonomy, genetics, ecology, and ethonobotany provide *ex situ* conservation, extend knowledge and idea on plant-human relationships, and may provide some information on biosafety.

Paradigm shift and collaboration

Long before the Convention on Biological Diversity (CBD), collaborative field works on PGRFA have been done on researcher-researcher bases, institute-institute bases, by means of bilateral relations and/or through coordination initiatives of international organizations. The International Board for Plant Genetic Resources (IBPGR) was established in 1974 to play an important role to promote and assist the worldwide efforts on PGRFA and reformed to be the International Plant Genetic Resources Institute (IPGRI) in 1991. The FAO International Undertaking on Plant Genetic Resources for Food and Agriculture (IU) was adopted as an instrument to promote international harmony in matters regarding access to PGRFA in 1983, when PGRFA had been generally considered as common property of human beings and to be freely accessible until effectuation of CBD in 1993. We have experienced a drastic paradigm shift on PGRFA, since the CBD assigns sovereign rights to the country of origin and requires that Prior Informed Consent (PIC) is received from the country in which access to organisms is requested. Cartagena Protocol effectuated in 2004 is important as a biosafety protocol which is closely related with PGRFA.

Because of the difficulty in determining origins of PGRFA and for their secured accessibility for crop improvement, amendments of IU had been discussed in order to be well harmonized with CBD, and then, International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGR) was settled and effectuated in June 29, 2004 to ensure access to and equitable benefit sharing for PGRFA.

At present, we understand that Material Transfer Agreement (MTA) should be settled whenever PGRFA is provided. It was a good change that many countries have begun to pay attention to PGRFA as national heritage. But, at the same time, it has become more difficult to plan and organize collaborative field studies internationally. Without consideration of IPR, we are not able to access PGRFA.

International cooperation of Japan on PGRFA and the NIAS Genebank Project

Roughly speaking, overseas field studies of Japan on PGRFA might be divided into two categories. One has academic purposes and is done mainly by universitiies scientists supported by MEXT Grant-in-Aid or else, and another has pragmatic purposes, for example, collecting materials for crop improvement and is done by agricultural research institutes like National Institute of Agrobiological Sciences (NIAS). NIAS operates the Genebank Project as the central coordinating institute (center bank) for plant, animal and microorganism genetic resources in cooperation with different research institutes (sub-banks) in Japan (Okuno et al. 2004).

Among various kinds of activities in the Genebank Project, overseas collaborative exploration with material transfer and field research cooperation without material transfer are essential as international collaboration. More than 70 missions have been dispatched overseas since 1975, of which two thirds are in cooperation with Asian countries (Kawase 2004). Recently we are enhancing field research cooperation without material transfer. The NIAS Genebank Project has made good collaboration with IPGRI (IBPGR) on exploration and field research for possible *in situ* (on-farm) conservation. Other activities on PGRFA as international cooperation are collaboration for gene bank establishment projects implemented through Japan International Cooperation Agency (JICA) in Sri Lanka, Chile, Pakistan and Myanmar. Unfortunately this kind of cooperation is not implemented at present. During the JICA project term or even after that, NIAS Genebank Project has been positively engaged in collaborative explorations together with other cooperation on PGRFA activities.

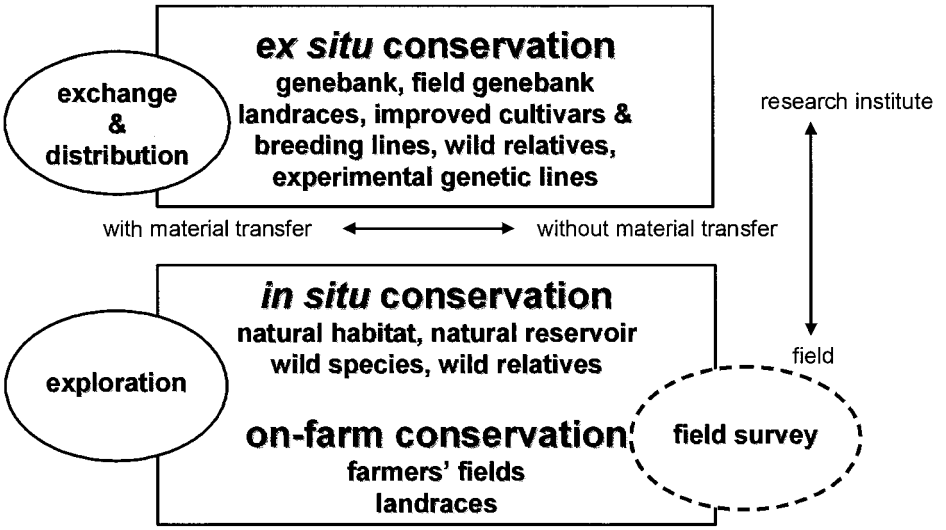


Fig. 1. A schematic drawing of possible types of access to PGRFA under CBD.

Trust–building relationships in collaboration on PGRFA

Considering the values of PGRFA not only for breeding purpose but also their multiple values for people and huge related research fields, all scientists, gene bank staff, relevant administrative officials and farmers should pay much attention on compliance, accountability, ethics as well as serious consideration of intellectual property right for trust–building relationships for fruitful international cooperation.

For example, I would like to take up collaborative field studies for discussion among various kinds of access of PGRFA. Based on the understanding of potential of, the ownership of and benefit sharing from PGRFA, certain documentation should be prepared for international transaction in the movement of PGRFA. In prior to collaborative field study, we have to reach mutual agreement such as the Memorandum of Agreement (MOA), Memorandum of Understanding (MOU), and Germplasm Acquisition Agreement (GAA), although it often takes time to reach the agreement.

When the collected materials are to be transferred overseas, a material transfer agreement (MTA) should be settled following the rules of the country. Export permit and quarantine procedures are also needed. After the field study, all passport data, characterization data, and evaluation data on the PGRFA should be shared by both parties. It is cardinal to point out that further consideration of the harmonization and governance of the international discussion as there are new developments also by the involvement of Trade-Related Aspects of Intellectual Property Rights (TRIPS) on WTO and the World Intellectual Property Organization (WIPO) on the materials and knowledge derived from PGRFA.

Conclusion

I would like to propose more close international collaboration on PGRFA particularly in Asian countries. We will have to start consideration and discussions about feasibility of responsibility sharing of *ex situ* conservation in Asia although it may be difficult to realize immediately. I believe that fruitful collaborative field study between countries is a good first step, and we will have to make sure of compliance, accountability, and ethics through the

collaboration. The NIAS Genebank Project of Japan wishes continuously to cooperate particularly with other countries.

References

Kawase M (2004) International cooperation by Japan on conservation and utilization of agricultural plant genetic resources. Farming Japan 38: 24-31.

Okuno K, K Shirata, T Niino, M Kawase (2005) Plant genetic resources in Japan: platforms and destinations to conserve and utilize plant genetic diversity. JARQ 39: 231-237.

Watanabe K, V Ramanatha Rao, M Iwanaga (1998) International trends on the conservation and use of plant genetic resources. Plant Biotechnology 15: 115-122.

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(独)農業生物資源研究所
ジーンバンク

茨城県つくば市観音台2−1−2
電話：029−838−7458

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